Adhesomes: Specific Granules Containing Receptors for Laminin, C3bi/Fibrinogen, Fibronectin, and Vitronectin in Human Polymorphonuclear Leukocytes and Monocytes

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Abstract. We have localized several major extracellular matrix protein receptors in the specific granules of human polymorphonuclear (PMN) and monocytic leukocytes using double label immunoelectron microscopy (IEM) with ultrathin frozen sections and colloidal-gold conjugates. Rabbit antibodies to 67-kD human laminin receptor (LNR) were located on the inner surface of the specific granule membrane and within its internal matrix. LNR antigens co-distributed with lactoferrin, a marker of specific granules, but did not co-localize with elastase in azurophilic granules of PMNs. Further, CD11b/CD18 (leukocyte receptor for C3bi, fibrinogen, endothelial cells, and endotoxin), mammalian fibronectin receptor (FNR), and vitronectin receptor (VNR) antigens were also co-localized with LNR in PMN specific granules. A similar type of granule was found in monocytes which stained for LNR, FNR, VNR, CD18, and lysozyme. Activation of PMNs with either PMA, f-met-leu-phe (fMLP), tumor necrosis factor (TNF), or monocytic leukocytes with lipopolysaccharide (LPS), induced fusion of specific granules with the cell membrane and expression of both LNR and CD18 antigens on the outer cell surface. Further, stimulation led to augmented PMN adhesion on LN substrata, and six- to eightfold increases in specific binding of soluble LN that was inhibited by LNR antibody. These results indicate that four types of extracellular matrix receptors are located in leukocyte specific granules, and suggest that up-regulation of these receptors during inflammation may mediate leukocyte adhesion and extravasation. We have thus termed leukocyte specific granules adhesomes.

EXTRAVASATION of polymorphonuclear leukocytes (PMNs)[1] and monocytes and their entrance into inflammatory tissue sites requires that they adhere to and penetrate the capillary endothelium, the basement membrane, and the stromal connective tissue (14, 42, 60). The hematogenous spread of metastatic cells occurs via a similar mechanism (3, 32). Certain cell surface receptors for extracellular ligands play central roles in these adhesion-dependent processes, and the molecular mechanisms underlying these interactions are being elucidated (27, 36, 48, 49).

The dominant adhesive ligand of basement membranes is laminin (LN) (36), whereas fibronectin (FN) and vitronectin (VN) are attachment ligands of the connective tissue stroma (23, 48, 49); other important adhesive molecules such as ICAM-1 and ELAM-1 are expressed on the endothelial cell surface (8, 9, 13, 35). Several types of laminin receptors (LNRs) have been described. A LNR with a molecular mass of 67 kD has been identified in neoplastic cells, PMNs, macrophages, and myocytes (25, 31, 37, 46, 60, 61, 68, 78). This receptor consists of a single polypeptide exhibiting saturable LN binding of moderate affinity (K_d ~nM) (2, 25, 46). Additional LN-binding proteins include 180 kD and 110–120 kD species found in neural and epithelial cells (30, 58), and several recently described integrins with LN-binding activity (20, 24, 28, 59, 63). Of these LNRs, 67-kD LNR appears to be the dominant laminin binding protein in PMNs and macrophages (25, 37, 78). Surface expression of 67-kD LNR in PMNs is elevated by inflammatory agonists such as PMA or f-met-leu-phe (fMLP) (78); high levels of 67-kD LNR are also expressed in oncogenically transformed cells (3, 21, 68, 79), suggesting that increased adhesion to basement membranes occurs during diapedesis.

Receptors for FN and VN are integrins; a superfamily of heterodimeric cell surface receptors composed of closely similar or identical beta subunits and alpha subunits that are apparently unique for each receptor (27, 49). Integrins include a subgroup of receptors which mediate leukocyte aggregation and adhesion to vascular endothelial cells: Mac-1, LFA-1, and p150,95 (1, 22, 29, 51, 72). Their specific alpha subunits have been defined as CD11a (LFA-1), CD11b (Mac-1), and CD11c (p150,95), and each receptor contains an

1. *Abbreviations used in this paper*: ECMR, extracellular matrix receptor; fMLP, f-met-leu-phe; FN, fibronectin; FNR, fibronectin receptor; IEM, immunoelectron microscopy; LN, laminin; LNR, laminin receptor; LPS, lipopolysaccharide; PMN, polymorphonuclear; TNF, tumor necrosis factor; VN, vitronectin; VNR, vitronectin receptor.

identical beta subunit: CD 18 (27, 29). LFA binds ICAM-1, while ligands for Mac-1 include C3bi, fibrinogen, and endotoxin (35, 75–77). As with LNR, inflammatory stimuli induce rapid expression of Mac-1 and p150,95 on the granulocyte surface (22, 38, 62, 64).

Although the molecular mechanisms of leukocyte interaction with endothelial cells, basement membranes, and stromal connective tissue are being elucidated, little high resolution structural data is available on the subcellular distribution of relevant adhesive receptors. Further, the effects of several different physiological inflammatory mediators such as fMLP, tumor necrosis factor (TNF), and bacterial lipopolysaccharide (LPS) upon the expression and/or subcellular redistribution of these receptors has not been examined extensively at the ultrastructural level. In this study, dual-label immunoelectron microscopy (IEM) was used to determine the distribution of 67-kD LNR, CD18, fibronectin receptor (FNR), and vitronectin receptor (VNR) relative to known subcellular markers in resting and activated PMNs and monocytes. We report that all four adhesion receptors are located in the leukocyte specific granule which becomes translocated to the cell surface after activation; specific granules have thus been termed "adhesomes."

Materials and Methods

Preparation and Activation of Human Leukocytes

Peripheral blood was obtained from normal human volunteers and PMNs isolated on discontinuous Ficoll-Hypaque density gradients (Pharmacia Fine Chemicals, Piscataway, NJ). The cells were 98% pure (contaminated with eosinophils detected by electron microscopy) and 99% viable (determined by lactate dehydrogenase liberation into the medium [26]). After washing with ice-cold calcium/magnesium-free Hanks' balanced salt solution (HBSS) buffered with 20 mM Hepes (pH 7.2), PMNs were activated by various agents. For fMLP, PMNs were suspended at 2×10^6 cells/ml and preincubated with 5 μg/ml cytochalasin *D* in calcium/magnesium containing HBSS for 10 min followed by washing and treatment with 1 μM fMLP for 20 min at 37°C. At this point the cells were either washed with HBSS for fixation (see below), or with binding buffer (HBSS containing 2 mM calcium [magnesium-free] and 2% human serum albumin [New York Blood Center, New York]) for LN binding experiments. Similarly, PMNs were activated with 50 or 500 ng/ml PMA (0.1–1.3 μM) or with ionophore A23187 (1.0 μM) for 20 min. A recombinant form of human TNF alpha (rTNF) (Genzyme Corp., Boston, MA) was used to activate either suspended PMNs, or cells plated (1×10^7 PMNs per dish) into 90-mm-diam tissue culture dishes (No. 25020; Corning Glass Works, Corning, NY) coated with purified murine LN for 1 h. The degree of PMN activation was determined by measuring the amount of myeloperoxidase or vitamin B_{12} binding activity liberated, respectively, from azurophilic or specific granules as previously described (34, 47), and possible cell lysis was quantified by measuring release of lactate dehydrogenase after stimulation (26). Monocytes were isolated from leukophoresed human peripheral blood using Lymphocyte Separation Medium (Organon Teknika Corp., Durham, NC) and purified by elutriation (70); monocyte-containing fractions were identified by immunofluorescent staining with Leu M-3 antibody (Becton-Dickinson, Mountain View, CA) and analysis by flow cytometry. Adherent cultures of purified monocytes were established in medium containing 1% FBS and activated with 1 μg/ml LPS (*E. coli*, strain 055.B5P; Sigma Chemical Co., St. Louis, MO) for 20 h as previously described (55).

Proteins

LN was purified from Englebreth-Holm-Swarm sarcomas propagated subcutaneously in germ-free C57J/B6 mice by salt extraction and gel filtration on Sepharose Cl-6B with 2 M GnHCl as recently described (43). Additional LN samples and original Englebreth-Holm-Swarm tumor-bearing mice were gifts of Drs. Hynda Kleinman and George Martin (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). The LN consisted of intact 400- and 200-kD alpha and beta chains and was essentially free of nidogen; purity was estimated to be >98% on silver-stained SDS-PAGE under reducing conditions. For soluble LN-binding assays, this ligand was centrifuged at 13,000 *g* for 15 min to remove putative aggregates, and radioiodinated with Bolton-Hunter reagent (New England Nuclear, Boston, MA) according to the manufacturer's directions. Uncoupled reagent was separated from iodinated LN using a TSK 400 HPLC column. Specific activity of iodinated ligand ranged from 1 to 3 μCi/μg, and 98–99% of the radioactivity was TCA precipitable. The radioligand was electrophoresed and autoradiographed to assess its purity and integrity. Only two heavily labeled major bands representing the alpha and beta chains of LN were observed. 67-kD LNR was purified from human PMN specific granules (12) on a LN–Sepharose affinity column as previously described (30, 68). FN was isolated from outdated human plasma (New York Blood Center, New York) using gelatin–Sepharose chromatography.

Antibodies

The following antibodies used for IEM at the indicated concentrations were kindly provided by the investigators listed: affinity-purified rabbit IgG (10 μg/ml) to a LNR fusion protein (fp9) expressing the 295 COOH-terminal amino acid sequence of human 67-kD LNR (52) and the corresponding preimmune serum (Drs. B. Segui-Real and Y. Yamada, National Institute of Dental Research, National Institutes of Health, Bethesda, MD); rabbit antiserum to purified native human 67-kD LNR (1:200) (69) (Dr. Lance Liotta, National Cancer Institute, National Institutes of Health, Bethesda, MD); murine monoclonal IgGs 60.3 and 60.1 (85 μg/ml) recognizing the CD18 and CD11b components, respectively, of the Mac-1 receptor (5, 66) (Dr. John Harlan, Harbor View Medical Center, Seattle, WA); affinity-purified rabbit IgGs against the $alpha_5$ subunit of human FNR and the $alpha_v$ chain of VNR, as well as purified placental FNR and VNR (56) (Drs. James Gailit and Erkki Ruoslahti, La Jolla Cancer Research Center, La Jolla, CA); and rabbit antiserum (anti–GE No. 610019) to purified human sputum elastase (1:30) (Dr. Joan Dawes, University of Edinburgh, Edinburgh, Scotland, UK). Purified rabbit IgGs to human lacto ferrin and to human lysozyme were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY) and used at a 1:30 dilution. These antibodies were detected indirectly with secondary antibodies or protein A conjugated to colloidal gold: affinity-purified goat anti–rabbit IgG linked to 5-nm gold particles (garG5), affinity-purified goat anti-mouse IgG attached to 10-nm gold (gamG10), and pA linked to either 5- or 10-nm gold (pAG5; pAG10) (Janssen Life Science Products, Piscataway, NJ).

Immunoelectron Microscopy

Resting or activated leukocytes in HBSS with 0.1% HSA and 20 mM Hepes (pH 7.2) were fixed for IEM with 3.5% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M Na-cacodylate (pH 7.2), and 0.1 M sucrose for 30 min at 23°C. Since leukocytes (particularly PMNs) contain several potent proteases which disrupt cellular ultrastructure and antigenicity during preservation, the following inhibitors were dissolved in our fixative: 1 mM EDTA, 4 mM Na-tetrathionate, 30 μM pepstatin, and 4 mM PMSF. These protease inhibitors markedly improved the ultrastructural preservation of our leukocyte preparations. To allow access of our immunoprobes to intracellular compartments, pellets of fixed leukocytes were cut into ultrathin (~80 nm) frozen sections at −95°C as previously described (55). Briefly, fixed cells were embedded in 7% acrylamide and the blocks infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.2). To improve the cryopreservation, specimens were injected mechanically at a velocity of 3 m/sec into various cryogens and rapidly frozen at their melting point with a KF-80 immersion cyrofixation apparatus (Reichert-Jung Optische Werke, Vienna, Austria): Freon 22 (−155°C), ethane (−171°C), or propane (−190°C). This method is much more reproducible than manual freezing techniques (57), and we observed that either ethane or propane produced the most consistent cryopreservation. Reduction of free aldehydes using $NaBH_4$, adsorption with a clarified solution of nonfat milk, and indirect staining of intracellular antigens exposed on the surfaces of thin cryosections mounted on formvar-coated nickel grids were performed as previously described (55). For controls, the grids were incubated with purified Englebreth-Holm-Swarm sarcoma LN or human plasma FN (100 μg/ml in 0.1 M $NaHCO_3$ [pH 8.5]), before labeling with LNR antibodies. Alternatively, extracellular matrix receptor antibodies were pretreated with saturating concentrations (110–170 μg/ml) of relevant or irrelevant purified receptor, centrifuged at 15,000 *g*, and the supernatants used for IEM staining. Further controls were preimmune or nonimmune rabbit or mouse IgG. The following protocols were

used in double-labeling experiments: (*a*) rabbit antibody/garG5/mouse antibody/gamG10 (alternatively, primary and secondary antibodies were applied as clarified mixtures); (*b*) rabbit antibody/pAG5/pA (50 μg/ml; Sigma Chemical Co.)/mouse or rabbit antibody/pAG10. Between each step the sections were washed seven times with 0.1 M Tris-HCl (pH 7.8). After immunostaining, the labeled leukocyte sections were fixed with 2% glutaraldehyde and negatively stained with 1% phosphotungstate (pH 7.0). Specimens were analyzed and photographed in a JEOL 100CX II transmission electron microscope operating at 80 kV.

Results

Immunoblotting of Human PMNs

Immunoblotting experiments were conducted to determine the PMN proteins recognized by the 67-kD LNR antibodies used in this study (Fig. 1). Whole-cell extracts, isolated specific granules (lane *2*), and affinity-purified LNR (lane *3*) were immunoblotted with antibodies to fp9 (an *E. coli* fusion protein expressing the COOH-terminal 295-amino acid residues of human 67-kD LNR [52]), to purified native human 67-kD LNR (69), or to lactoferrin. With LNR/fp9 antibodies, specific granules (lane *5*) and isolated LNR (lane *6*) exhibited a single reactive 67-kD protein; no staining of a 32-kD band (52) was observed (lanes *5* and *6*). LNR/fp9 IgG did not recognize human serum albumin (lane *4*). Similar results were obtained with extracts of unfractionated PMNs and antibodies to purified native 67-kD LNR (not shown). Lactoferrin antibodies labeled a 77-kD protein of specific granules (lane *7*).

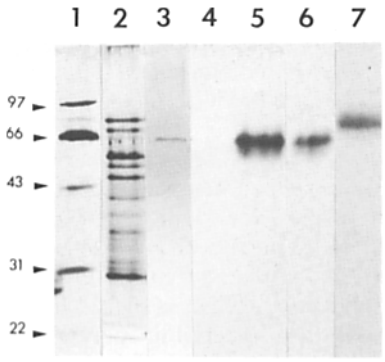


Figure 1. Immunoblotting of 67-kD LNR fusion protein (fp9) IgG on isolated PMN specific granules and purified LNR. Lanes *1–3* are silver-stained PAGE gels of molecular mass markers $\times 10^{-3}$ (lane *1*), PMN specific granules from the 1.07–1.09 density range of a Percoll gradient (lane *2*), and purified LNR extracted from specific granules and isolated by LN–Sepharose chromatography (lane *3*). Lanes *4–6* are autoradiograms of immunoblotted samples stained with LNR fp9 IgG followed by ^{125}I-protein A. Lane *4*, purified human serum albumin; lane *5*, specific granule fraction shown in lane *2*; lane *6*, purified LNR depicted in lane *3*. Lane *7* is the specific granule fraction (lane *2*) immunoblotted with lacto ferrin antibodies.

Distribution of LNR in Resting Human PMNs

Antibodies to native 67-kD LNR or to LNR/fp9 were both localized in the electron lucent granules of unstimulated human PMNs (Fig. 2, *A* and *B*). (Because LNR/fp9 IgGs have little likelihood of containing antibodies to putative mammalian contaminants, this reagent was used to localize 67-kD LNR in most experiments.) LNR antigens were situated on the inner surface of the lucent granule membrane (Fig. 2 *B*, *arrowheads*) and within its internal matrix; electron dense granules and the plasmalemma were not stained (Fig. 2 *B*). However, sparse LNR/fp9 staining was detected on the plasma membranes of PMNs fixed with a formaldehyde solution lacking glutaraldehyde (not shown). LNR/fp9 labeling of lucent granules was eliminated by preincubating PMN cryosections with purified LN before immunostaining (Fig. 2 *C*); similar treatment with FN did not inhibit LNR staining (Fig. 2 *D*). This demonstrates the immunospecificity of our staining since LN presumably binds LNR sites on the cryosection surface thus preventing access to LNR antibodies. Similarly, antibodies to CD18 and CD11b (the beta and alpha subunits of the Mac-1 complex) were localized in PMN lucent granules (Fig. 2, *E* and *F*), and 67-kD LNR antigens were also detected in the electron lucent granules of human eosinophils (Fig. 2 *G*).

LNR and CD18 Antigens Are Co-localized within PMN-specific Granules

Double-label IEM was performed to determine if 67-kD LNR and CD18 antigens were localized in the same lucent granule, and whether they co-localized with established markers of PMN-specific or azurophilic granules. With colloidal gold conjugates of either species specific secondary antibodies or protein A (5 nm for LNR; 10 nm for CD18), 67 kD LNR and CD18 epitopes were co-distributed in the lucent granules of resting human PMNs (Fig. 3 *A*). Both antigens were especially concentrated on the inner surface of the granule membrane (Fig. 3 *B*), but were also present within its central matrix. Further, LNR co-localized with lactoferrin antigens (Fig. 3, *C* and *D*) and did not co-distribute with elastase epitopes in the dense azurophilic granules (Fig. 3, *E* and *F*), thus establishing that 67-kD LNR is located within the specific granules of human PMNs.

Immunolocalization of LNR and CD18 in Stimulated Human PMNs

Parameters for optimal stimulation of PMNs were determined with in vitro assays for liberation of myeloperoxidase (from azurophilic granules) and vitamin B_{12} binding protein (from specific granules) into the culture medium; PMNs were pelleted immediately after stimulation and fixed for IEM (Table I). Treatment of suspended PMNs with 500 ng/ml PMA, or with 5 μg/ml cytochalasin *D* and 1 μM fMLP stimulated significant secretion of the specific granule marker. For cells plated on a LN-coated substratum, 10 nM rTNF alpha produced a similar effect. Activation with these stimuli produced very little PMN lysis as evidenced by the low levels of lactate dehydrogenase release. Since 67-kD LNR and CD18 epitopes were co-localized in specific granules of resting PMNs, we studied the subcellular distribution of the latter receptors after stimulation with these agonists (Fig. 4). PMNs exhibited fusion of LNR-containing specific granules with the plasmalemma (Fig. 4 *A*), and moderate amounts of 67-kD LNR antigens were observed on the outer cell surface (Fig. 4 *C*) after activation with rTNF alpha. Similarly, stimulation with PMA, or cytochalasin *D* and fMLP (not shown) induced expression of LNR and the contents of specific granules at the PMN surface (Fig. 4 *B*). LNR epitopes were also

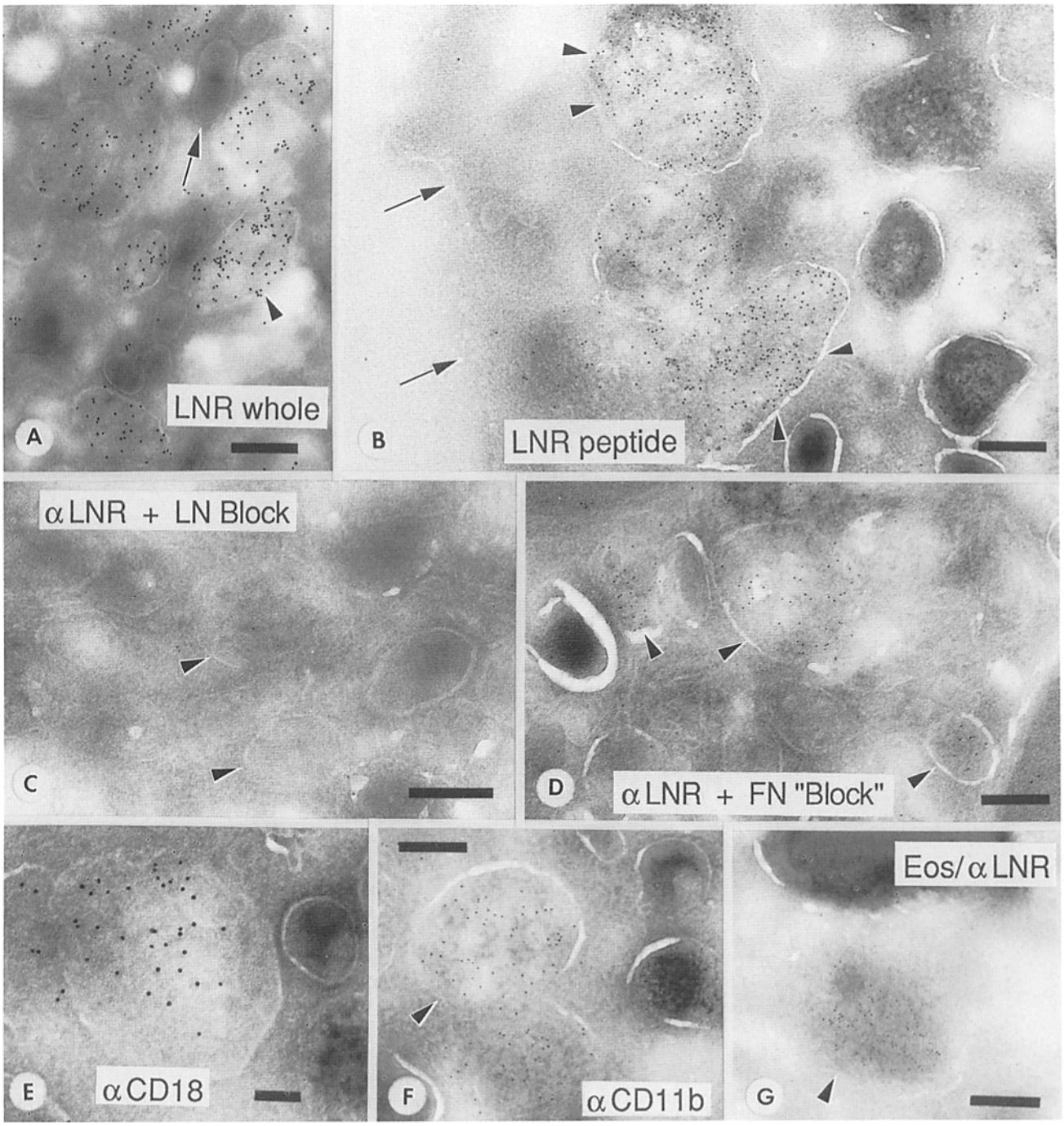


Figure 2. IEM localization of 67-kD LNR, Mac-1 beta (CD18), and Mac-1 alpha (CD11b) subunits on ultrathin cryosections of resting human PMNs and eosinophils (*Eos*) prepared as described in Materials and Methods. Sections were stained with the indicated primary antibodies followed by garG10 (*A*), pAG5 (*B, C, D, F,* and *G*) or gamG10 (*E*). (*A*) Antibodies to intact native LNR were located in electron lucent granules (*arrowhead*) but not in dense granules (*arrow*) of the human PMN. (*B*) LNR/fp9 antigens (affinity-purified IgG) were also found within lucent granules and were localized on the inner surface of its membrane (*arrowheads*) and within its internal matrix; the plasmalemma (*arrows*) was unstained. (*C*) Labeling of LNR/fp9 antigens in PMN lucent granules (*arrowheads*) was prevented by pre-incubating the sections with purified LN, but not with FN (*D*). A similar distribution of CD18 (*E*) and CD11b (*F*) antigens was found in PMNs. Likewise, eosinophils had lucent granules (*arrowhead*) with LNR/fp9 antigens (*G*). Bars: (*A–D, F,* and *G*) 200 nm; (*E*) 100 nm.

Figure 3. Double-label IEM staining of LNR/fp9 and CD18 antigens relative to each other and to known markers of specific granules (lacto ferrin) and azurophilic granules (elastase) on ultrathin cryosections of unactivated human PMNs. Samples were prepared and immuno-stained using protocol *b* described in Materials and Methods. *A* and *B* were double labeled for LNR/fp9 (G5) and CD18 (G10) antigens. (*A*) Electron lucent granules (*arrowheads*) were positive for CD18 and LNR/fp9, whereas dense granules (*arrow*) were unstained. (*B*) Both LNR and CD18 were localized to the inner membrane surface (*arrowheads*) and internal matrix of lucent granules; the cell membrane (*arrow*) was unlabeled. *C* and *D* were stained for LNR/fp9 (G5) and lactoferrin (G10). LNR and lactoferrin antigens were co-localized in lucent granules (*C* and *arrowheads* in *D*) while the the dense granules were unlabeled (*D, arrow*). *E* and *F* were labeled with antibodies to LNR (G5) and elastase (G10). Dense granules were positive for elastase (*E* and *arrow* in *F*) whereas specific granules were stained for LNR/fp9 (*F, arrowhead*). Bars: (*A–C, E,* and *F*) 200 nm; (*D*) 100 nm.

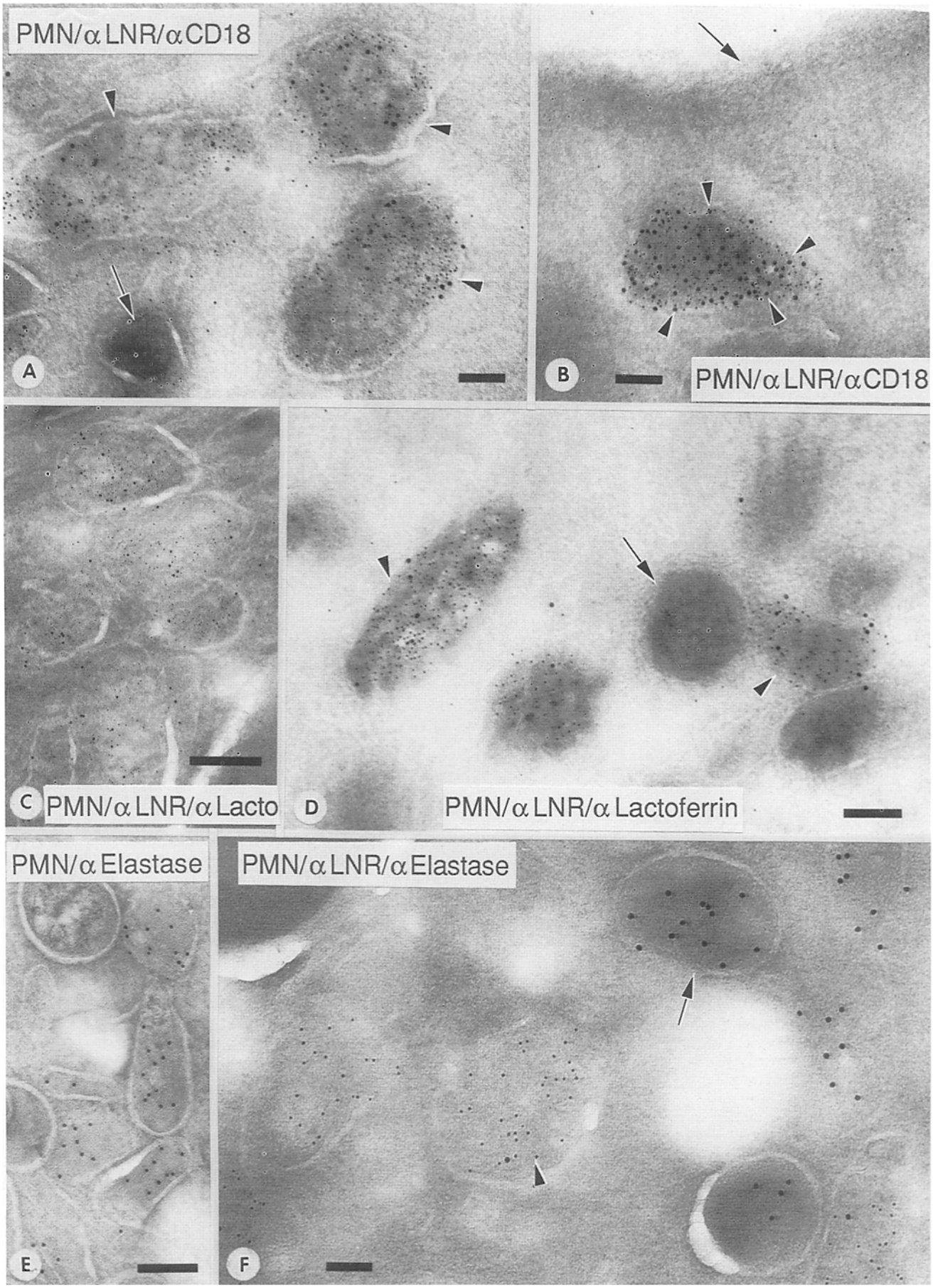

PMN/α LNR/α CD18
A
B
PMN/α LNR/α CD18
C
PMN/α LNR/α Lacto
D
PMN/α LNR/α Lactoferrin
PMN/α Elastase
PMN/α LNR/α Elastase
E
F

*Table I. Liberation of PMN Subcellular Markers after Stimulation with Various Agonists**

Stimuli	Myeloperoxidase‡	B_{12}-binding§	LDH‖
	%	%	%
50 ng/ml PMA	1.6	4.8	0.05
500 ng/ml PMA	6.2	24.7	0.13
1 μM FMLP	2.3	9.8	0.12
5 μg/ml cytochalasin D and 1 μM fMLP	60.5	38.4	0.10
DMSO control	2.8	2.8	0.13
10 nM rTNFα	1.3	16.6	0.14
Laminin substrate and 10 nM rTNFα	2.6	43.8	0.19
Laminin substrate and buffer	2.4	5.2	0.09

* Human PMNs intended for IEM were isolated from peripheral blood on Ficoll-Hypaque gradients and resuspended in HBSS containing 20 mM Hepes (pH 7.4) and 0.1% human serum albumin. Suspensions of 2×10^6 cells/ml were activated with the indicated agonists for 20 min at 37°C with continuous agitation (except for treatment with TNF which was for 1 h). After activation, the PMNs were pelleted for fixation, and enzyme activities were assayed in the supernatants. PMNs (10^7) adherent to LN-coated 10-cm-diam culture dishes were also treated with TNF for 1 h, and marker enzymes were measured in the culture media.

‡ Myeloperoxidase activity was measured to evaluate release of azurophilic granules. Equivalent PMN suspensions were also lysed with 0.5% NP-40 to determine the total amount of endogenous myeloperoxidase.

§ Soluble vitamin B_{12} binding activity was measured to quantify liberation of specific granules.

‖ Lactate dehydrogenase release was used to quantify PMN lysis.

detected within glycocalyx material closely associated with microvilli of activated PMNs (Fig. 4 *D*, *arrow*). In double labeling experiments, both 67-kD LNR and CD18 antigens were co-expressed as mixtures on the cell surface adjacent to specific granules fusing with the plasmalemma (Fig. 4 *E*), and also as isolated clusters elsewhere along the cell membrane (Fig. 4 *F*). Activated PMNs did not exhibit liberation of all of their specific granules (Fig. 4, *A*, *C*, *E*).

Binding of LN to Stimulated PMNs

To assess the relevance of LN binding to PMN function, adhesion of PMNs to LN-coated substrata was measured in relation to cellular activation. Stimulation with fMLP or PMA resulted in increased PMN attachment to surface-bound LN in a dose-response manner, while unactivated cells did not adhere well (Fig. 5). A soluble LN-binding assay was also established to further quantify the association of LN with the PMN surface. PMNs activated with cytochalasin *D*/fMLP exhibited an 8.5-fold increase in specific binding of soluble LN relative to unstimulated cells, and PMA treatment increased this binding 6.6 times (Table II). Inclusion of 67-kD LNR/fp9 antibodies in the binding buffer caused a 4.2-fold reduction of specific LN binding compared to nonimmune serum, thus supporting the functional specificity of the LNR/fp9 antibodies.

Liberation of Specific Granule Components from Activated PMNs

Since 67-kD LNR and lactoferrin antigens were co-localized in specific granules, we asked whether PMN stimulation induced release of one or both of these components into the culture medium. Cells were activated with cytochalasin *D*/fMLP or PMA, and the release of 67-kD LNR and lactoferrin was monitored with immunoblotting and densitometry of clarified culture supernatants (Table III). Significant amounts of lactoferrin appeared in the media after activation with either agonist, while 67-kD LNR was not released.

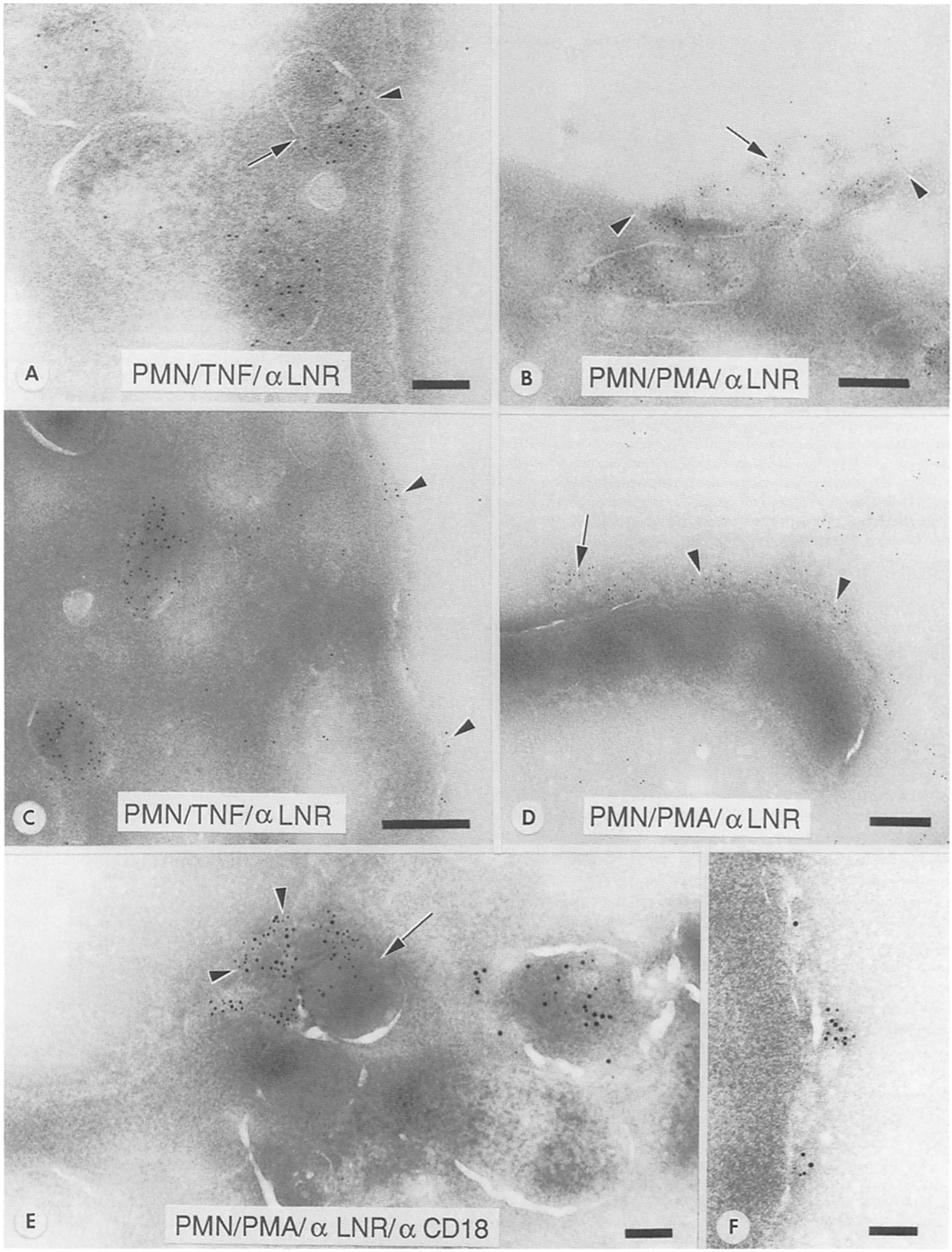
Distribution of LNR in Human Monocytes

Resting human monocytes isolated from peripheral blood by elutriation contained electron lucent and dense cytoplasmic granules similar to those of PMNs (Fig. 6). As with the polymorphonuclear granulocytes, monocytic lucent granules exhibited intense labeling with 67-kD LNR/fp9 antibodies, while the dense granules and the cell membrane were unstained (Fig. 6 *A*). Alternatively, both types of granules were positive for lysozyme epitopes (Fig. 6, *B* and *D*). In double staining experiments, both CD18 and lysozyme were co-distributed with 67-kD LNR in monocytic lucent granules (Fig. 6, *C* and *D*). The Golgi apparatus of these cells was unstained for LNR and CD18 antigens (Fig. 6 *E*). Upon activation with LPS, clusters of 67-kD LNR and CD18 epitopes were localized on the outer surface of the plasmalemma.

Detection of Other Integrins in Lucent Granules of Leukocytes

Since CD11b and CD18 antigens were observed in PMN and monocyte lucent granules along with 67-kD LNR, we asked whether other integrin molecules were co-localized at these sites. Using antibodies specific for the $alpha_5$ subunit of mammalian FNR, or for the $alpha_v$ chain of VNR, these additional receptors were located in lucent granules of PMNs (Fig. 7, *A* and *D*) and monocytes (not shown). In each case, the staining was eliminated by pretreatment of the antibodies with purified relevant receptor (Fig. 7, *B* and *E*), but not with irrelevant integrins (Fig. 7, *C* and *F*). Double-staining experiments were then conducted to determine the relationships between the distributions of 67-kD LNR, CD18, FNR and VNR antigens in PMNs and monocytes (Fig. 8). Both FNR and VNR were colocalized with LNR (Fig. 8, *A* and

Figure 4. Activation of human PMNs with 10 nM rTNF (*A* and *C*), 1.3 μM PMA (*B* and *D–F*), or cytochalasin D plus fMLP (not shown; see Table I) causes expression of LNR/fp9 and CD18 antigens on the cell surface. Cryosections of stimulated PMNs were stained with LNR/fp9 affinity-purified IgG and pAG5 (*A–D*), or double labeled with antibodies recognizing LNR/fp9 and CD18 antigens after protocol *a* described in Materials and Methods (*E* and *F*). (*A*) Stimulation-induced fusion of LNR containing specific granules (*arrow*) with the plasmalemma (*arrowhead*). (*B*) LNR-positive specific granule matrix (*arrow*) was sometimes found extending outward from the site of granule fusion with the cell membrane (*arrowheads*). Other activated PMNs exhibited uniform LNR/fp9 antigen labeling on the cell membrane (*C* and *D*, *arrowheads*), and LNR clusters on portions of the glycocalyx on microvilli (*D*, *arrow*). (*E*) Mixtures of LNR/fp9 (G5) and CD18 (G10) antigens (*arrowheads*) were seen on the cell membrane adjacent to double-stained specific granules (*arrow*) apparently fusing with the plasmalemma. (*F*) Double-labeled clusters of LNR and CD18 antigens were also randomly distributed on the surfaces of activated PMNs. Bars: (*A*, *E*, and *F*) 100 nm; (*B–D*) 200 nm.


A
PMN/TNF/α LNR
B
PMN/PMA/α LNR
C
PMN/TNF/α LNR
D
PMN/PMA/α LNR
E
PMN/PMA/α LNR/α CD18
F

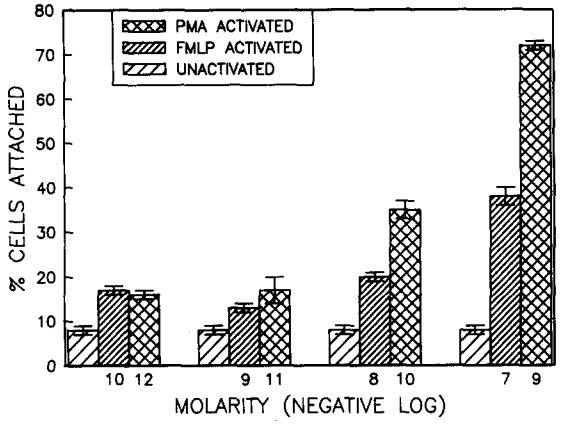


Figure 5. Adhesion of stimulated human PMNs to LN-coated substrata as a function of agonist dosage. PMNs were isolated from peripheral blood on a Ficoll-Hypaque gradient, activated with the indicated concentrations of agonists for 20 min at 37°C, and seeded (2×10^4 cells per well) into Costar microtiter wells that were precoated with a 10-μg/ml solution of LN purified from Engelbreth-Holm-Swarm sarcomas. After 1 h of incubation at 37°C, unattached cells were washed away with a Cetus Pro/Pette, and the cells fixed with methanol and stained with hematoxylin. Adherent PMNs were observed with an inverted microscope at 195×, and the total number of attached cells per well was counted with an automated image analyzer. Data shown represents the means of three determinations, and the bars are the standard errors.

*Table II. Specific Binding of Soluble Laminin to Human PMNs Increases Due to Cellular Activation, and Is Inhibited by LNR/fp9 Antibodies**

Experiment No.	Stimuli	LN bound, cpm	Relative change
1	Unactivated control‡	357	–
1	Cytochalisin D + fMLP§	3,041	+8.5
2	Unactivated control‖	1,500	–
2	PMA + medium¶	9,900	+6.6
2	PMA + nonimmune serum**	14,000	+9.3
2	PMA + anti-LNR/fp9‡‡	3,300	−4.2

* Purified EHS sarcoma was iodinated to a specific activity of 1.9 μCi/μg (experiment No. 1) or 1.3 μCi/μg (experiment No. 2) as described in Materials and Methods.
‡ For experiment No. 1, 1×10^6 human PMNs were incubated in 300 μl binding buffer containing 728 ng radiolabeled LN ± 100-fold excess unlabeled LN for 1 h at 4°C. Unbound LN was separated from labeled PMNs by centrifugation at 900 *g* through a 0.5-M sucrose cushion. The tubes were frozen on dry ice and the tips containing the cell pellets were cut off and counted. Specific LN binding was determined by subtracting the counts bound to PMNs incubated with radiolabeled LN plus unlabeled ligand from those bound to cells treated with radioligand alone.
§ Cells were activated with 5 μg/ml cytochalasin D and 1 μM fMLP for 20 min at 37°C before incubation with LN.
‖ In experiment No. 2, 2.5×10^6 PMNs were incubated with 670 ng ^{125}I-LN ± 100-fold excess cold ligand.
¶ Stimulation was performed with 50 ng/ml PMA for 20 min at 37°C.
** Nonimmune rabbit serum was added to the binding buffer at a final dilution of 1/100.
‡‡ The binding medium was supplemented with rabbit LNR/fp9 antiserum at a final concentration of 1/100.

*Table III. Densitometric Analysis of 67-kD LNR and Lactoferrin Released by Human PMNs after Activation**

Stimuli	Density of immunoblotted protein relative to control	
	67-kD LNR	Lactoferrin
Control	1.0	1.0
Cytochalasin D and 1 μM fMLP	−36.0	+685.7
500 ng/ml PMA	−4.0	+133.3

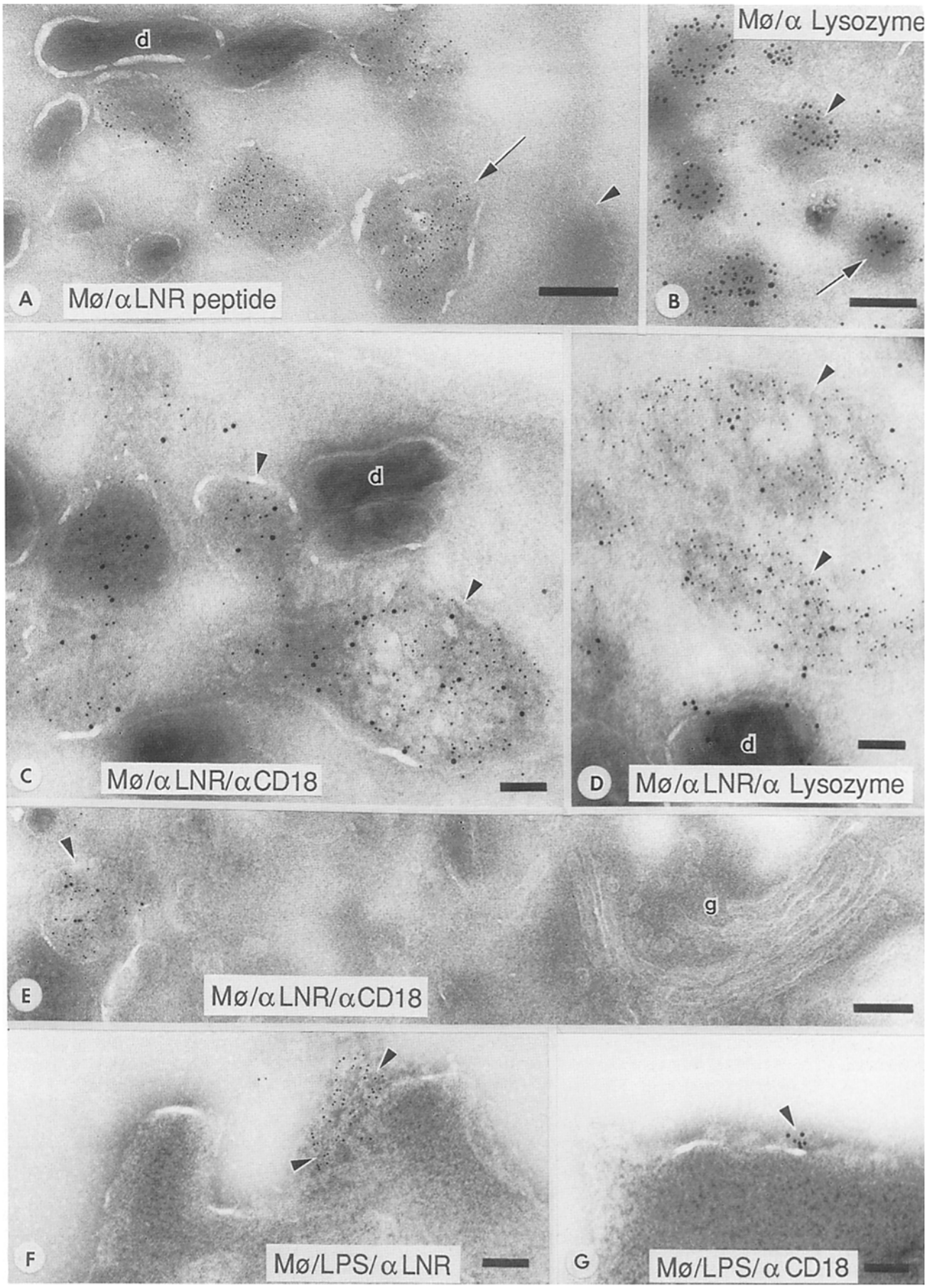
* PMNs were isolated from human peripheral blood, suspended at 10^7 cells/ml in MEM containing 5% sucrose, 5 μg/ml *N*-methoxysuccinyl-ala-ala-proval chloromethyl ketone (an inhibitor of PMN elastase), 20 mM Hepes (pH 7.2), and activated as described in Table II. After activation, the cells were centrifuged at 10,000 *g* and the supernatants collected and concentrated 10-fold. Aliquots of media were electrophoresed on SDS-PAGE gels, immunoblotted with LNR/fp9 or lactoferrin antibodies, stained with radioiodinated protein A, and autoradiographed as described in Fig. 1. For each sample, the staining density at 67 kD (LNR) or 77 kD (lactoferrin) was measured with an LKB laser densitometer fitted with an analytical integration system, and expressed as a percentage of the corresponding control band.

C), with CD18 (Fig. 8, *D* and *E*), and with each other (Fig. 8 *F*) in the lucent granules of PMNs and monocytes (Fig. 8, *G* and *H*). Staining of integrins but not of 67-kD LNR antigens was eliminated by treating the primary antibodies with purified relevant integrin (Fig. 8 *B*).

Discussion

We have located antigens of 67-kD LNR, CD11b, CD18, and the alpha chains of FNR and VNR in the specific granules of human PMNs through the use of dual label IEM on ultrathin frozen sections. PMN specific granules were identified by the presence of lactoferrin (a well-established marker of specific granules), the absence of elastase immunostaining (50), and their electron lucent appearance. Human monocytes exhibited similar granules that were positive for all four matrix receptors as well as lysozyme. Our observations are summarized in Table IV. Since 67-kD LNR definitely co-distributed with lactoferrin, there can be little doubt that this receptor is packaged in specific granules as opposed to tertiary granules as suggested in subcellular fractionation studies (78). After PMN activation (with PMA, fMLP, or rTNF), or stimulation of monocytes using LPS, specific granules were observed fusing with the plasmalemma and both LNR and CD18 antigens were detected at the cell surface. These receptor antigens were co-localized as clusters within the glycocalyx, and they were also broadly distributed over the

Figure 6. IEM distribution of LNR/fp9, lysozyme, and CD18 antigens in resting (*A–E*) or activated (*F* and *G*) human monocytes. (*A*) LNR/fp9 antigens (pAG5) were localized to lucent granules (*arrow*) similar to those seen in PMNs, but not on the cell membrane (*arrowhead*) or in dense granules (*d*). (*B*) Both dense (*arrow*) and lucent (*arrowhead*) granules were stained for lysozyme antigens (garG10). (*C*) LNR/fp9 (G5) and CD18 (G10) antigens were co-distributed in lucent granules of double labeled monocytes stained using protocol *b* described in Materials and Methods. Dense granules (*d*) were unlabeled. (*D*) Lucent granules showed a similar co-localization of LNR/fp9 (G5) and lysozyme IgG (G10) in lucent granules, but dense granules (*d*) were only positive for lysozyme. (*E*) Monocyte labeled with reagents used in *C*; while a lucent granule was positive for LNR and CD18 (*arrowhead*), the Golgi apparatus (*g*) was unstained. Clusters of LNR/fp9 (*F, arrowheads*) and CD18 (*G, arrowhead*) antigens were observed on the outer cell surface after 20 h of LPS stimulation. Bars: (*A, C, D, F,* and *B*) 100 nm; (*B* and *E*) 200 nm.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A
Mø/αLNR peptide
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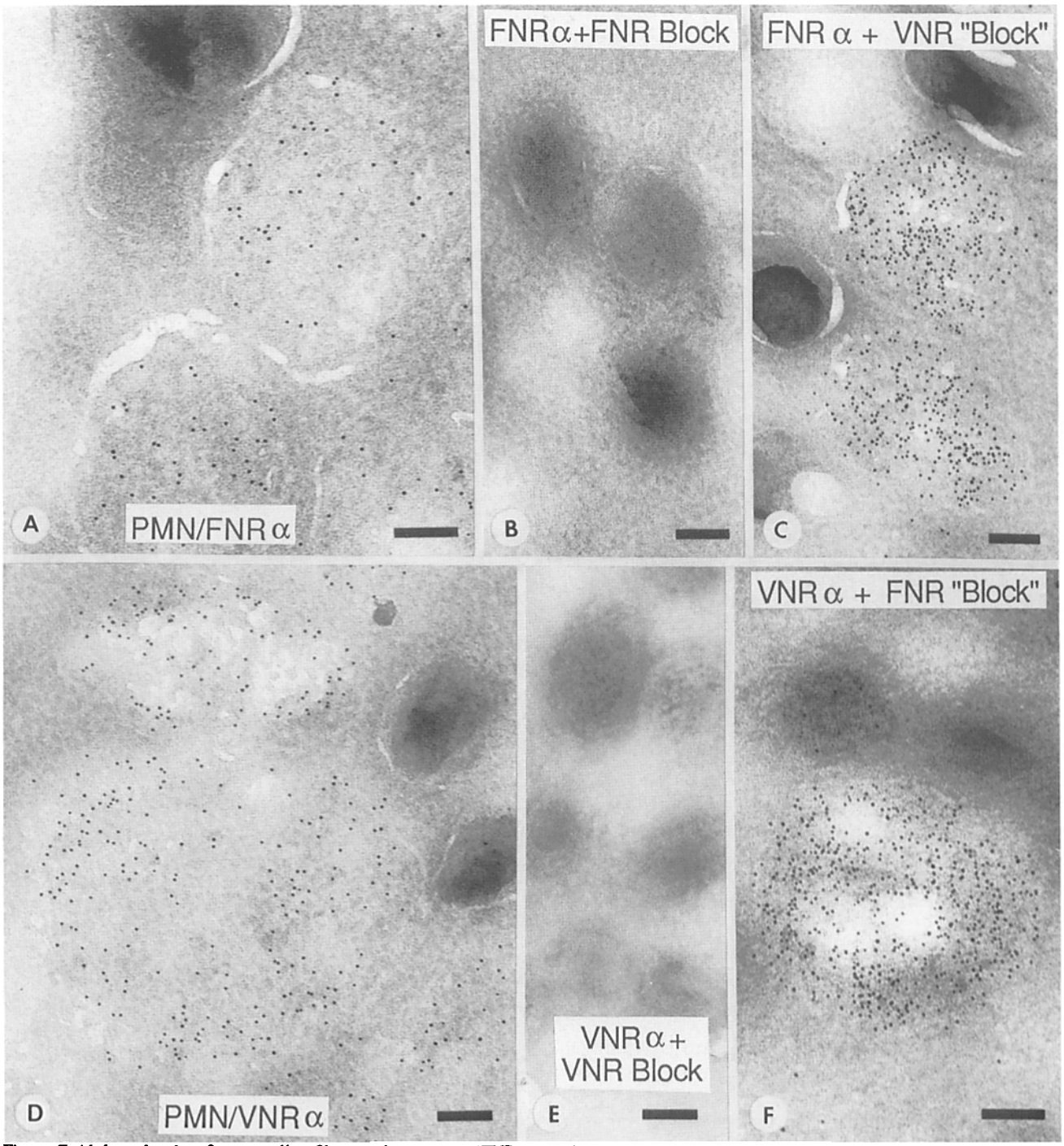

Figure 7. Alpha subunits of mammalian fibronectin receptor (*FNR*) and vitronectin receptor (*VNR*) were localized in the specific granules of resting human PMNs frozen with liquid propane. (*A*) Section stained with rabbit IgG specific for FNR alpha$_5$ chain and then pAG5. (*B*) Incubation of FNR IgG with purified FNR prevented immunostaining of specific granules, while treatment with VNR did not inhibit FNR labeling (*C*). (*D*) Labeling with VNR alpha chain IgG was similarly restricted to specific granules, and inhibited by pretreatment with purified VNR (*E*) but not with FNR (*F*). Bars: (*A*, *C*, *D*, and *F*) 100 nm; (*B* and *E*) 200 nm.

remainder of the cell surface. Concomitant with this surface expression of LNR and CD18 epitopes, we observed increased binding of soluble LN and enhanced cellular adhesion to LN substrata in activated PMNs. These events were accompanied by liberation of myeloperoxidase, vitamin B_{12} binding protein, and lactoferrin, but not LNR antigens into the culture medium. Since four types of adhesion-promoting extracellular matrix receptors were co-expressed in leukocyte specific granules, we propose that these granules be termed adhesomes.

Several novel approaches were taken in the preparation of human leukocytes for high resolution IEM study. Since these cells contain numerous proteolytic enzymes, fixation was performed with a mixture of protease inhibitors. In addition,

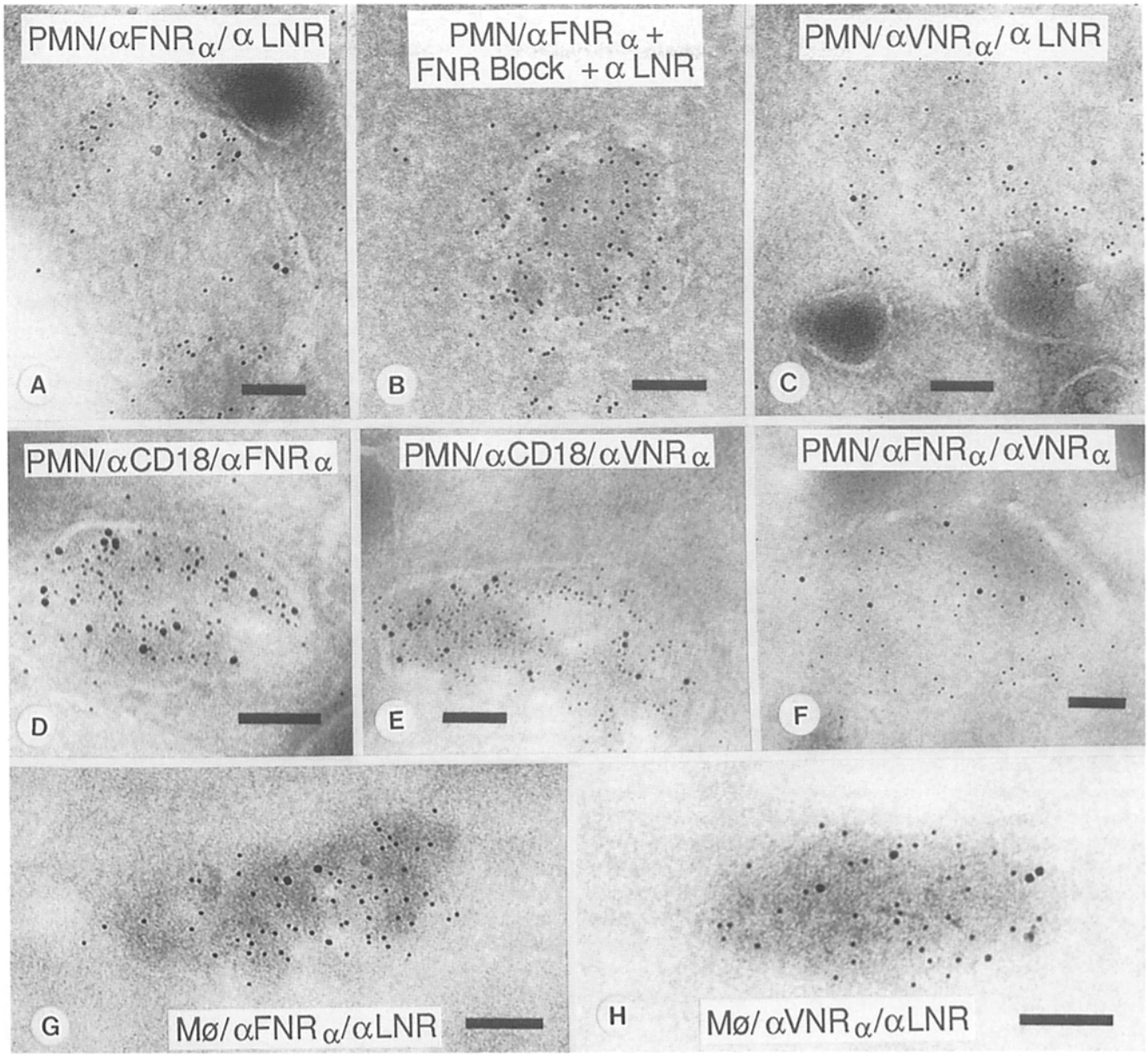

Figure 8. Double IEM labeling of unactivated human PMN specific granules (*A–F*) or monocyte lucent granules (*G* and *H*) for 67-kD LNR/fp9 or CD18, and FNR alpha$_5$ or VNR alpha-chains with a procedure similar to protocol *b* described in Materials and Methods; the cryogen was liquid propane. (*A*) PMN specific granule was positive for both LNR/fp9 (G5) and FNR alpha$_5$ (G10) antigens; the dense (azurophilic) granule was unstained. (*B*) Incubation of FNR alpha$_5$ antibodies with purified FNR prevented staining of specific granules with pAG10, but did not block LNR/fp9 labeling (pAG5). (*C*) VNR alpha-(G10) and LNR/fp9 (G5) antigens were co-localized in PMN-specific granules but not in azurophilic granules. CD18 (G10) co-distributed with FNR alpha$_5$ (G5) in *D* and VNR alpha-(G5) in *E*, and both FNR alpha$_5$ (G10) and VNR alpha-(G5) also co-localized within PMN specific granules in *F*. (*G*) Monocyte lucent granule double labeled for FNR alpha$_5$ (G10) and LNR/fp9 (G5) antigens. (*H*) VNR alpha-(G10) was also present along with LNR/fp9 (G5) in the monocyte lucent granule. Bars, 100 nm.

cells were frozen by injection into liquid cryogen with an apparatus that insures very reliable cryopreservation (57). Furthermore, antibodies of well-defined specificity were used for extracellular matrix receptor (ECMR) localization in this study. 67-kD LNR was detected with affinity-purified rabbit IgG recognizing the COOH-terminal 295 amino acids of human LNR expressed in fp9, an *E. coli* fusion protein (37, 52, 69). This antibody labels a single 67-kD species in immunoblots of PMN or specific granule homogenates, and does not cross-react with human serum albumin. FNR and VNR were stained with rabbit antibodies that specifically recognize the alpha subunits of their respective receptors (56), and the immunolabeling was inhibitable by adsorption with purified relevant but not irrelevant receptor. CD18 localization was performed with monoclonal antibody 60.3 that recognizes the common beta subunit of the leukocyte adhesion complex (5, 51) and blocks PMN attachment to endothelial cell monolayers in vitro and diapedesis in vivo (22, 72). Therefore, the IEM data reported here are very reliable.

The lack of 67-kD LNR or CD18 IEM labeling on the surfaces of resting PMNs and monocytes was somewhat unexpected because recent immunofluorescent microscopic ex-

Table IV. Distribution of ECMRs in Human Leukocyte Granules

Cell	Specific granules	Azurophilic granules
PMN	LNR (67 kD) anti-whole anti-peptide	Elastase
	CD11b, CD18	None
	FNR_α	None
	VNR_α	None
	Lactoferrin	None
Monocyte	LNR (67 kD)	None
	CD18	None
	FNR_α	None
	VNR_α	None
	Lysozyme	Lysozyme

periments have localized LNR/fp9 antigens on the surfaces of unstimulated thioglycollate elicited murine peritoneal macrophages (37) and CD18 epitopes on the plasma membranes of resting human PMNs (38). Because antigens of extracellular matrix receptors are known to be inactivated by glutaraldehyde (17), this discrepancy may be explained by the fact that our leukocytes were fixed with glutaraldehyde, while those of the latter studies were treated with formaldehyde or unfixed. We have in fact detected sparse LNR and CD18 immunostaining on the cell membranes of resting PMNs fixed with formaldehyde alone, and moderate levels of 67-kD LNR and CD18 labeling were observed on the plasma membranes of activated PMNs and monocytes preserved with glutaraldehyde. Previous IEM studies of the intracellular distribution of integrins using a polyclonal antiserum recognizing CD11c (alpha subunit of p150,95) and CD18 antigens on frozen sections of human leukocytes showed that their epitopes were localized in peroxidase negative vacuoles of monocytes (38), and along the membranes of PMN specific granules (4). We likewise observed ECMR antigens on the membranes of these granules (Figs. 2 *B* and 3 *B*), but also detected ECMR within their electron lucent matrix. This difference may be attributed to improved ultrastructural preservation. Because FNR, VNR, CD11b, and CD18 are transmembrane proteins and 67-kD LNR has the properties of a tightly bound peripheral membrane protein (52), it was surprising to find their epitopes distributed in the granule matrix and upon its inner membrane surface. Several explanations may account for the location of ECMRs within the adhesome matrix. Given recent EM observations of highly purified human FNR which show that the combined length of its "head" and "tails" is approximately fivefold greater than the thickness of a membrane (41), we estimate that ~2/3 of the adhesome matrix might be occupied by extended integrin molecules presenting antigens for immunolabeling. Thus, tangential sectioning of obliquely oriented adhesome membranes could be responsible for the centralized appearance of ECMRs in these granules. Previous IEM studies of secretory vesicles have also documented a central location for other integral membrane proteins such as vesicular stomatitis virus membrane glycoprotein G and epidermal growth factor receptor (7, 11). Further, we have recently localized antibodies against the intracytoplasmic domain of CD11b to the outer membrane surfaces of PMN adhesomes in the absence of internal labeling (Singer, I. I., and S. D. Wright, unpublished observations). Together, these staining patterns document the expected transmembrane orientation of the extracellular and cytoplasmic domains of the integrin Mac-1. Despite the presence of 67-kD LNR antigens within the adhesome matrix, we have not been able to detect a substantial release of LNR into the culture medium concurrent with abundant lactoferrin secretion after PMN activation. These results strongly suggest that ECMRs present in the adhesome are tightly bound to its membranes after fusion with the cell surface.

Fusion of specific granules containing several ECMRs with the leukocyte plasmalemma after activation suggests that ECMR up-regulation may be responsible for increases in ligand binding and cellular adhesion observed after treatment with agonists. This suggestion is supported by a large number of studies documenting increased surface expression of leukocyte integrins and LNR in activated PMNs, monocytes, and platelets (6, 33, 38, 62, 64, 67, 68). However, ECMR up-regulation probably does not by itself fully account for the effects of agonists on the adhesive behavior of leukocytes. For example, in time course experiments of activated human PMNs, the kinetics of CD18 up-regulation do not coincide precisely with increases in cellular adhesion (33, 44, 64, 71). Also, PMN aggregation and attachment to endothelial cell monolayers have been dissociated from increased surface expression of CD11b/CD18 complex (16, 44, 65). IEM of CD11b distribution on PMNs has shown that stimulation with PMA induces formation of CD11b clusters, and that the time-course of this receptor aggregation is coincident with enhanced binding of complement-coated erythrocytes; receptor disaggregation also correlated with decreased ligand binding at later times (18). Similarly, PMA treatment of murine macrophages induced cell attachment to LN-coated substrata and clustering of surface 67-kD LNR detected using immunofluorescent microscopy with the LNR antibodies of the present study (37). Likewise, we have demonstrated that stimulation of human PMNs with either PMA, rTNF, or fMLP, or monocytes with LPS, leads to fusion of adhesomes with the plasmalemma concomitant with increased surface expression of ECMR aggregates consisting of co-clustered LNR and CD18 antigens. These results suggest that aggregation also plays a role in the enhancement of ECMR function and regulation of cellular adhesion in activated leukocytes. Other alterations such as ECMR phosphorylation, conformational changes, or interaction with the cytoskeleton or other surface membrane components might also regulate ECMR function (15, 37, 64, 72).

Packaging of 67-kD LNR, CD11b, CD18, FNR, and VNR within specific granules, and co-distribution of LNR and CD18 in clusters on the surfaces of activated cells may help to explain how the extracellular matrix regulates some aspects of leukocyte function. First, attachment of macrophages to surfaces coated with FN or LN activates their Mac-1 receptors which then engage in increased phagocytosis of C3bi-coated particles (10, 45, 71). This effect is reversible and also dependent upon the interaction of substrate-bound FN with FNR since it is blocked by RGD peptides (73, 74). Because FNR and CD18 are co-distributed in adhesomes, we suggest that FNR might also be located in close proximity to CD18 in ECMR clusters seen on the cell membranes of activated monocytes. We speculate that cross-linking of FNRs

after cellular attachment to an FN substrate might induce conformational changes in neighboring Mac-1 receptors leading to their activation. Second, inflammatory agonists such as fMLP or TNF induce maximal respiratory burst and H_2O_2 production in PMNs only when the cells are spread upon extracellular matrix-coated substrata, or endothelial cell monolayers; the process requires microfilament assembly during stimulation (39). This effect is accompanied by the release of the specific granule components lactoferrin and vitamin B_{12} binding protein into the medium, while contents of azurophilic granules are not secreted (34). Further, CD18 antibodies that block attachment of PMNs to C3bi or fibrinogen also inhibit TNF-induced respiratory burst on surfaces coated with LN, fibrinogen, or thrombospondin (40). Perhaps the close spatial association of CD18 and LNR on the surface of the activated PMN provides an explanation for this phenomenon. In addition, since *b*-cytochrome, an electron transporter involved in generating the PMN respiratory burst (19), is localized with ECMRs in specific granules (adhesomes) and translocated to the plasma membrane after PMN activation (12), we hypothesize that this up-regulation may be ECMR-driven. As FNR and VNR link the extracellular matrix to the actin cytoskeleton at cell-to-substratum adhesion sites (53, 54, 56), perhaps patching and cross-linking of LNR, FNR, or Mac-1 receptors by their immobilized ligands induces polymerization of actin microfilaments (via phosphorylation), and this network translocates specific granules/adhesomes to the cell surface. Whatever the mechanism, the adhesome undoubtedly serves as a secretory unit that commandeers several ECMRs to the PMN surface simultaneously. In this manner, the coordinated expression of ECMRs that facilitate cellular adhesion to the endothelial surface (Mac-1), basement membranes (LNR), and the surrounding connective tissue stroma (FNR, VNR) is ensured during extravasation of PMNs and monocytes in response to chemotaxins and inflammatory stimuli.

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