Stimulus-dependent Secretion of Plasma Proteins from Human Neutrophils

Niels Borregaard, Lars Kjeldsen, Kåre Rygaard,* Lone Bastholm,* Morten H. Nielsen,* Henrik Sengeløv, Ole W. Bjerrum, and Anders H. Johnsen‡

*Granulocyte Research Laboratory, Department of Medicine and Hematology L-4042, University Hospital, *University Institute of Pathological Anatomy, ‡Department of Clinical Chemistry, University Hospital, DK-2100 Copenhagen, Denmark*

Abstract

In search for matrix proteins released from secretory vesicles of human neutrophils, a prominent 67-kD protein was identified in the extracellular medium of neutrophils stimulated by the chemotactic peptide, FMLP. The protein was purified to apparent homogeneity and partially sequenced. The sequence of the first 32 NH_2-terminal amino acids was identical to the sequence of albumin. mRNA for human albumin could not be detected in bone marrow cells, nor could biosynthetic labeling of albumin be demonstrated in bone marrow cells during incubation with [^{14}C]leucine.

Immunofluorescence studies on single cells demonstrated the presence of intracellular albumin in fixed permeabilized neutrophils. Light microscopy of immunogold-silver–stained cryosections visualized albumin in cytoplasmic "granules." The morphology of these was determined by immunoelectron microscopy as vesicles of varying form and size. Subcellular fractionation studies on unstimulated neutrophils demonstrated the presence of albumin in the low density pre-γ and γ-regions that contain secretory vesicles, but are devoid of specific granules and azurophil granules. Albumin was readily released from these structures during activation of neutrophils with inflammatory mediators. Immunoblotting demonstrated the presence of immunoglobulin and transferrin along with albumin in exocytosed material from stimulated neutrophils. This indicates that secretory vesicles are unique endocytic vesicles that can be triggered to exocytose by inflammatory stimuli. (*J. Clin. Invest.* 1992. 90:86–96.) Key words: albumin • exocytosis • granules • secretory vesicles • subcellular fractionation

Introduction

Neutrophils are highly specialized cells equipped with a battery of proteolytic and digestive enzymes capable of destroying extracellular matrix and bacterial debris. These enzymes are stored in the matrix of granules of which two kinds have been recognized: the lysosomal-like azurophil granules and the specific granules (1). In addition, the membrane of specific granules contains receptors for chemotactic factors (2), adhesion molecules (3), and elements of the electron transport chain functioning as an NADPH oxidase of prime importance for killing of microorganisms (4, 5).

Mobilization of granules is an important aspect of neutrophil activation, since this results in recruitment of functional proteins to the plasma membrane and release of destructive enzymes to the environment.

It has been recognized for long that stimulation of neutrophils by inflammatory mediators can result in a severalfold increase in the number of plasma membrane proteins such as complement receptor 3 (6) and decay accelerating factor (7). Yet these stimuli are incapable of mobilizing specific granules to the extent that can explain the upregulation of these proteins in the plasma membrane (8).

A special type of granules identified by their content of gelatinase has been suggested as the source of membrane material readily incorporated into the plasma membrane, since these have been reported to be mobilized more quickly and extensively than specific granules (9). The presence of gelatinase in a separate granule subset has recently been questioned by the demonstration of co-localization of gelatinase and lactoferrin in specific granules using immunoelectron microscopy (10).

We have identified an easily mobilizable intracellular compartment termed secretory vesicles in neutrophils (8, 11). These vesicles are identified by their content of alkaline phosphatase that can only be measured in the presence of detergent, in contrast to plasma membrane-bound alkaline phosphatase. These vesicles are readily mobilized to the plasma membrane by stimuli that do not cause significant exocytosis of specific granule content, e.g., nanomolar concentrations of the chemotactic tripeptide FMLP. Mobilization of secretory vesicles is accompanied by release of tetranectin, a protein recently identified in plasma and in all secretory human cells studied (12). Based on their kinetics of mobilization, secretory vesicles qualify as an intracellular store of both cytochrome b_{558} and adhesion proteins (8, 11, 13).

To further investigate the content of secretory vesicles, we decided to examine the protein profile of material that was exocytosed from neutrophils after stimulation with FMLP. This would permit identification of the most prominent protein(s) released from secretory vesicles, and hopefully give further insight into the origin and function of these structures.

Methods

Isolation of neutrophils. Peripheral blood was obtained by venipuncture of healthy volunteers, anticoagulated with 25 mM sodium citrate, and mixed at room temperature with an equal volume of 2% Dextran T-500 (Pharmacia-LKB, Uppsala, Sweden) in 0.9% NaCl to ease the sedimentation of erythrocytes. The leukocyte-rich supernatant was centrifuged at 200 *g* for 10 min and the cells resuspended in ice-cold 0.9% NaCl. Mononuclear cells were separated from polymorphonuclear cells and residual erythrocytes by centrifugation through Lymphoprep (Nyegaard, Oslo, Norway) (14).

Address correspondence to Dr. Niels Borregaard, Granulocyte Research Laboratory, Department of Medicine and Hematology L, University Hospital, Rigshospitalet afsnit 4042, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark.

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The resulting granulocyte-erythrocyte pellets were resuspended in ice-cold H_2O for 30 s to lyse erythrocytes. Isotonicity was subsequently restored with 1.8% NaCl. Granulocytes were pelleted at 200 *g* for 6 min, and resuspended in Krebs-Ringer phosphate: 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.

Isolation of bone marrow cells. 10 ml bone marrow was aspirated under local anesthesia from the superior posterior iliac crest and immediately anticoagulated by adding 100 U/ml heparin. Mononuclear cells and polymorphonuclear cells were separated by centrifugation on Lymphoprep.

Biosynthesis of albumin in bone marrow cells. 10^7 mononuclear bone marrow cells were resuspended in 2 ml leucine-free minimum essential medium (Eagle) (Gibco Laboratories, Life Technologies Ltd., Grand Island, NY) containing 10% dialyzed fetal calf serum (Gibco Laboratories) and incubated at 37°C for 60 min. This was followed by adding 25 μCi of L-[^{14}C] leucine/ml (NEN DuPont, Boston, MA). After additional 2 h incubation, the cells were washed twice in saline and resuspended in RIPA buffer: 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 100 μg/ml leupeptin, 100 μg/ml pepstatin, 100 KIE/ml aprotinin, 30 mM Hepes, pH 7.3, and left on ice for 30 min. Insoluble material was pelleted by centrifugation 220,000 *g* for 12 min in an Airfuge (Beckman Instruments Co., Palo Alto, CA). The supernatant was incubated for 1 h at 4°C with 20 μl rabbit anti-albumin (A 001; Dakopatts A/S, Glostrup, Denmark), after which 40 μl Protein A-Sepharose (50 mg/ml) (Pharmacia-LKB) was added. The sample was then rotated end over end for 1 h after which the Protein A-Sepharose was sedimented by centrifugation and washed three times in RIPA buffer, once in 150 mM NaCl, 10 mM Tris pH 6.8, and finally once in 50 mM Tris pH 6.8. The pellet was then resuspended in 50 μl H_2O, mixed with 75 μl electrophoresis sample buffer, boiled for 5 min, and subjected to SDS-PAGE on a 5–20% gradient gel under reducing conditions (15). The gels were fixed in 7.5% acetic acid, 40% ethanol for 1 h, submerged in Amplify (Amersham International, Amersham, UK) for 1 h, dried, and mounted on Kodak X-Omat S with an intensifying screen. The films were developed after exposure for 1–2 wk at −80°C.

Subcellular fractionation. Neutrophils were resuspended in KRP buffer containing 5 mM diisopropyl fluorophosphate (Aldrich Chemical Co., Inc., Milwaukee, WI) and kept on ice for 10 min. After centrifugation at 200 *g* for 10 min, the cells were resuspended in 13 ml disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM $ATPNa_2$, 3.5 mM $MgCl_2$, 10 mM Piperazine *N,N′*-bis2[ethane-sulfonic acid] [Pipes],[1] pH 7.2) containing 0.5 mM PMSF and disrupted by nitrogen cavitation as described (4). Nuclei and intact cells were sedimented by centrifugation at 400 *g* for 15 min (P_1) and 10 ml of the postnuclear supernatant (S_1) was applied on top of a 28-ml two-layer Percoll density gradient (1.05/1.12 g/ml) containing 0.5 mM PMSF and centrifuged as described (4). This resulted in generation of four separate regions that could be identified visually. The bottom band (α-band) containing azurophil granules, the intermediate band (β-band) containing specific granules, the top band (γ-band) containing plasma membranes and secretory vesicles, and the clear supernatant (S_2) containing cytosol.

Azurophil granules were identified by myeloperoxidase (16) that was quantitated by spectral analysis using an absorption coefficient of the 472-nm peak of 75 $mM^{-1}cm^{-1}$ (17). Specific granules were identified by vitamin B_{12}-binding protein (18), which was measured as described by Gottlieb et al. (19), as detailed in reference 4.

Plasma membranes were identified by HLA class I, assayed by a mixed ELISA (Melisa) (20), or by alkaline phosphatase measured in the absence of detergent (8, 11). Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) as substrate in a 1 mM $MgCl_2$, 50 mM sodium barbital buffer, pH 10.5 (21). Secretory vesicles were identified by alkaline phosphatase that could only be measured in the presence of 0.2% Triton X-100 (latent alkaline phosphatase) (8, 11).

Immunoblotting. Protein was transferred from SDS-polyacrylamide slabs to 0.2-μm nitrocellulose filters (Bio-Rad Laboratories, Richmond, CA) essentially as described by Towbin et al. (22), in 192 mM glycine, 25 mM Tris pH 8.3, 20% (vol/vol) methanol, using a trans-blot vertical system (Bio-Rad Laboratories). After transfer, additional protein binding sites were blocked by incubation in 2% Tween-20 in PBS for 30 min. Primary antibodies were applied in PBS containing 0.05% Tween and recognized by peroxidase-conjugated swine anti-rabbit antibody (P217 Dakopatts). The nitrocellulose filters were washed in 50 mM Tris, pH 7.6, and developed in the same buffer containing 10 mg/ml DAB-chromogen (Dakopatts) and 0.03% H_2O_2.

RNA extraction and Northern blotting. Human liver tissue was obtained from autopsies and stored at −80°C until RNA extraction. RNA was extracted from liver and leukocytes by the single-step acid guanidinium thiocyanate-phenol-chloroform method (23). The concentration of RNA was determined by spectrophotometry. RNA samples were electrophoresed through a denaturing 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (GeneScreen Plus; NEN DuPont) in 10× standard saline citrate (SSC) (1× SSC is 150 mM sodium chloride and 15 mM sodium citrate). Radiolabeled probes were prepared by the random priming method (24) using [α-^{32}P]dCTP and a commercial kit (both from Amersham International). The human serum albumin (HSA) probe was a 1.9-kb EcoRI-HindIII cDNA fragment of the cloned human albumin gene (25) containing the entire coding sequence of human albumin, and the human β-actin probe was a 2.1-BamHI fragment of the plasmid pHFβA-1 (26). Membranes were prehybridized at 42°C in 50% formamide, 1% SDS, 1 M NaCl, 5% dextran sulfate, and 100 μg/ml denatured salmon testes DNA, and hybridized to the denatured probes for 18–20 h in the same buffer. Maximal washing stringency was 65°C in 2× SSC, 1% SDS for 1 h. Membranes were exposed to x-ray film at −80°C with an intensifying screen for 1–3 d.

Purification of 67-kD protein. Neutrophils were isolated as described above from 10 U of freshly prepared buffy coats and resuspended in KRP at a concentration of 3×10^7 cells/ml at 37°C on a shaking waterbath. After 5 min, 10^{-8} M FMLP was added from a 1 mM stock in ethanol. After additional 15 min, the cells were pelleted by centrifugation and the supernatant was lyophilized after dialysis against H_2O and centrifugation at 10,000 *g* for 10 min. The material was resuspended in 10 ml 16.5% polyethylene glycol 6000 (E. Merck, Darmstadt, Germany), 5 mM Pipes, pH 7.0, and rotated end over end for 1 h at 4°C. The precipitate was collected by centrifugation at 10,000 *g* for 10 min and the supernatant, enriched in 67-kD protein, was

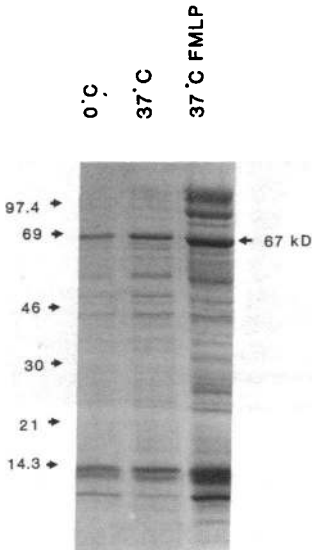

Figure 1. SDS-PAGE protein profile of material exocytosed in response to FMLP stimulation. Neutrophils, 3×10^7 cells/ml, were incubated in 11 ml KRP at 0°C or at 37°C in the absence or presence of 10^{-8} M FMLP. After 15 min, the cells were sedimented by centrifugation and the supernatants, S_0, were dialyzed against H_2O and concentrated by Speed-Vac to 0.5 ml. 50 μl of each supernatant was electrophoresed under reducing conditions on a 5–20% SDS-polyacrylamide gel with a 3% stacking gel

1. *Abbreviation used in this paper:* Pipes, piperazine *N, N′*-bis2-[ethane-sulfonic acid].

dialyzed overnight against 10 mM Tris, pH 9.0, and applied to a DE 52 column (Whatman International, Ltd., Maidstone, England) equilibrated in 10 mM Tris, pH 9.0 (bed vol 2.5 ml). The column was subsequently washed with 40 ml 20 mM Pipes, pH 6.0, and the 67-kD protein and a 92-kD protein were eluted with 150 mM NaCl, 20 mM Tris, pH 7.0. 2 ml-containing peak amounts of 67 kD protein as assessed by SDS-PAGE was applied to a Sephadex G-150 (Pharmacia-LKB) column (bed vol 300 ml) equilibrated in 10 mM Tris, 50 mM NaCl, pH 7.0 (flow rate 6 ml/h). Fractions containing peak amounts of 67 kD protein (~ 0.5 mg) were pooled, dialyzed against H_2O, lyophilized, and processed for NH_2-terminal amino acid sequencing.

Protein was determined using a kit (Bio-Rad Laboratories).

Sequence analysis. The amino acid sequence of the purified protein was determined using an automatic protein sequencer (475A; Applied Biosystems, Inc., Foster City, CA) equipped with an on-line HPLC system for detection of the amino acid phenylthiohydanthoin (PTH) derivatives. The PTH-derivatives were separated on a C18-DB column (5-7943; Supelco Inc., Supelco Park, PA). All chemicals and solvents were sequence- or HPLC-grade delivered by Applied Biosystems, Inc.

Amino acid analysis. The purified protein was hydrolyzed for 20 h in 6 N HCl gas phase at 110°C under vacuum in pyrolyzed tapered microvials (100 μl; Hewlett-Packard Co., Waldbronn, Germany). The hydrolysate was dried and redissolved in 8 μl of 0.4 M sodium borate, pH 10.4. Amino acid analysis was performed on 6 μl using a Aminoquant analyzer (Hewlett-Packard Co.) (27) (precolumn derivatization with *o*-phthaldialdehyde followed by 9-fluorenylmethylchloroformate, both reagents supplied by Hewlett-Packard Co.).

ELISA. Albumin was quantitated by an ELISA using a rabbit polyclonal antibody for catching (Dakopatts A 001) diluted 1:5,000 in 50 mM $Na_2CO_3/NaHCO_3$, pH 9.6. 100 μl was applied in each well (96 wells immunoplates; Nunc, Roskilde, Denmark) and incubated at room temperature overnight. 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% [vol/vol] Triton X-100). Additional binding sites were blocked by a 1-h incubation with 200 μl buffer B (500 mM NaCl, 3 mM KCl, 8 mM Na_2PO_4/KH_2PO_4, pH 7.2, 1% [vol/vol] Triton X-100, 1% [wt/vol] BSA [Sigma Chemical Co.]). After washing three times in buffer A, 100 μl samples were applied along with serial dilutions of a HSA (Sigma Chemical Co.) standard (250 ng/ml) and incubated for 1 h. All dilutions of samples and standard were made in buffer B. After three additional washes in buffer A, 100 μl of affinity purified, biotinylated (see below) rabbit anti-HSA antibody was applied from a stock (0.12 mg/ml) diluted 1:10,000 in buffer B and incubated for 1 h, followed by washing three times in buffer A. 100 μl peroxidase-conjugated avidin (Dakopatts P 347) diluted 1:2,000 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 was developed after 30 min incubation in sodium phosphate citric acid buffer containing 0.04% (wt/vol) *o*-phenylenediamine and 0.03% H_2O_2, and stopped by addition of 100 μl 1 M H_2SO_4. Absor-

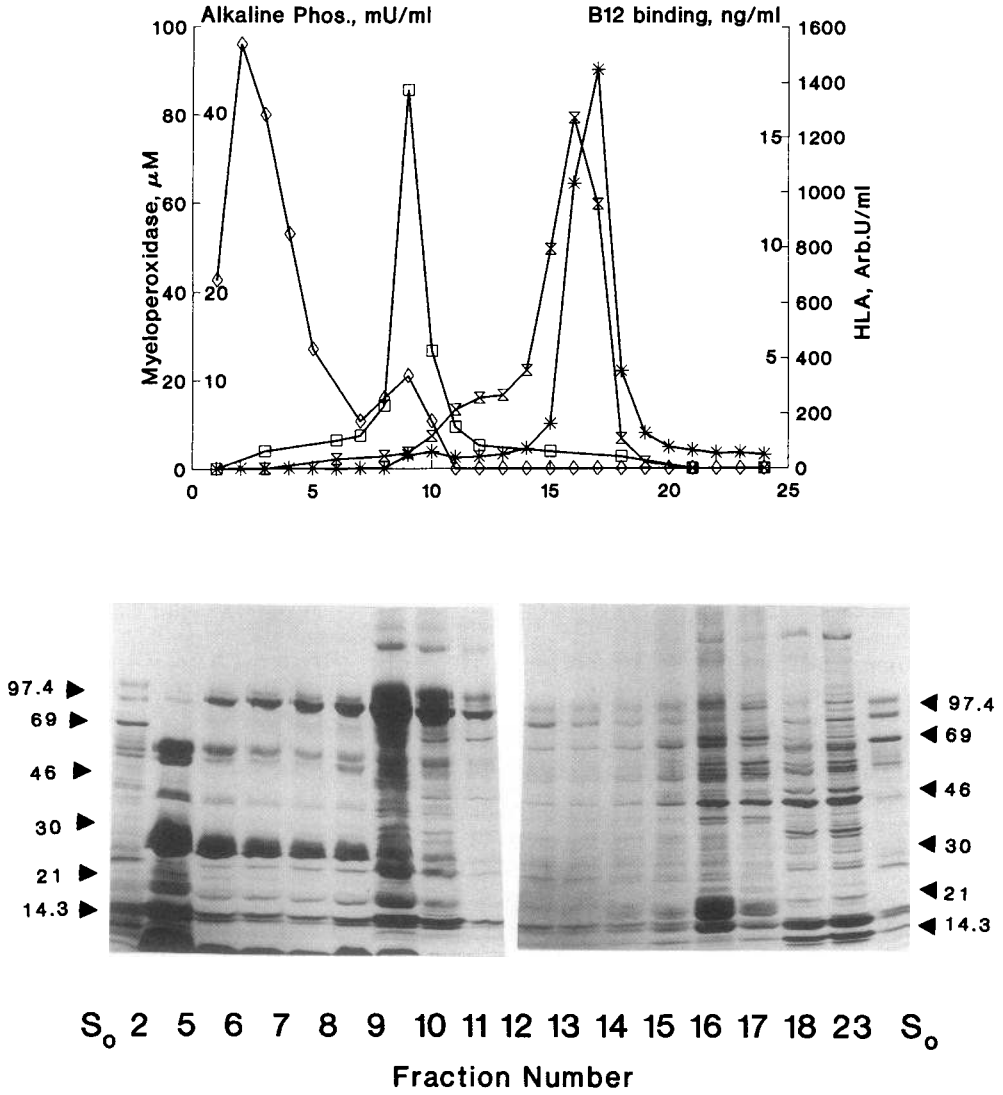

Figure 2. SDS-PAGE protein profile of subcellular fraction of unstimulated neutrophils. 5 × 10^8 neutrophils were cavitated in 13 ml disruption buffer. 10 ml of the postnuclear supernatant S_1 was applied on a 28-ml two-layer Percoll gradient and centrifuged. Fractions of 1.5 ml were collected from the bottom of the gradient. The profile of markers for azurophil granules (myeloperoxidase), specific granules (vitamin B_{12}-binding protein), secretory vesicles (latent alkaline phosphatase), and plasma membranes (HLA class I) is given on top. 400 μl of each fraction was centrifuged for 20 min in an Airfuge to pellet the Percoll. The biological material was collected as a disc, resuspended in 100 μl saline and mixed with 100 μl electrophoresis sample buffer. 150 μl from each fraction was applied to electrophoresis under reducing conditions in a 5–20% polyacrylamide slab gel with a 3% stacking gel (15). Myeloperoxidase, ◇; vitamin B_{12}-binding protein, □; latent alkaline phosphatase, ⧖; HLA class I, ★.

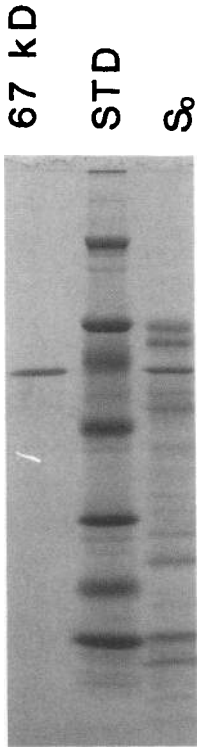

Figure 3. SDS-PAGE of purified 67 kD protein. A 5–20% SDS polyacrylamide gradient gel was run under reducing conditions with a 3% stacking gel. (Lane *A*) 3 μg of 67 kD protein purified from extracellular medium of FMLP-stimulated neutrophils from buffy-coat preparations; (Lane *B*) Molecular mass standards: 14.3 kD; 21 kD; 30 kD; 46 kD; 69 kD; 97.4 kD; 200 kD; (Lane *C*) 25 μg protein from S_0.

bance at 492 nm was read in a Multiscan Plus automatic ELISA reader (Labsystems, Helsinki, Finland).

Affinity purification and biotinylation. 16 mg rabbit anti-HSA (Dakopatts A 001) was dialyzed overnight in PBS and applied to a CNBr-activated Sepharose column (Pharmacia-LKB) to which 4 mg HSA had been coupled according to the manufacturer's instruction. After washing until the eluate displayed background absorbance at 280 nm, bound antibodies were eluted with 3 M KSCN in PBS. The protein peak, judged by 280 nm absorbance, was collected and dialyzed overnight against PBS for use in immunoelectron microscopy or against 100 mM Na_2HCO_3, 150 mM NaCl for biotinylation. Biotinylation was performed essentially as described (28).

Immunofluorescence studies on single cells. Isolated neutrophils were resuspended and fixed at 10^7 cells/ml in 2% paraformaldehyde, 0.1% glutaraldehyde in PBS for 10 min at 4°C. The cells were washed twice in PBS and permeabilized for 20 min with 0.2% Triton X-100 where appropriate. Fc receptors were blocked by incubating the cells in PBS containing 1 mg/ml human immunoglobulin (State Serum Institute, Copenhagen) for 1 h followed by washing. Labeling of cells with monoclonal antibodies was performed by incubating 100 μl cells 10^7/ml in PBS with 50 μl monoclonal antibody or murine preimmune serum for 1 h at 4°C. After washing twice in PBS by centrifugation, 50 μl fluorescein-conjugated rabbit anti-mouse antibody (Dakopatts) was added and the cells incubated for 30 min at 4°C. After two additional washes, the cells were resuspended in PBS and analyzed in a FACScan® (Becton-Dickinson Immunocytometry Sys., Mountain View, CA).

Table I. Amino Acid Analysis of the 67-kD Protein Compared with Human Albumin

Amino acid	Mole %	
	h-Albumin*	67 kD protein
Asx	9.9	9.8
Glx	15.1	15.1
Ser	4.2	4.2
His	2.9	3.1
Gly	2.2	4.1
Thr	5.3	4.8
Ala	11.5	10.4
Arg	4.0	4.8
Tyr	3.2	2.8
Val	7.1	6.8
Met	1.1	1.4
Ile	1.5	1.8
Phe	5.5	5.3
Leu	11.1	10.7
Lys	10.8	10.4
Pro	4.6	4.3

The analysis was performed on 6 pmol protein. Mole % is expressed as % of all amino acids except Cys and Trp, which were not determined.
* Reference 32.

Table II. Sequence Analysis of the NH_2-terminus of the 67-kD Protein Compared with Human Albumin

No.	Amino acid		Net pmol
	h-Albumin*	67 kDa protein	
1	Asp	?	—
2	Ala	Ala	228
3	His	His	75
4	Lys	Lys	258
5	Ser	?	—
6	Glu	Glu	172
7	Val	Val	224
8	Ala	Ala	242
9	His	His	90
10	Arg	Arg	202
11	Phe	Phe	191
12	Lys	Lys	234
13	Asp	Asp	158
14	Leu	Leu	136
15	Gly	Gly	126
16	Glu	Glu	107
17	Glu	Glu	102
18	Asn	Asn	132
19	Phe	Phe	151
20	Lys	Lys	132
21	Ala	Ala	86
22	Leu	Leu	110
23	Val	Val	102
24	Leu	Leu	74
25	Ile	Ile	116
26	Ala	Ala	142
27	Phe	Phe	126
28	Ala	Ala	135
29	Gln	Gln	101
30	Tyr	Tyr	94
31	Leu	Leu	151
32	Gln	Gln	116

The analysis was performed on 300 pmol protein. Net pmol is the yield of the PTH-derivative corrected for background and lag.
* Reference 32.

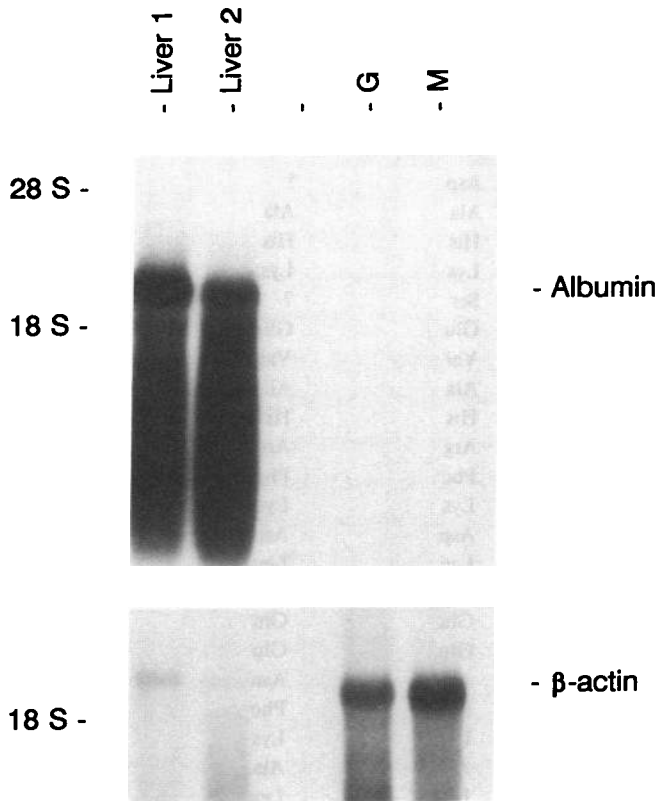

Figure 4. Northern blotting of albumin mRNA expression in human liver (positive control) and in granulocytes. 15 μg of total RNA was loaded per lane. The smearing seen in the liver sample is due to partial degradation of the RNA that was obtained from autopsies. The same blots were stripped and reprobed with a human β-actin specific probe in order to ascertain the quality of the RNA isolated from the granulocytes. Molecular weight was determined with reference to the 18 and 28S rRNA bands. (*G*) mature granulocytes isolated from bone marrow; (*M*) mononuclear cells isolated from bone marrow.

Immunostaining of semi- and ultrathin cryosections. Neutrophils, 10^7 cells/ml, were fixed at 4°C in 2% paraformaldehyde and 0.5% glutaraldehyde in PBS for 20 min, then washed in 0.1 M phosphate buffer, pH 7.3, resuspended for 20 min in 15% gelatine (E. Merck) in 0.1 M phosphate buffer at 37°C, and pelleted at 4,100 *g* for 2 min. The gelatine was hardened for 18 h at 4°C, and blocks (1 × 1 × 1 mm) of the pellet were cryoprotected by immersion for 45 min in 2.3 M sucrose with 1% paraformaldehyde in PBS before they were frozen in liquid nitrogen. Semithin and ultrathin cryosections were made on a RMC MT 6000 X-L and RMC MT 7CR 21 cryo-ultramicrotomes. Semithin sections were collected on glass slides, ultrathin sections on Formvar-coated nickel grids. A two-layer indirect immunostaining procedure was used for light and electron microscopy (29, 30). In short, sucrose was removed by washing, and free aldehyde groups were quenched with 0.02 M glycine. Primary antibody used for light microscopy was monoclonal anti-HSA (lot 128 F 4806; Sigma Chemical Co.) diluted 1:10, whereas primary antibody for immunoelectron microscopy was affinity-purified rabbit anti-HSA 10 μg/ml. Rabbit preimmune serum (10 μg/ml IgG) was used as control. After 60 min incubation with primary antibody at room temperature, sections were washed and exposed to secondary antibodies conjugated to colloidal gold for 60 min. For light microscopy goat anti-mouse IgG conjugated to 1 nm gold (AuroProbe One PRN 470-473; Amersham International) diluted 1:60, and for electron microscopy goat anti-rabbit IgG with 5 nm gold particles (AuroProbe EM GAR G5, RPN 420; Amersham International) diluted 1:50 were used. The antibodies were diluted in PBS containing 0.8% BSA, 0.1% fish skin gelatine (IGSS quality; Amersham International), and 1% goat serum (State Serum Institute). The sections were washed before and after incubation with antibodies in the same PBS diluent without goat serum. Semithin sections were examined by epipolarized light in an Olympus BH-2 light microscope after silver-enhancement of the gold labels for 25 min in the dark with a physical developer containing 14.2% gum arabic, 0.8% hydrochinone, 2.4% citric acid monohydride, 2.2% trisodium citrate dihydrate, and 0.7% silverlactate (29). Ultrathin sections were contrasted with uranyl acetate in methyl cellulose before they were examined in a JEOL 100 C electron microscope.

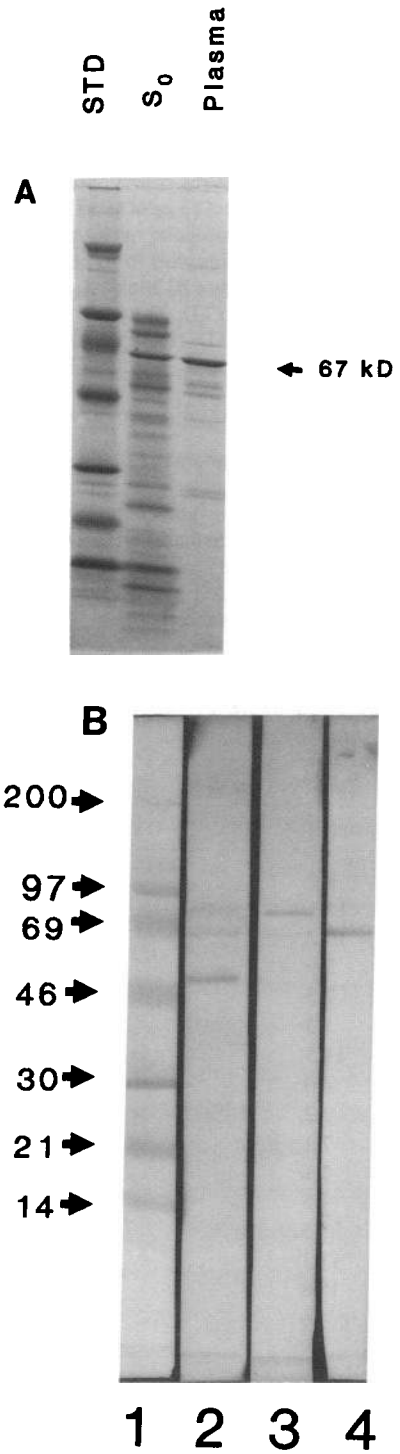

Figure 5. (*A*) SDS-PAGE protein profiles of plasma and material exocytosed from neutrophils stimulated by 10^{-8} M FMLP. 50 μg protein from S_0 (same as Fig. 1) and 12.5 μg normal human plasma were electrophoresed under reducing conditions on a 5–20% SDS-polyacrylamide gradient gel with 3% stacking gel. (*B*) Western blots of S_0. S_0 was transferred from SDS-PAGE on 5–20% gradient gels to nitrocellulose membranes. (Lane *1*) MW standards: 14.3 kD; 21 kD; 30 kD; 46 kD; 69 kD; 97.4 kD; 200 kD; (lane *2*) 2.25 μg protein applied and probed with rabbit anti-IgG (Dakopatts A 423); (lane *3*) 2.25 μg protein applied and probed with rabbit anti-transferrin (Dakopatts A 061); (lane *4*) 0.34 μg protein applied and probed with rabbit anti-albumin (Dakopatts A001).

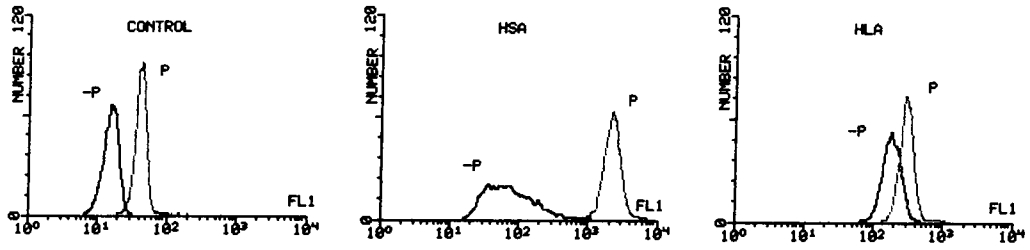

Figure 6. Detection of intracellular albumin by immunofluorescence of single cells. Neutrophils were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde. Half the cells (*P*) were permeabilized with 0.2% Triton X-100, and the other half (−*P*) left unpermeabilized. Both were labeled with either murine preimmune IgG (Control) (mouse IgG, diluted 1:49; Becton-Dickinson Immunocytometry), monoclonal anti-human albumin (HSA) (lot 128F-4806 diluted 1:20; Sigma Chemical Co.) or monoclonal anti-HLA (M736 diluted 1:20; Dakopatts A001). Panels are histograms with log fluorescence on abscissa and cell number on ordinate. Mean fluorescence values: (*Control* −*P*) 16; (*Control P*) 42; (*HSA* −*P*) 99; (*HSA P*) 2212; (*HLA* −*P*) 204; (*HLA P*) 341.

Quantitation of FITC-Dextran. Fluorescence of FITC-Dextran 70 (Sigma Chemical Co.) was measured in a Scanning Fluorescence spectrophotometer (F-4000, excitation 485 nm, emission 515 nm; Hitachi Ltd., Tokyo, Japan).

Results

Proteins released by FMLP stimulation. The protein profile of concentrated extracellular medium (S_0) from control cells and FMLP-stimulated cells is shown in Fig. 1. It is apparent that a variety of different proteins are secreted by merely incubating the cells at 37°C, but much more is released after stimulation by FMLP. Some of these proteins may be released from their membrane anchor, e.g., Fc-receptor III (31), others exocytosed from intracellular stores, such as secretory vesicles and specific granules. To discriminate between these possibilities, subcellular fractionation of unstimulated cells was performed to localize the source of exocytosed proteins. The SDS-PAGE profile (Fig. 2) indicated that a 67-kD protein qualify as a potential marker for secretory vesicles by the criteria that the protein should be present in fractions of unstimulated neutrophils known to contain latent alkaline phosphatase, the protein should not be present in significant amounts in specific or azurophil granules, and the protein should be a prominent band in S_0 from FMLP-stimulated neutrophils (Fig. 1).

Identification of the 67-kD protein. The 67-kD protein was purified to apparent homogeneity from S_0 from FMLP-stimulated neutrophils, as shown in Fig. 3. Briefly, concentrated S_0 was precipitated by 16.5% polyethylene glycol. The supernatant that contained the majority of the 67-kD protein together with minor small molecular weight proteins and a prominent 92-kD protein was applied to anion exchange chromatography (DE 52) and eluted in 150 mM NaCl, 20 mM Tris, pH 7.0. The eluate contained two major bands of 67 and 92 kD. These were separated on Sephadex G-150. The sequence of the first 32 NH_2-terminal amino acids of the purified protein was determined, and analysis of the amino acid composition was performed. As shown in Table I and II, the 67-kD protein is identical to albumin (32).

Origin of albumin. To determine whether albumin had been taken up from plasma or synthesized, like other granule proteins, a cDNA probe was used to detect mRNA for human albumin in both mononuclear and polymorphonuclear bone marrow cells. No mRNA could be detected, as shown in Fig. 4. Likewise, incubation of mononuclear bone marrow cells with [^{14}C]leucine did not result in incorporation of radioactivity in albumin when subsequently probed by immunoprecipitation with monoclonal antibody or polyclonal antibodies to human albumin (data not shown). This indicates that albumin is incorporated into neutrophils or their precursors by an endocytic process. To determine whether this is specific for uptake of albumin, we compared the protein profile of plasma and concentrated S_0 (Fig. 5 *A*). It is evident that most bands identified in plasma can also be observed in S_0. Immunoblotting demonstrated the presence of IgG, transferrin, and albumin in S_0 (Fig. 5 *B*). In addition, S_0 contains a few prominent bands not ob-

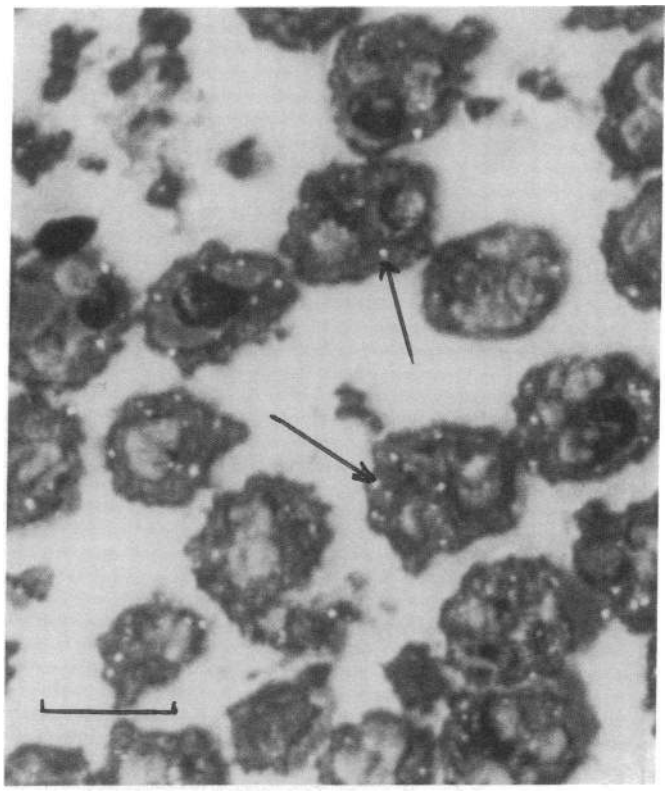

Figure 7. Epipolarized light micrograph of fixed, cryosectioned neutrophils incubated with mouse monoclonal anti-HSA as primary antibody and 1 nm gold-conjugated rabbit anti-mouse as secondary antibody. The gold labeling was silver enhanced. When viewed in epipolarized light the silver is seen as bright spots. These show that immunolabeling for albumin is confined to granule-like structures scattered in the cytoplasm (*arrows*). ×1,800. Bar, 10 μm.

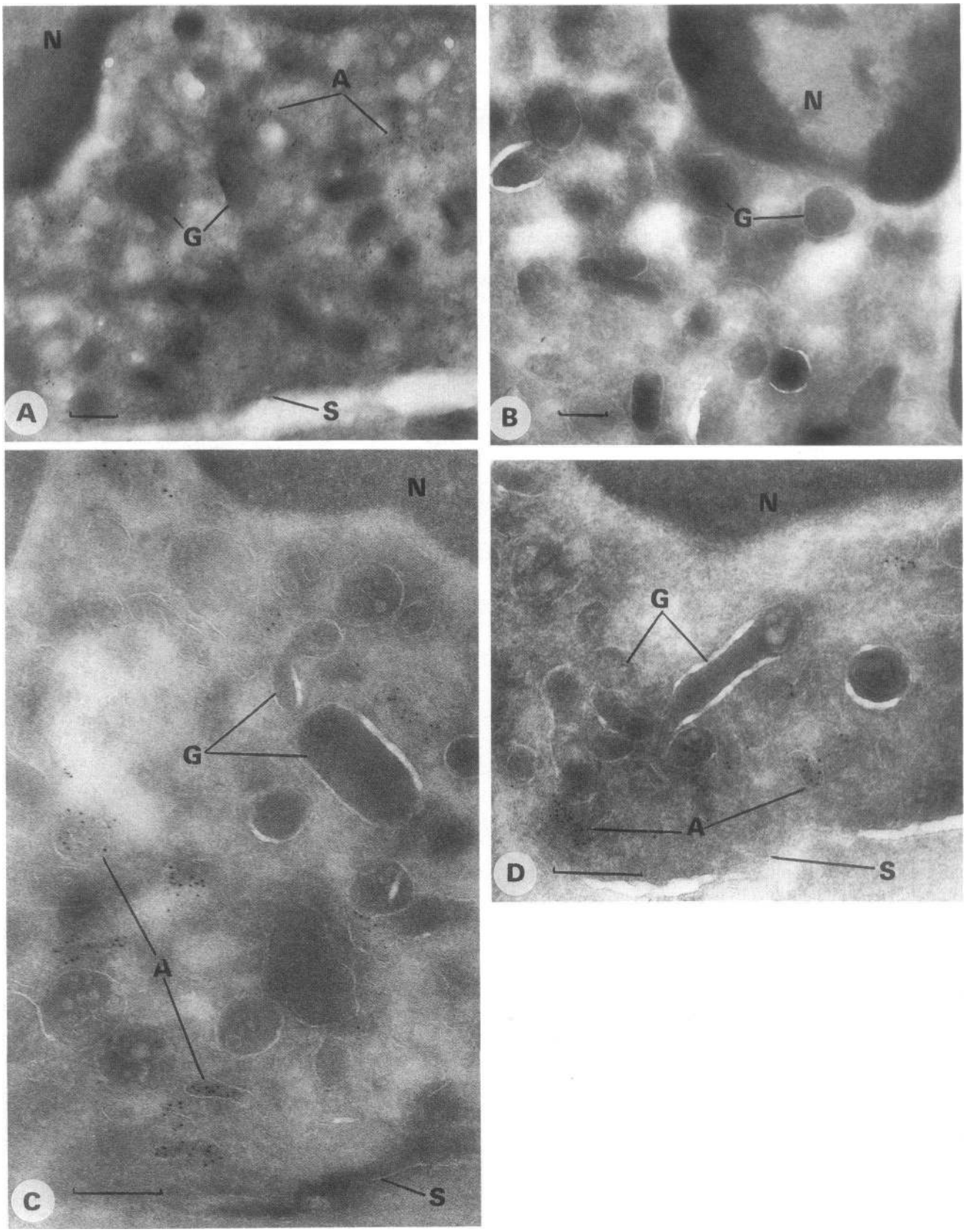

Figure 8. (*A–D*) Immunogold electron microscopy of fixed ultrathin cryosectioned neutrophils, incubated with affinity-purified rabbit anti-HSA and 5 nm gold-conjugated anti-rabbit IgG (*A, C, D*), or, as control, preimmune rabbit serum before immunogold (*B*). At lower magnification immunolabeling for albumin (*A*) is present as clusters of gold particles (*A*) in the cytoplasm between the neutrophil granules (*G*). No labeling is observed when preimmune serum is used (*B*). At higher magnification (*C, D*) it is evident that albumin labeling (*A*) is confined to membrane-limited structures distinct from specific and azurophil granules (*G*). *N* denotes the nucleus, *S* the cell surface membrane. *A, B,* ×34,000; *C, D,* ×65.000. Bar, 250 nm.

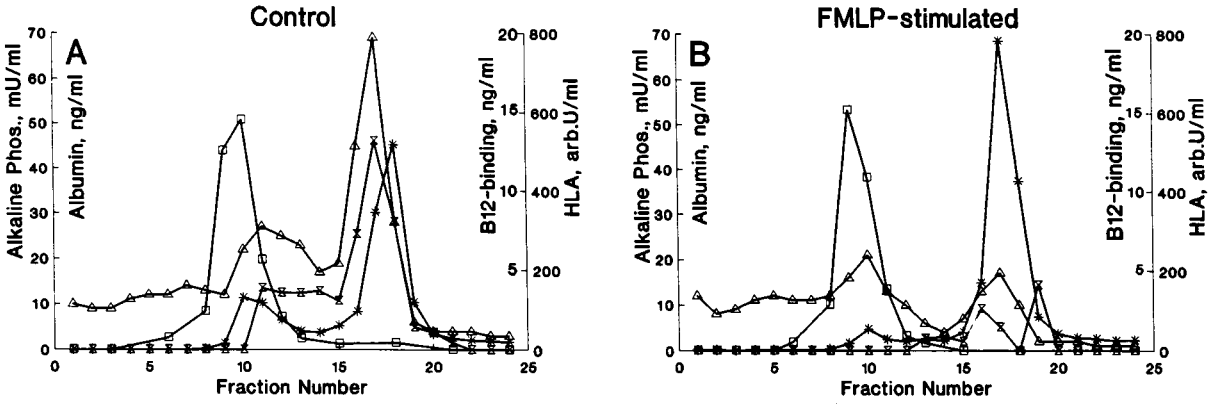

Figure 9. Subcellular localization of albumin in unperturbed and FMLP-stimulated neutrophils. 6×10^8 neutrophils were incubated at 3×10^7 cells/ml in KRP. One half was kept at 0°C, the other half incubated at 37°C in the presence of 10^{-8} M FMLP. After 15 min incubation, the cells were pelleted by centrifugation, disrupted by nitrogen cavitation, and fractionated on two-layer Percoll gradients. *A* is control cells kept at 0°C. *B* is FMLP-stimulated cells. Vitamin B_{12}-binding protein, □; Latent alkaline phosphatase, ⧖; Albumin, △; HLA, ★.

served in plasma when comparable amounts of protein are applied. Most of these bands seem to represent proteins from specific granules, as observed in Fig. 2.

Subcellular localization of albumin. Since albumin was the dominating plasma-related protein released in S_0 by FMLP treatment, we chose to study the localization and release of albumin in more detail. To verify the intracellular localization of albumin in normal neutrophils, immunofluorescence studies were performed on fixed neutrophils before and after permeabilization. As shown in Fig. 6, permeabilization of neutrophils resulted in increased fluorescence of anti-albumin antibodies, indicating that intracellularly localized antigen was made available to the antibody. As control, no significantly increased fluorescence was observed when an antibody to HLA class I antigen was used as a marker for the plasma membrane. Epipolarized light microscopy of immunogold-silver stained cryosections (Fig. 7) showed labeling for albumin in scattered cytoplasmic granule-like structures with no apparent preference for any part of the cell. Immunoelectron microscopy of ultrathin cryosections (Fig. 8) demonstrated labeling for albumin in distinct intracellular membrane-limited organelles, quite similar to the alkaline phosphatase containing secretory vesicles that have recently been morphologically described (33). This was confirmed by subcellular fractionation of cavitated neutrophils on two-layer Percoll gradients. Fig. 9 shows that the major part of albumin co-localizes with latent alkaline phosphatase and is separate from the plasma membrane marker HLA. Since plasma membrane vesicles and secretory vesicles have very similar density, their peaks are only separated by one fraction. This was, however, invariably observed in all of four experiments of which the experiment in Fig. 9 is typical, and can also be observed in Fig. 2. After stimulation with FMLP, this pool of albumin is exocytosed. It should be noted that although the exocytosis of secretory vesicles and the associated albumin pool is almost complete, only minimal exocytosis of specific granules occurs. This is illustrated by Fig. 10, which gives the release of albumin and the specific granule marker vitamin B_{12}-binding protein along with upregulation of alkaline phosphatase in response to a range of concentrations of FMLP, platelet activating factor (PAF), and LTB_4.

Endocytosis of FITC-dextran. Unstimulated neutrophils have been reported to take up albumin and FITC-dextran by fluid phase endocytosis to varying degrees (34–36). This process is reported to be accelerated by FMLP stimulation (35). Fig. 11 shows that FMLP stimulation results in increased cell association of FITC-dextran. However, the majority of the FITC-dextran is not sedimented by centrifugation and is found on top of the density gradient that contains cytosol and extracellular buffer. Since FITC-dextran does not enter the cytosol, this indicates that the FITC-dextran, albeit cell associated, is released to the external medium when the cells are disrupted. Thus, the FITC-dextran is either released from phagocytic vacuoles that all break during disruption of cells. This is less likely, since no significant breaking of any other organelle is observed during nitrogen cavitation. Alternatively, the cell-associated FITC-dextran is localized extracellularly, and therefore released when cells are disrupted.

In addition to the FITC-dextran on top of the gradient, a smaller peak of FITC-dextran is associated with more dense structures deep in the gradient. It should be noted that the fluid phase endocytosis marker did not concentrate in fractions either in unstimulated cells or in stimulated cells that contain latent alkaline phosphatase.

Discussion

Normal neutrophils were shown to contain albumin in intracellular stores that are easily mobilized by weak secretagogues. The fact that small amounts of albumin was released by incubating cells in the absence of stimulus probably reflects the high sensitivity to exocytosis of the albumin-containing vesicles. In this respect, the kinetics of exocytosis of albumin is similar to the kinetics of upregulation of CR-1 and CR-3 in response to isolation and warming of isolated neutrophils (7), as also previously noted for secretory vesicles (11). Judged by the protein profile of exocytosed material and by our previous demonstration of similar intracellular localization of another plasma protein, tetranectin (8), albumin seems to be associated with most other plasma proteins.

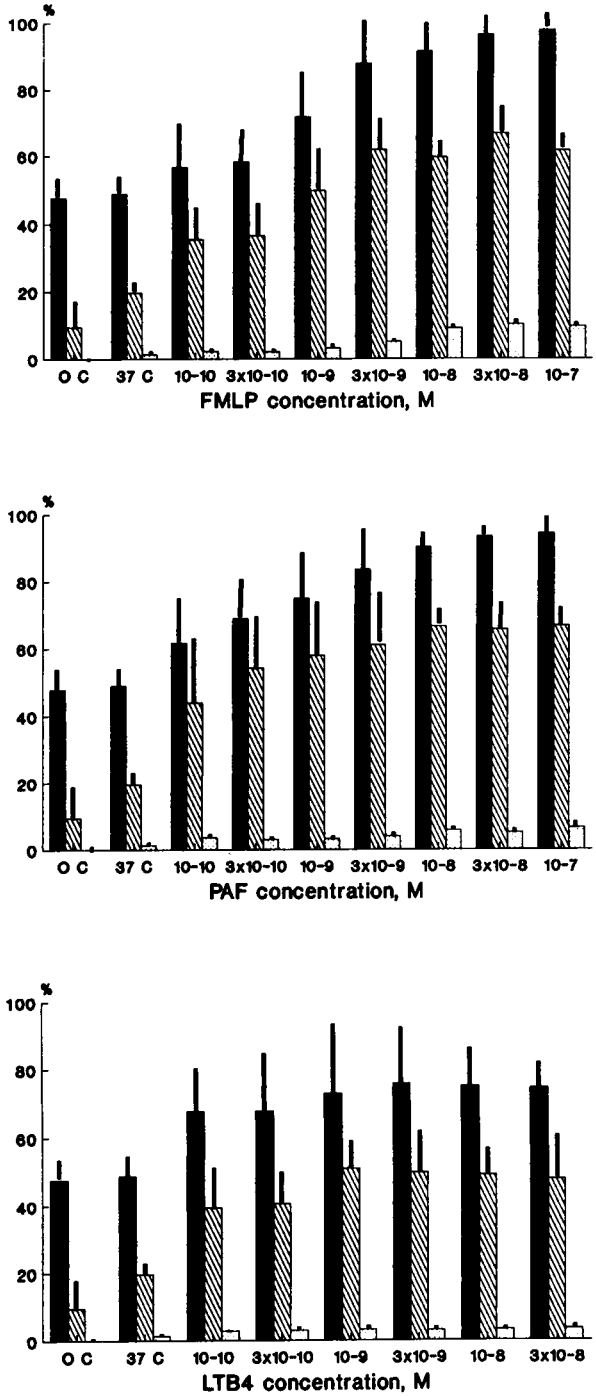

Figure 10. Exocytosis of albumin in response to stimulation with inflammatory mediators. Neutrophils, 3×10^7/ml in KRP, were incubated at 37°C for 20 min with additions as indicated. Control cells were incubated either on ice or at 37°C. The incubation was terminated by adding 2 vol ice-cold KRP. Cells were sedimented by centrifugation 200 *g* for 10 min and resuspended to their original volume. Release was expressed as content in supernatant as percentage of content in resuspended cells plus supernatant. Alkaline phosphatase was measured in the presence and absence of 0.2% Triton X-100 and expressed as activity in the absence in percentage of activity in the presence of detergent. Results are mean of three independent experiments (standard deviation is given as |). FMLP was added from a 10^{-3} M stock in ethanol. LTB_4 (Sigma Chemical Co.) was added from a 10^{-3} M stock in ethanol. PAF (Sigma Chemical Co.) was added from a 10^{-3} M stock in PBS. Closed bar is alkaline phosphatase. Hatched bar is albumin. Stippled bar is vitamin B_{12} binding protein.

Our previous work on secretory vesicles (8, 11, 13), and the fact that we are able to separate plasma membrane vesicles from secretory vesicles by free-flow electrophoresis (37) fully support that secretory vesicles are intracellular structures that fuse with the plasma membrane after stimulation. The findings that albumin co-localizes with secretory vesicles on subcellular fractionation and is exocytosed in parallel with exocytosis of secretory vesicles strongly indicate that albumin is localized in secretory vesicles. Since biosynthesis of albumin was not demonstrated, the plasma protein must have been taken up by an endocytic process. This points to the endocytic origin of secretory vesicles. It is therefore not surprising that secretory vesicles contain alkaline phosphatase and cytochrome b_{558}, and probably several other membrane proteins also found in the plasma membrane (8, 11, 13). However, the membrane of secretory vesicles does not contain HLA class I antigen (20). Thus, some segregation of membrane proteins must take place during formation of secretory vesicles.

A recent study has shown uptake of albumin into a tubular system and multivesicular bodies in FMLP-stimulated neutrophils. The membrane of these structures was shown to contain CR1 that originated from small intracellular vesicles, distinct from specific and azurophil granules, before stimulation (38). The ultrastructure of secretory vesicles has recently been described as alkaline phosphatase containing intracellular vesicles that fuse into a tubular system that communicates with the plasma membrane after FMLP stimulation (33). It is therefore likely that the fluid phase markers whose uptake has been claimed to be increased by FMLP stimulation, although apparently located in intracellular vesicles, are associated with this tubular system, and therefore still extracellular. Our studies on subcellular localization of FITC-dextran support this notion. It is, furthermore, evident that stimulation with FMLP does not result in generation of endocytic vesicles containing latent alkaline phosphatase. Thus, the stimulated release of serum proteins and exocytosis of secretory vesicles cannot be interpreted as a consequence of an acceleration of a classical endocytosis-exocytosis cycle.

The finding in neutrophils of a stimulus-dependent extensive release of vesicles of endocytic origin bears striking similarity to α-granules of platelets that contain plasma proteins that are endocytosed by megakaryocytes (39, 40). Some endocytic vesicles in neutrophils undoubtedly represent the traditional constitutive pinocytotic communication between the pericellular environment, lysosomes, and Golgi (41). The small part of intracellular albumin not released by FMLP may be located here where we also find the FITC-dextran that can be sedimented by centrifugation (Figs. 9 and 11), but a significant part of endocytic vesicles in human neutrophils, as defined by their content of plasma proteins, represent unique mobilizable secretory vesicles that are lost after stimulation by weak secretagogues, and not regained within the time frame of formation of normal endocytic vesicles (15–45 min) (42). It should be noted that no proteolytic modification of albumin could be detected as a consequence of endocytosis.

Important questions to be answered about endocytic secretory vesicles are: when during myelopoiesis are these vesicles formed, in the bone marrow like α-granules of platelets or in the circulation? Do secretory vesicles play a role for the egress of mature neutrophils from the bone marrow and/or for margination of neutrophils in blood vessels by regulating the content of adhesion proteins in the plasma membrane? Has the

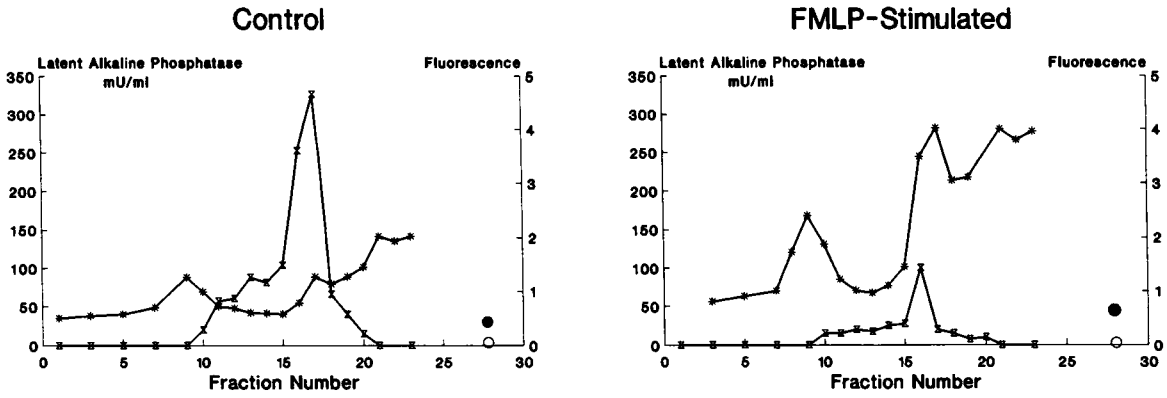

Figure 11. Subcellular localization of cell-associated FITC-dextran. 1.4×10^9 neutrophils were incubated at 5×10^7 cells/ml in KRP at 37°C in the presence of 5 mg/ml FITC-dextran. After 5 min the sample was divided, 10^{-8} M FMLP (Sigma Chemical Co.) was added to one half, the other served as control. After additional 15 min the incubation was stopped by dilution with 2 vol ice-cold KRP and the cells pelleted by centrifugation. The supernatants were siphoned off and the pellets resuspended in 10 ml ice-cold KRP. This was repeated four times. The pellets were finally resuspended in 13 ml relaxation buffer and subjected to subcellular fractionation. Each fraction was analyzed for alkaline phosphatase in the presence or absence of 0.2% Triton X-100. The difference is given as latent alkaline phosphatase Σ; 500 μl was removed from each fraction, 0.2% Triton X-100 was added, and Percoll was removed by centrifugation 150,000 *g* for 20 min in an Air-fuge (Beckman Instrs.). The supernants were diluted with 2 vol PBS and fluorescence activity measured (arb. units): (*) Fluorescence of relaxation buffer containing 0.2% Triton X-100 diluted with 2 vol PBS; (○) Fluorescence of supernatant from the last wash before cavitation diluted with 2 vol PBS containing 0.13% Triton X-100; (●) Results of one of two essentially similar experiments are shown.

existence of stimulus-dependent mobilizable endocytic vesicles been overlooked in other cells, like fibroblasts, endothelial cells, and macrophages? The fact that the plasma protein tetranectin has been demonstrated in all such cells (12) indicates that this may be the case.

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