Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators

Niels Borregaard,* Lars Kjeldsen,* Henrik Sengeløv,* Michael S. Diamond,† Timothy A. Springer,† H. Clarke Anderson,‡ Takashi K. Kishimoto,§ and Dorothy F. Bainton||

The Granulocyte Research Laboratory, Department of Hematology, University Hospital, Rigshospitalet, Copenhagen, Denmark; †The Center for Blood Research, Harvard Medical School, Boston, MA; ‡Department of Pathology and Oncology, The University of Kansas Medical Center, School of Medicine, Kansas City; §Boehringer Ingelheim Pharmaceuticals Inc., Department of Immunology, Ridgefield, CT; and ||Department of Pathology, University of California, San Francisco

Abstract: The localization of the adhesion protein L-selectin in human neutrophils was determined by subcellular fractionation and immunoelectron microscopy and compared with the localization of Mac-1 ($\alpha_m\beta_2$) and alkaline phosphatase, the marker for secretory vesicles. L-selectin was found to be localized exclusively on the plasma membrane of unstimulated cells and also of stimulated cells, although markedly diminished. This was in contrast to Mac-1, which was also localized in secretory vesicles and in specific/gelatinase granules as shown previously [Sengeløv, H., et al. *J. Clin. Invest.* (1993) 92, 1467–1476]. Stimulation of neutrophils with inflammatory mediators such as tumor necrosis factor (TNF), platelet-activating factor (PAF), or f-Met-Leu-Phe (fMLP), induced parallel up-regulation of the surface membrane content of alkaline phosphatase and Mac-1 and down-regulation of L-selectin, as evidenced by flow cytometry. Preimbedding immunoelectron microscopy confirmed that L-selectin was present mainly on tips of microvilli in unstimulated cells and showed that alkaline phosphatase and Mac-1 were randomly distributed on the surface membrane of fMLP-stimulated cells. These studies indicate that the transition of neutrophils from L-selectin-presenting cells to Mac-1-presenting cells induced by inflammatory mediators is mediated by incorporation of secretory vesicle membrane, rich in Mac-1 and devoid of L-selectin, into the plasma membrane. *J. Leukoc. Biol.* 56: 80–87; 1994.

Key Words: *fMLP · GM-CSF · IL-8 · LTB_4 · PAF · secretory vesicles · TNF*

INTRODUCTION

The dynamic process of neutrophil-endothelial interaction, a key event in inflammation in general and in defense against invading microorganisms in particular, has been clarified by the demonstration both in vitro and in vivo of the role of selectins in mediating the initial rolling contact of neutrophils with endothelial cells [1–7] and of β_2 integrins and ICAMs in mediating the subsequent irreversible adhesion [5, 8–15]. The immediate response of endothelial cells to inflammatory mediators involves translocation of P-selectin from intracellular stores (Weibel-Palade bodies) [16] to the surface of endothelial cells [17–19] and subsequent synthesis and insertion into the plasma membrane of E-selectin [20, 21] and ICAM-1 [22], but the dynamic presentation of L-selectin and Mac-1 ($\alpha_m\beta_2$) on the surface of neutrophils is less well understood. It has been demonstrated that L-selectin is present on the tips of microvilli [2, 23], a localization probably mediated through the cytoplasmic tail of L-selectin [24] and seemingly ideal for mediating the initial rolling contact of neutrophils with P-selectin on endothelial cells, whereas Mac-1, when present, is distributed randomly over the surface membrane but excluded from microvilli of nonstimulated cells [23]. We have demonstrated that secretory vesicles, an organelle of neutrophils [25–28], monocytes, and eosinophils [29], contain Mac-1 and that the appearance of Mac-1 on the surface of intact neutrophils can be accounted for by incorporation of secretory vesicle membrane into the plasma membrane in response to stimulation with inflammatory mediators [29, 30]. Secretory vesicles are identified by their membrane content of alkaline phosphatase present on the luminal surface and albumin present in the matrix. Because this organelle which is largely ignored in studies of neutrophil activation [31], is the most rapidly and most easily mobilized organelle of neutrophils [32], it is potentially very important as a rapidly mobilizable source of neutrophil surface proteins. We therefore decided to address the question whether secretory vesicles contain L-selectin in addition to Mac-1 and thus might play a role in continued rolling contact of neutrophils with endothelium by replenishing L-selectin lost from the surface by shedding [33, 34], or whether secretory vesicles are devoid of L-selectin, in which case a highly selective role of secretory vesicles in the transition of neutrophils from rolling, L-selectin-presenting cells to adherent, Mac-1-presenting cells must be suspected.

MATERIALS AND METHODS

Human neutrophils were isolated from freshly drawn blood as previously described [35]. Sedimentation of red cells was induced by dextran T-500 (Pharmacia, Uppsala, Sweden), and the leukocyte-rich supernatant was centrifuged on Lymphoprep (Nygaard, Oslo, Norway). Residual erythro-

Abbreviations: fMLP, *N*-formylmethionyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage colony-stimulating factor; KRP, Krebs-Ringer phosphate; LTB_4, leukotriene B_4; PAF, platelet-activating factor; TNF, tumor necrosis factor; ZAS, zymosan-activated serum.

Reprint requests: Niels Borregaard, The Granulocyte Research Laboratory, Department of Hematology, Rigshospitalet L-4042, 9 Blegdamsvej, DK-2100 Copenhagen Ø, Denmark.

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cytes were lysed by hypotonic shock and the neutrophils resuspended in buffer as indicated. All procedures were carried out at 4°C except sedimentation of red cells.

Stimulation of neutrophils was performed at 37°C at a cell concentration of 3×10^7 cells/ml for subsequent subcellular fractionation, or at 10^7/ml for subsequent fluorescence-activated cell sorter (FACS) analysis, 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 volumes 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 and for subcellular fractionation.

Subcellular fractionation was performed as previously described [35]. In short, neutrophils were resuspended in KRP containing 5 mM diisopropyl fluorophosphate (Aldrich Chemical Co., Milwaukee, WI) and kept on ice for 10 min. After centrifugation at 200*g* for 10 min the cells were resuspended in 15 ml of disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 0.5 mM phenylmethanesulfonyl fluoride, 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 [35]. 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.

Marker proteins

Azurophil granules were identified by myeloperoxidase measured by enzyme-linked immunosorbent assay (ELISA) using as catching antibody rabbit antimyeloperoxidase antibody (Dakopatts A 398, Glostrup, Denmark) after affinity

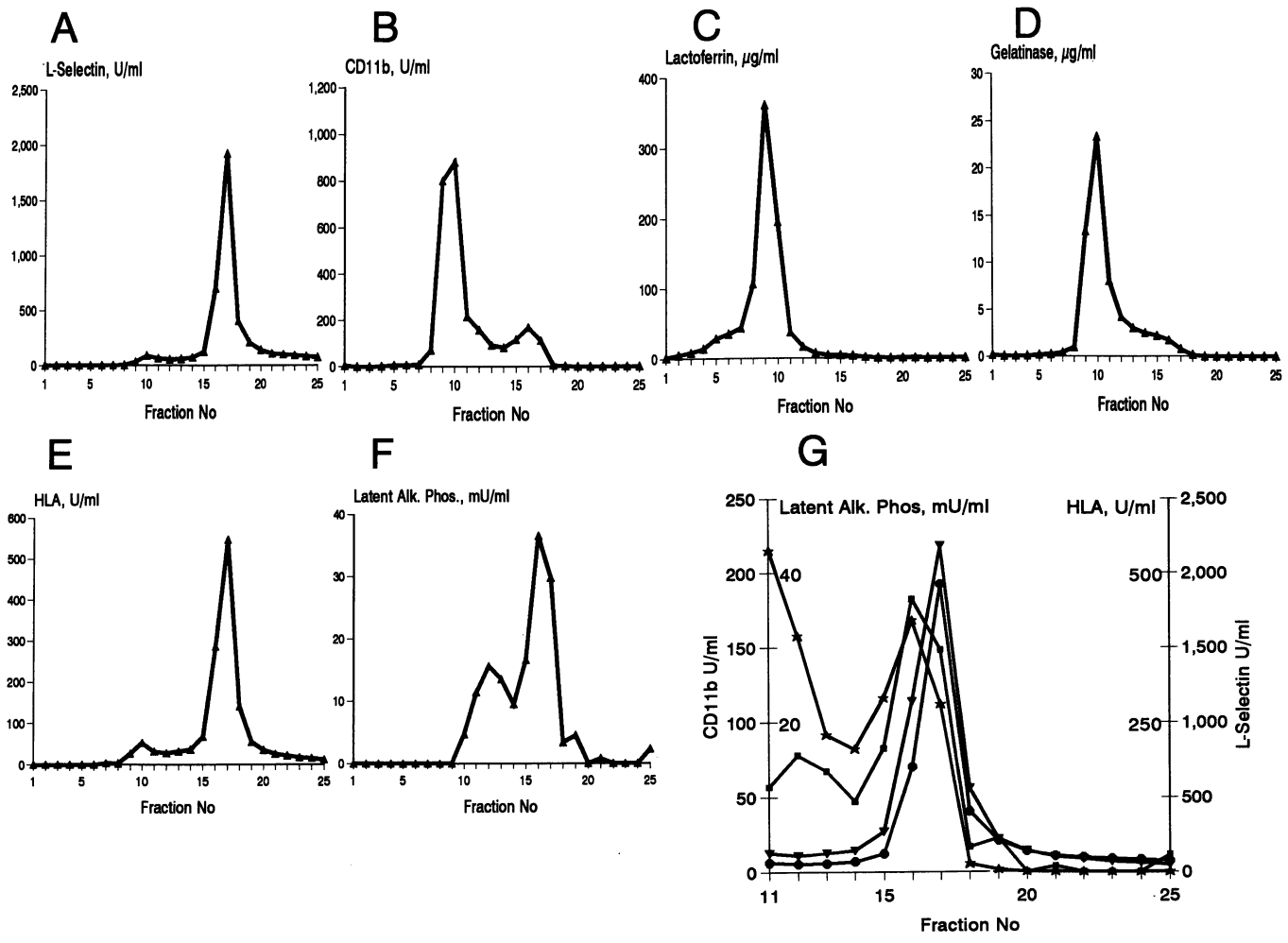

Fig. 1. Subcellular localization of L-selectin and Mac-1. Isolated neutrophils were divided in two equal parts, one was kept at 4°C (this figure) and the other stimulated with 10^{-8} M fMLP at 37°C (Fig. 2). Subcellular fractionation was then performed in parallel. (One representative experiment out of three is shown.) (A) L-selectin; (B) Mac-1; (C) lactoferrin (a marker of specific granules); (D) gelatinase (a marker of gelatinase granules); (E) HLA (a marker of plasma membranes); (F) latent alkaline phosphatase (a marker of secretory vesicles); (G) is an insert where the results for fractions 11-20 (light membranes) are shown by the following symbols: Mac-1 (*), L-selectin (●), latent alkaline phosphatase (■), and HLA (▼). Note difference between this figure and Figure 2 in the left *y* axis of G.

purification on a myeloperoxidase-Sepharose column. The antibody was diluted 1:100. Rabbit antimyeloperoxidase (Dakopatts A 398) was biotinylated at 1 mg/ml and 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 [36]. Gelatinase granules were identified by gelatinase [36], measured by ELISA [37]. Secretory vesicles were identified by alkaline phosphatase [38] that could be measured only in the presence of 0.2% (v/v) Triton X-100 (latent alkaline phosphatase) [25, 26] and by albumin measured by ELISA [27]. Plasma membranes were identified by human leukocyte antigen (HLA) class I assayed by a mixed ELISA (MELISA) [39].

Mac-1 was quantitated by ELISA as described [30] using CBRM1/23, an IgG2a mouse monoclonal antibody against the COOH-terminal extracellular region of the α chain of Mac-1 as catching antibody and biotinylated LM2/1, an IgG1 mouse monoclonal antibody against the I domain of the α chain of Mac-1, as detecting antibody.

L-selectin was quantitated by ELISA using DREG 55 as catching antibody (2.5 μg/ml) and biotinylated DREG 200 (0.63 μg/ml) as detecting antibody. DREG 55 and 200 are characterized in ref. 40. One batch of normal human serum was used as standard in all experiments in 2-fold serial dilutions starting with a 20-fold diluted sample (55 arb. U/ml).

ELISA

ELISA was performed using the same general procedure independent of the antibodies except as specified in previous publications regarding MELISA [39] and lactoferrin [36] where avidin-biotin interaction was not used. Immunoplates (Nunc, Roskilde, Denmark) were coated with 100 μl of 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 of buffer B (500 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 1% Triton X-100, 1% bovine serum albumin) for 1 h. After washing three times in buffer A, 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 [30]). After three additional washes in buffer A, 100 μl of biotinylated catching antibody was applied, appropriately diluted in buffer B and incubated for 1 h followed by washing three times in buffer A. Then 100 μl of peroxidase-conjugated avidin (Dakopatts, P347) in buffer B was 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 in-

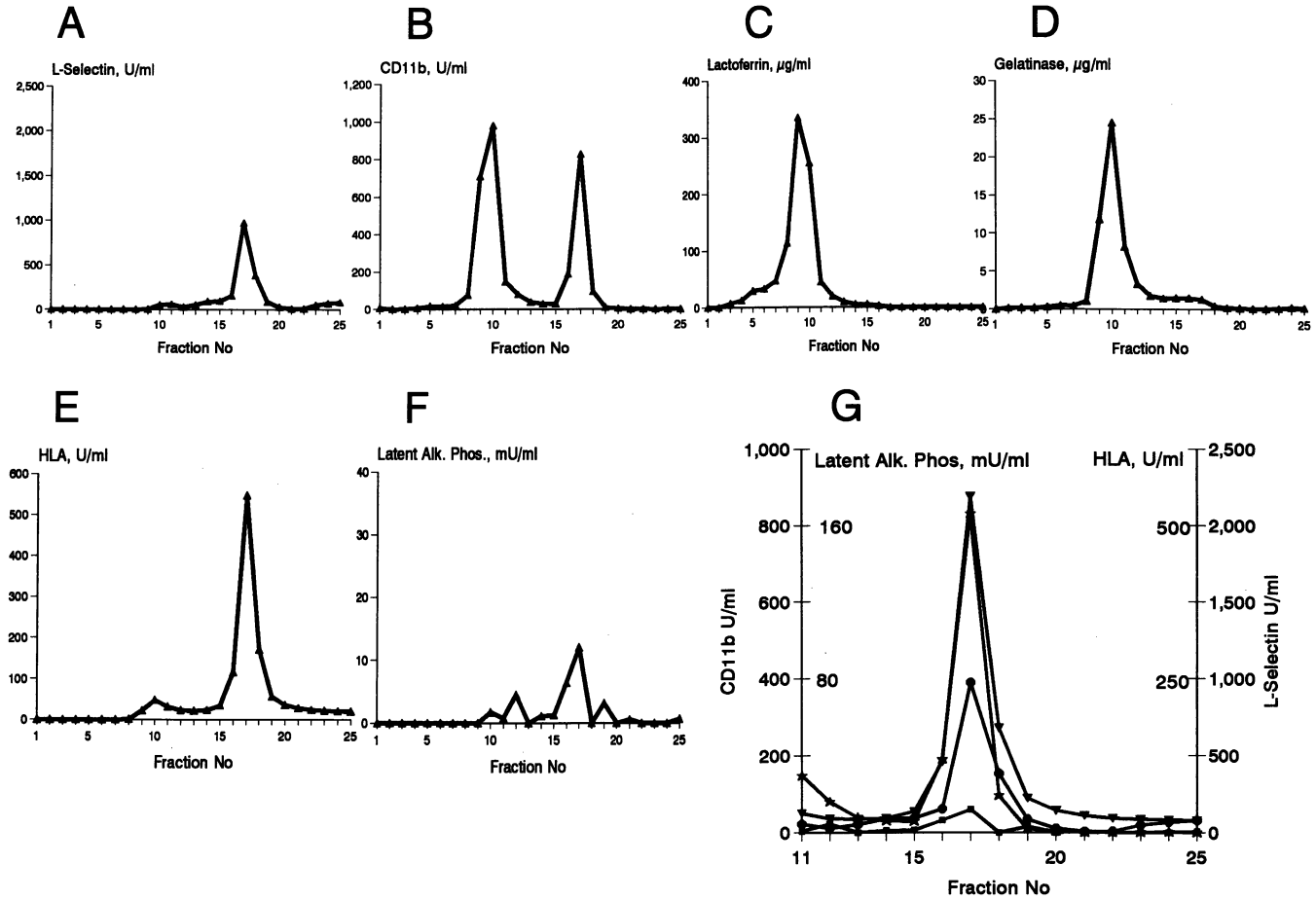

Fig. 2. Subcellular localization of L-selectin and Mac-1. Isolated neutrophils were divided in two equal parts, one kept at 4°C (Fig. 1) and the other stimulated with 10^{-8} M fMLP at 37°C (this figure). Subcellular fractionation was then performed in parallel. (One representative experiment out of three is shown.) See legend of Figure 1 for explanation of A–G.

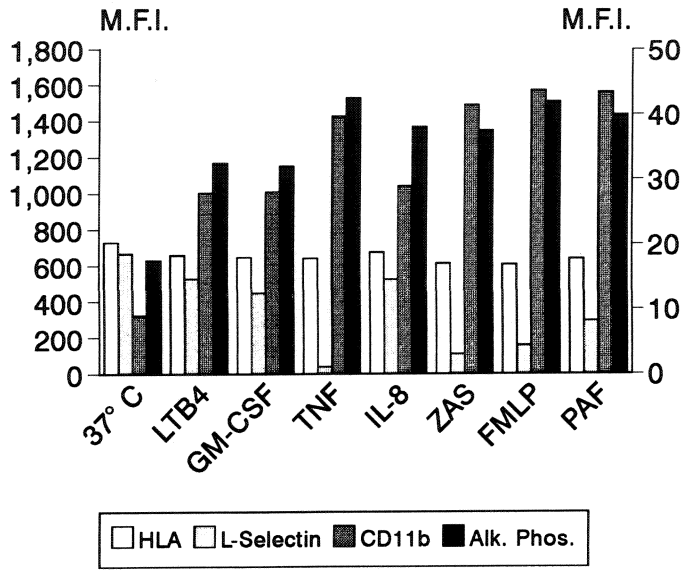

Fig. 3. Immunofluorescence. The surface membrane content of HLA (open bar, 1. *y* axis), L-selectin (light dotted bar, 1. *y* axis), Mac-1 (heavy dotted bar, 1. *y* axis), and alkaline phosphatase (solid bar, 2. *y* axis) was determined by FACS using monoclonal antibodies and given as mean fluorescence intensity. Cells were either kept at 4°C or 37°C or stimulated at 37°C for 15 min with each of the following: 10^{-8} M LTB_4, 100 U/ml GM-CSF, 500 U/ml TNF, 0.2 μg/ml IL-8, 5% ZAS, 10^{-8} M fMLP, and 10^{-8} M PAF. These doses were found to be individually maximally effective in causing up-regulation of Mac-1 and alkaline phosphatase. Results are means of three independent experiments.

cubation in sodium phosphate-citric acid buffer containing 0.04% (w/v) *o*-phenylenediamine and 0.03% H_2O_2 and was stopped by addition of 100 μl of 1 M H_2SO_4. Absorbance was read at 492 nm in a Multiscan Plus automatic ELISA reader (Labsystems, Helsinki, Finland).

FACS analysis

Isolated neutrophils were either kept at 4°C or 37°C or stimulated at 37°C as indicated. Labeling of cells was performed by incubating 100 μl of cells at 1 × 10^7/ml with 50 μl of monoclonal antibody (LM2/1, DREG 200, 1B12, an IgG1 murine antibody against human osteosarcoma alkaline phosphatase [41, 42], anti-HLA class 1, Dakopatts M736) or murine preimmune IgG1 (Becton-Dickinson, Mountain View, CA) for 30 min at 4°C. After washing twice, 50 μl of 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 phosphate-buffered saline (PBS) containing 1% formaldehyde and analyzed in a FACScan (Becton-Dickinson). Mean fluorescence intensity for specific antibodies was corrected for nonspecific fluorescence by subtracting mean fluorescence for preimmune IgG1.

Immunoelectron microscopy

Because of fixation sensitivity of the antigens recognized by monoclonal antibodies CBRM1/29 [43] (Mac-1) and 1B12 (alkaline phosphatase), detection of these two antigens on the

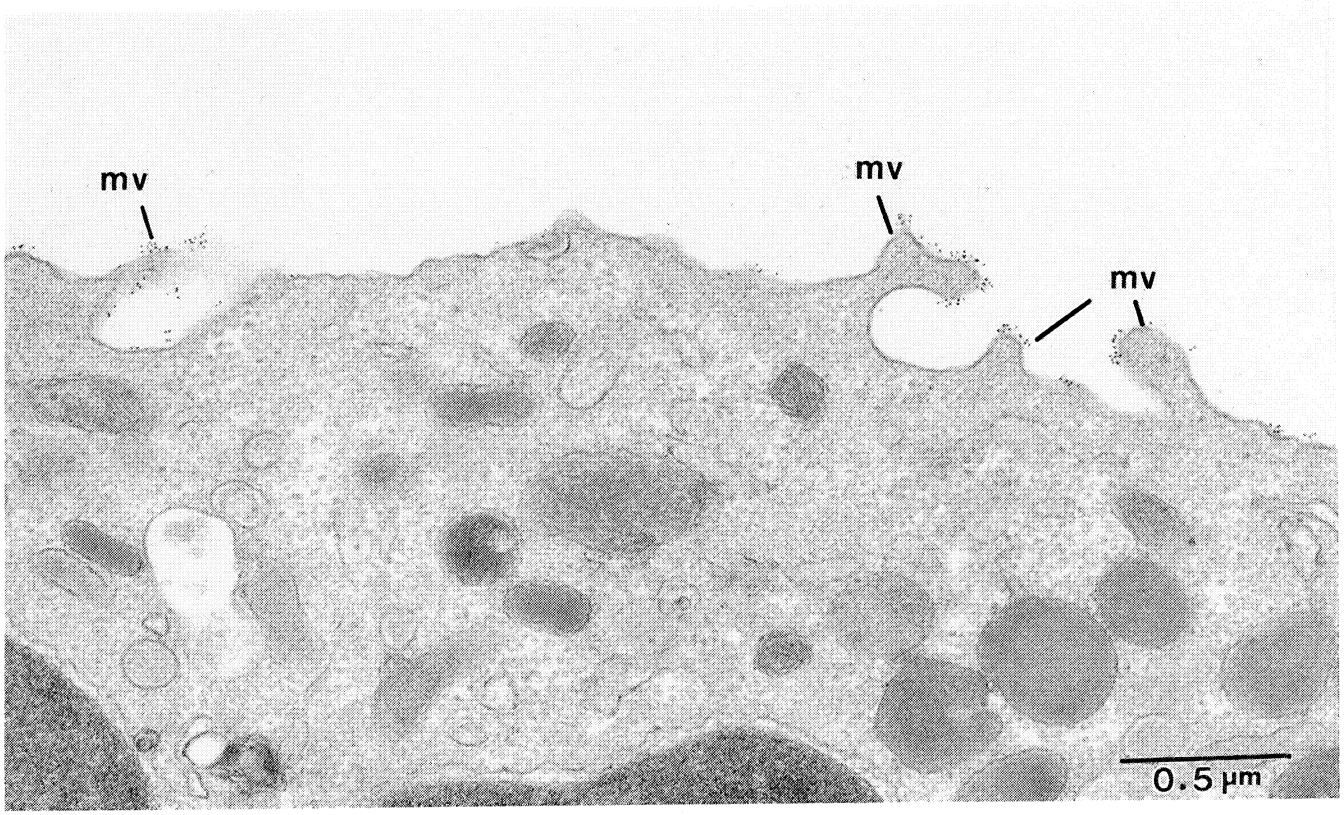

Fig. 4. L-Selectin on the surface of neutrophils. Unstimulated neutrophils were fixed in 2% formaldehyde, 0.05% glutaraldehyde, washed, and incubated with the monoclonal antibody DREG-200 against L-selectin. A bridge of rabbit anti-mouse antibody was applied, and immunolabeling with protein A gold-5 was finally performed. Transmission electron micrograph of a portion of a neutrophil is given. As can be seen, the majority of the immunogold label is on the tips of microvilli (mv). ×45,000.

plasma membrane was performed by incubating unfixed cells at 4°C for 45 min with either CBRM1/29 (1 mg/ml) diluted 1:100 or 1B12 (1 mg/ml) diluted 1:20 followed by fixation in 2% paraformaldehyde, 0.05% glutaraldehyde for 30 min at room temperature. Rabbit anti-mouse antibody (Zymed) diluted 1:200 was then added, followed by protein A-gold-5 (1:50 dilution). Immunogold labeling of L-selectin was performed before fixation as described above or could be performed after fixation by adding mAb DREG 200 diluted 1:200 (stock 2 mg/ml). The cells were subsequently refixed in 1.5% glutaraldehyde for 45 min at 22°C, washed in sodium cacodylate buffer, postfixed in 1% OsO_4 in veronal-acetate buffer, stained with aqueous 1% uranyl acetate, dehydrated in ethyl alcohol, and embedded in Epon. Detailed morphometric analyses of these localizations are the subject of a separate paper.

Source of stimuli

N-Formylmethionyl-leucyl-phenylalanine (fMLP) (Sigma), 1 mM in ethanol. Granulocyte-macrophage colony-stimulating factor (GM-CSF; Sandoz Pharmaceuticals, East Hanover, NJ), 10^4 U/ml in H_2O. Recombinant interleukin-8 (a generous gift from Dr. K. Thestrup-Pedersen, Department of Dermatology, Marselisborg Hospital, Aarhus, Denmark), 10 μg/ml in PBS. Leukotriene B_4 (LTB_4; Sigma), 1 mM in ethanol. Platelet-activating factor (PAF; Sigma), 1 mM in PBS, 0.5% (w/v) BSA (Sigma). Tumor necrosis factor (TNF; Amersham International, Amersham, UK), 10^4U/ml in H_2O.

RESULTS AND DISCUSSION

The distribution of L-selectin in subcellular fractions of unstimulated neutrophils (**Fig. 1**) indicates that L-selectin is present exclusively in the plasma membrane, whereas Mac-1 is localized with a main peak in specific/gelatinase granules and a second peak colocalizing with secretory vesicles. The distribution of secretory vesicles is bimodal in this two-layer gradient, with the main peak of the marker latent alkaline phosphatase localizing close to the plasma membrane marker HLA and L-selectin, while the rest of the secretory vesicles are found in a broad region from the peak in the light membrane region to the region containing specific granules. The separation of HLA and L-selectin from latent alkaline phosphatase and from Mac-1 is evident when focus is made on the light membrane region of the gradient as seen in Figure 1G. The localization of Mac-1 in secretory vesicles and L-selectin in the plasma membrane is further illustrated by the translocation in response to stimulation with fMLP (**Fig. 2**). Stimulation of intact cells with fMLP results in nearly selective and complete exocytosis of secretory vesicles. This is evidenced by the disappearance of latent alkaline

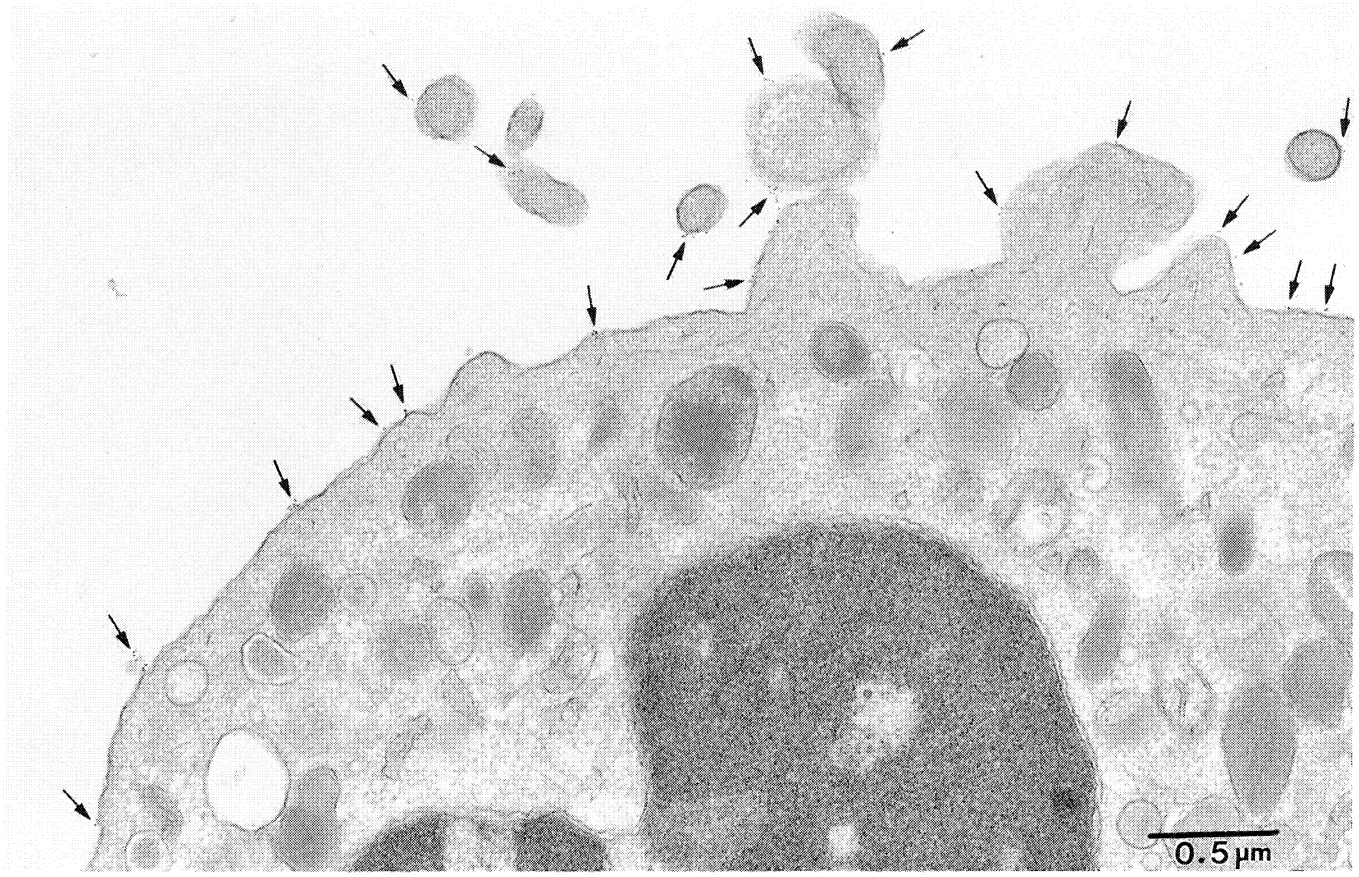

Fig. 5. Alkaline phosphatase on the surface of neutrophils. Neutrophils were stimulated with 10^{-7} M fMLP for 10 min at 37°C, chilled to 4°C, and labeled with monoclonal antibody 1B12 against alkaline phosphatase. The cells were then fixed in 2% formaldehyde, 0.05% glutaraldehyde, immunolabeled with rabbit anti-mouse antibody followed by protein A gold-5, and processed for transmission electron microscopy. Note that instead of the small microvilli found on the resting neutrophil (Fig. 4), the plasma membrane has become very irregular with large extensions or ruffles. The immunogold label (arrows) indicates the presence of alkaline phosphatase on the plasma membrane in a random distribution. ×30,000.

phosphatase (alkaline phosphatase present on the inside of secretory vesicles [26]). Now, complete colocalization of Mac-1 and L-selectin in the plasma membrane is observed as a result of translocation of Mac-1 from secretory vesicles to the plasma membrane, whereas the main part of Mac-1 present in specific/gelatinase granules is unchanged. The level of L-selectin is reduced as a result of stimulation-induced shedding, the mechanism of which is still unclear [33].

This demonstrates that secretory vesicles, although endocytic in origin [27], are not simply inside-out plasma membrane vesicles but are organelles organized with a selective membrane composition, highly enriched in Mac-1 and cytochrome b_{558} relative to the plasma membrane [29, 30] and devoid of HLA and L-selectin as demonstrated here, but sharing alkaline phosphatase with the plasma membrane. Thus, the incorporation of secretory vesicle membrane into the plasma membrane can be expected to affect profoundly the profile of proteins present in the plasma membrane rather than just adding more membrane with the same protein composition. Such changes in the protein profile of the plasma membrane can be observed and quantified by immunofluorescence on single cells.

The incorporation of secretory vesicle membrane into the plasma membrane is monitored with a monoclonal antibody against alkaline phosphatase. It is demonstrated (**Fig. 3**) that stimulation of neutrophils with inflammatory mediators that may be operative in controlling the neutrophil-endothelial interaction results in incorporation of secretory vesicle membrane into the plasma membrane, as evidenced by the parallel increase in the amounts of alkaline phosphatase and Mac-1 in the plasma membrane. It is observed that the amount of L-selectin is reduced following stimulation. This has previously been shown to be due to stimulation-induced shedding of L-selectin [33]. Although this occurs in parallel with exocytosis of secretory vesicles, these two phenomena do not seem to be causally related because stimuli that mobilize secretory vesicles efficiently may differ in their capacity to induce shedding of L-selectin (TNF versus PAF) (Fig. 3).

It has previously been shown by enzyme cytochemistry that alkaline phosphatase, the membrane marker of secretory vesicles [25–27], is localized in intracellular vesicles in unstimulated neutrophils [44] and that stimulation causes these to fuse to form intracellular tubular structures that are in communication with the cell surface [45]. Our results demonstrate that after mobilization of secretory vesicles, the

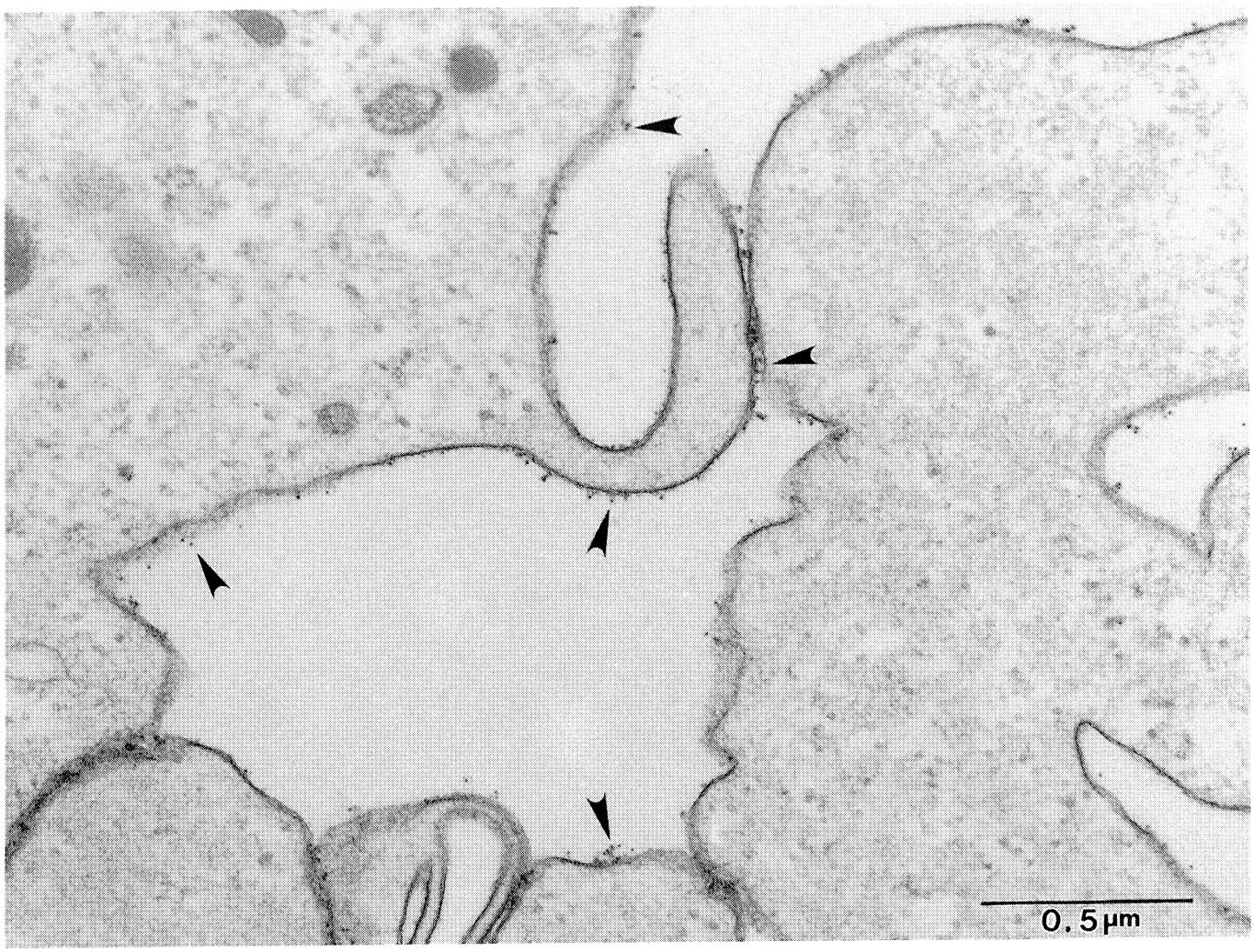

Fig. 6. Mac-1 on the surface of neutrophils. Neutrophils were stimulated with 10^{-7} M fMLP for 10 min at 37°C, chilled to 4°C, and labeled with monoclonal antibody CBR M1/29 against the α chain of Mac-1 (CD11b). The cells were then fixed in 2% formaldehyde, 0.05% glutaraldehyde and immunolabeled with rabbit anti-mouse antibody, followed by protein A gold-5, and processed for transmission electron microscopy. The immunogold label (arrowheads) is found randomly distributed on both ruffles and other parts of the plasma membrane. ×60,000.

associated alkaline phosphatase and Mac-1 become accessible to macromolecules such as antibodies and hence are incorporated into the plasma membrane, where they may interact with the surroundings of the cell. To further visualize this, intact cells were labeled with monoclonal antibody against L-selectin, alkaline phosphatase, or Mac-1, and the membrane topology of these proteins was determined by immunoelectron microscopy. **Figure 4** confirms the localization of L-selectin on the tips of microvilli by preimbedding immunoelectron microscopy of unstimulated cells as previously demonstrated both in frozen thin sections [2] and by scanning electron microscopy [23]. **Figures 5 and 6** show that both alkaline phosphatase and Mac-1 are randomly distributed on the surface of fMLP-stimulated cells. We found no indication that alkaline phosphatase was present in tubular structures as reported previously [45]. Also, we did not observe a patchy distribution of alkaline phosphatase in microinvaginations indicative of caveolae—the cholesterol-rich plasma membrane subdomains that have been shown to contain phosphatidylinositol-linked proteins in other cell types [46–48]. However, different technical approaches were used, and the data has to be interpreted cautiously in light of these differences.

In conclusion, we have demonstrated that L-selectin is present exclusively on the plasma membrane of neutrophils. Secretory vesicles which are endocytic in origin lack L-selectin. This organelle is rapidly incorporated into the plasma membrane of neutrophils and changes the protein profile of the surface membrane from an L-selectin-presenting cell to a Mac-1-presenting cell. The selective membrane protein composition and exquisite sensitivity to mobilization by inflammatory mediators make secretory vesicles likely candidates as the organelles that determine the ability of the neutrophil to interact firmly with endothelium for subsequent diapedesis.

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