

# LEUKOCYTE ADHESION RECEPTORS ARE STORED IN PEROXIDASE-NEGATIVE GRANULES OF HUMAN NEUTROPHILS

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Leukocytes must interact with vascular endothelial cells to be able to migrate from the circulation to sites of inflammation. These cellular adhesion reactions are mediated, in part, by surface molecules that belong to a family of structurally related glycoproteins: Mac-1, p150,95, and lymphocyte function-associated antigen 1 (LFA-1)<sup>1</sup> (1). These proteins are composed of noncovalently linked  $\alpha/\beta$  heterodimers that have distinct  $\alpha$  subunits with  $M_r$  of 170,000 (Mac-1, CD11b), 150,000 (p150,95, CD11c), and 180,000 (LFA-1, CD11a). The  $\beta$  subunit (CD18),  $M_r$  95,000, has been shown to be identical in all three proteins by physicochemical, immunochemical, and protein sequencing studies, and homologous to several adhesion receptors in other cell types (2, 3). The importance of this family of surface proteins in leukocyte function is underscored by the existence of a human genetic disease termed leukocyte adhesion deficiency (LAD). Patients with LAD are deficient in expression of all of these proteins and suffer from recurrent life-threatening bacterial infections because their leukocytes do not adhere to and migrate between endothelial cells to form an acute inflammatory exudate (1).

While LFA-1 appears on the cell surface very early in the maturation of neutrophils and monocytes, Mac-1 and p150,95 are found on more mature cells (4–6). In addition to being present on the plasma membrane, two of the glycoproteins, Mac-1 and p150,95, are stored in a latent intracellular pool in neutrophils and monocytes (7–11). Chemoattractants such as FMLP or phorbol esters trigger a rapid 5–10-fold increase in Mac-1 and p150,95 expression on the granulocyte cell surface. Because inhibitors of protein synthesis did not block this increase, it seemed to represent mobilization to the surface of a preexisting intracellular pool. However, these chemoattractants do not induce LFA-1 on granulocytes.

In the present study, we used immunocytochemical techniques on frozen thin sections of human leukocytes to determine the subcellular location of the Mac-1, p150,95, and LFA-1 leukocyte adhesion receptors (LAR). This method has

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<sup>1</sup> *Abbreviations used in this paper:* GAR-5, 5-nm goat anti-rabbit Ig immunogold complex; LAD, leukocyte adhesion deficiency; LAR, leukocyte adhesion receptors.

the advantage that all organelle compartments are accessible to antibody without the addition of permeabilizing agents that are deleterious to organelle structure, as seen by high resolution microscopy. Using this method, we have been able to demonstrate a latent pool of Mac-1 and p150,95 in the membranes of neutrophil-specific granules.

### Materials and Methods

*Preparation of Antiserum.* p150,95 was purified, and an antiserum to p150,95 was prepared as previously described (12, 13). Briefly, a lysate of hairy cell leukemia spleen cells was passed through a SHCL3 (anti-p150,95 mAb) immunoaffinity column. p150,95 was eluted at low pH, and 600 pmol were injected into a rabbit. After three boosts the rabbit was bled and serum was collected.

*Purification of Antiserum by Absorption Steps.* To preclude reactivity with any cytoplasmic antigens contaminating the p150,95 antigen preparation, the antiserum was absorbed with homogenized leukocytes from a patient severely deficient in Mac-1, LFA-1, and p150,95. The antiserum contained antibodies to both the p150,95  $\alpha$  subunit, and the  $\beta$  subunit common to p150,95, Mac-1, and LFA-1, and it therefore was capable of detecting all three glycoproteins (13). We, therefore, refer to the antigens detected by these antibodies as leukocyte adhesion receptors (LAR).

*Preparation of Human Leukocytes.* Normal human leukocytes, anticoagulated in heparin, were sedimented in dextran and washed in HBSS. The cells were fixed in 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C and were washed well in the same buffer containing 5% (wt/vol) sucrose. They were then incubated for peroxidase (14) to mark the peroxidase-positive azurophil granules (15). In other experiments,  $10^6$  cells were incubated with 50 ng PMA for 15 min at 37°C before fixation in order to cause degranulation.

*Immunocytochemical Techniques on Frozen Thin Sections.* The cell pellet was embedded in 2.1 M sucrose, frozen, and stored in liquid nitrogen. Sections were cut on a Reichert-Jung (Buffalo, NY) Ultracut E. The techniques used for preparing frozen thin sections have been previously described (16). The primary antibody was used at a 1:20 dilution. The 5-nm immunogold probe goat anti-rabbit Ig-gold (GAR-5) was used at a 1:50 dilution; the probes protein A-5 and A-10 were used at 1:20–50 dilution (Janssen Pharmaceutica, Beerse, Belgium). Nonimmune purified rabbit IgG was used as a control.

A double-labeling experiment to localize LAR and lactoferrin was performed using protein A, as described by Slot and Geuze (17). Rabbit antibody against lactoferrin (Accurate Chemical Co., Westbury, NY) was applied and labeled with protein A-10. Before the secondary antibody was applied, free protein A (0.05 mg/ml) was applied. Subsequently, antibodies to LAR were applied, followed by protein A-5. Sections were also labeled in reverse, using the large gold to label LAR and the small gold to label lactoferrin. The grids were then stained with uranyl acetate and embedded in methylcellulose as described by Tokuyasu (18) and modified by Griffiths (19).

The different secondary colloidal gold reagents were chosen because antibody-gold complex (GAR-5) stains in clumps, whereas the protein A-golds stain in a 1:1 ratio with the primary antibody. Protein A therefore gives the most precise localization, whereas GAR-5 is easier to detect because there are more grains per antibody site.

### Results

*Localization of LAR.* Our goal was to investigate the distribution of LAR within the organelles of resting polymorphonuclear neutrophilic leukocytes (PMN). We used a polyclonal antiserum to the p150,95  $\alpha/\beta$  complex because it was much more reactive than mAb to Mac-1 or p150,95 in our immunolocalization protocol. Immunoprecipitation showed that this antiserum crossreacted with Mac-1, p150,95, and LFA-1, since it recognizes the common  $\beta$  subunit (13).

Therefore, we designated it as an antibody to LAR. First, fixed leukocytes were incubated for peroxidase to mark one of the major granule populations, the azurophil (primary) granules (15). Frozen thin sections were then prepared and incubated with the antiserum and immunogold. Sections incubated with polyclonal antibody against LAR showed immunogold label along the membranes of ~80% of the peroxidase-negative specific granules (Fig. 1). Less abundant label was also seen on the plasma membrane (Fig. 1*a*), the perinuclear cisterna (Fig. 1*c*), and occasional small vesicles and multivesicular bodies (not illustrated). When the preliminary incubation for peroxidase was omitted, so that the contents of azurophil granules would appear lucent and any immunogold label could be more easily seen, no label for LAR was present on azurophil granules (not illustrated). Negligible gold labeling was found when the primary antiserum was replaced by normal rabbit serum.

*Double-labeling of LAR and Lactoferrin.* Because lactoferrin has been used in cell fractionation (20, 21) and immunocytochemical (22) studies to mark the specific (secondary) granules, we wished to determine whether the granules containing LAR also contained lactoferrin. Initially, thin sections were tested with antibody to lactoferrin alone and immunolabeled with a large gold probe, Protein A-10. Label for lactoferrin was present in the matrix of 80–90% of the peroxidase-negative specific granules (Fig. 2*a*). When the cells were double-labeled, using Protein A10 to label lactoferrin antibody and Protein A-5 to label antibody to LAR, the larger gold label for lactoferrin was present in the matrix of the specific granules, and the smaller gold-label for LAR was present along the membrane of these granules (Fig. 2*b*). The secondary gold labels were also reversed, so that the small gold labeled LAR and the large gold labeled lactoferrin, with similar results (Fig. 2*c*).

*Effect of PMA Stimulation on the Localization of LAR.* Cells stimulated with PMA before being immunolabeled for LAR (Fig. 3) showed vacuole formation and degranulation. The heavy immunogold labeling of the plasma membrane is consistent with the previous observation that PMA stimulation causes exocytosis of specific granules and increased cell surface expression of the Mac-1 and p150,95 LAR in neutrophils (7–11). Some of the vacuoles were also labeled, but more sparsely than the plasma membrane. Although there is great variation of labeling from cell to cell, counts of grains of gold indicated a 10-fold increase in plasma membrane density, over the resting cell. Note that most of the gold particles are on the exterior of the plasma membrane, whereas in specific granules (Fig. 1), most of the gold particles are located on the interior of the granule membrane.

*Localization of LAR in Other Leukocytes.* In eosinophils, label was present along the plasma membrane and was occasionally present in small intracellular vesicles (Fig. 4). No label was detected in eosinophil granules. In basophils, there was label on the plasma membrane, and a small amount in some intracellular granules (Fig. 5).

In monocytes, label for LAR was present on the plasma membrane and in peroxidase-negative granules and vesicles (9). Lymphocytes were labeled on the plasma membrane only, whereas platelets were negative (not illustrated).

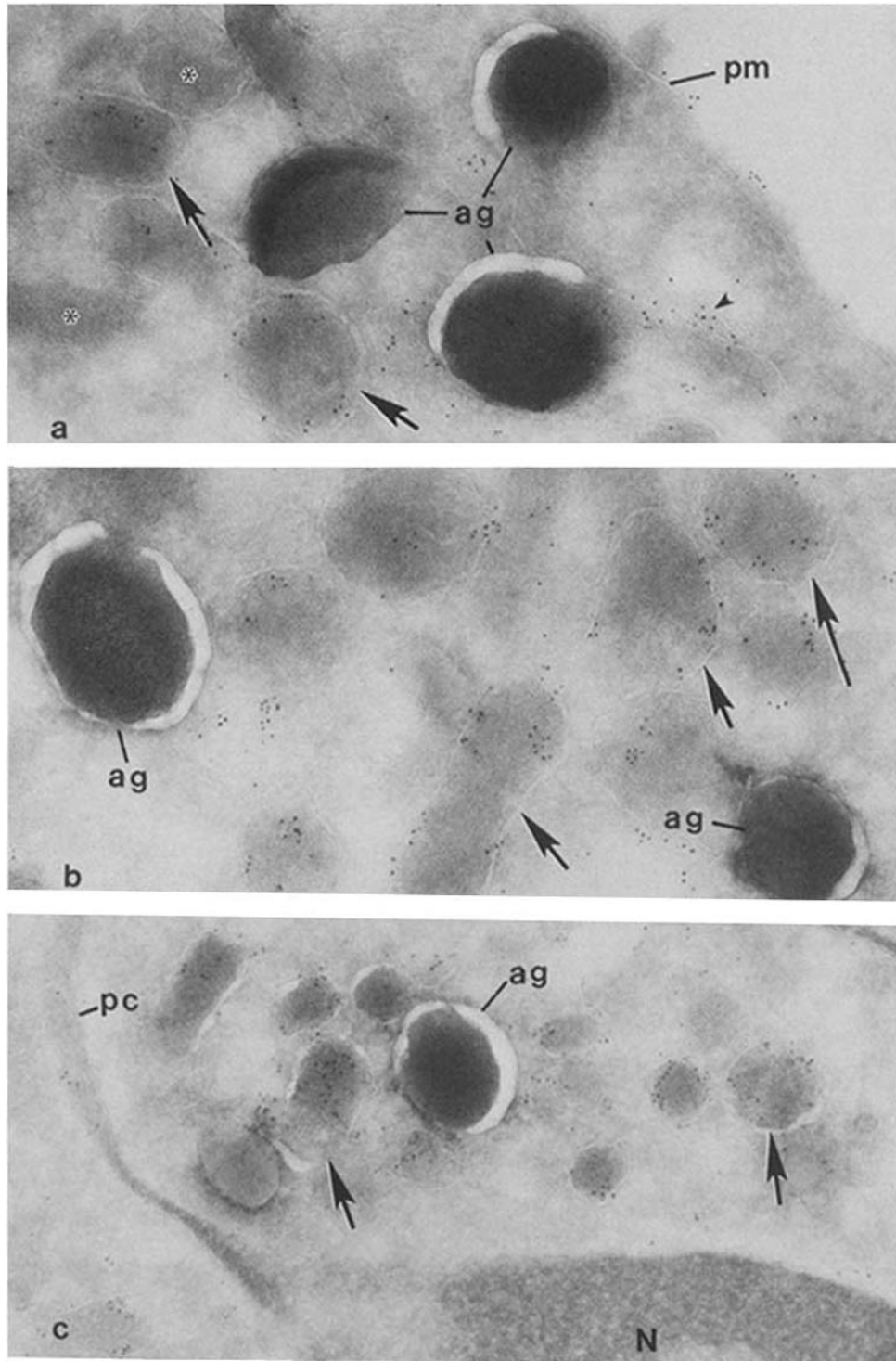


FIGURE 1. Electron micrographs of human neutrophils stained for peroxidase, incubated with rabbit polyclonal antibody to LAR on a frozen thin section, and labeled with GAR-5. The peroxidase-positive azurophilic granules (*ag*) contain dense reaction product, whereas the lighter, specific granules are peroxidase-negative. Most of the gold grains outline the membranes of the specific granules (*large arrows*), although some are present in the matrix of these granules. Because GAR-5 labels in clumps of two to six particles, label for an antibody site on the membrane may appear both on the membrane and in the matrix. Some of the peroxidase-negative granules are not labeled (\*). Some label is present on the plasma membrane (*pm*) (*a*) and in the perinuclear cisterna (*pc*) (*c*), and possibly other organelles (*small arrow*) (*N*, nucleus). Buffy-coat cells fixed in 0.25% glutaraldehyde for 1 h cold at 4°C, stained for peroxidase, and embedded in sucrose. (*a*)  $\times 70,000$ ; (*b*)  $\times 75,000$ ; (*c*)  $\times 56,000$ .

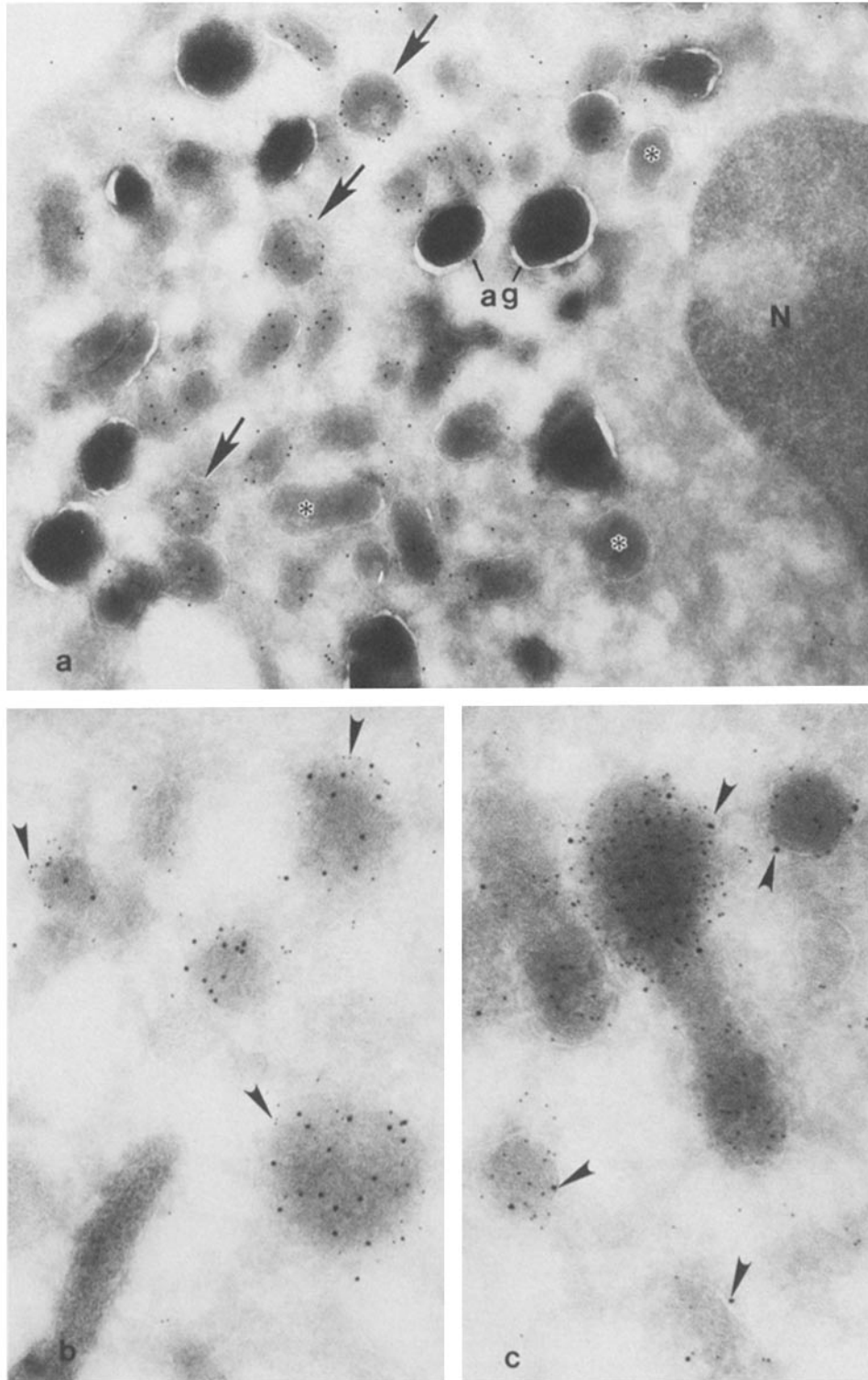


FIGURE 2. (a) Portion of a polymorphonuclear neutrophilic leukocyte (PMN) stained for peroxidase to mark the azurophil granules (ag), then immunolabeled for lactoferrin. Many of the peroxidase-negative specific granules have gold label within their matrix (arrows), but, several contain no label (\*). The few grains of gold that are not within definite organelles might represent grazing sections of granules in another plane of section.  $\times 54,000$ . (b) PMN double-labeled for lactoferrin and LAR. Peroxidase-negative granules contain the large gold label for lactoferrin within their matrices and the small gold label for LAR on their membranes (arrowheads).  $\times 69,000$ . (c) PMN double-labeled for LAR and lactoferrin. Peroxidase-negative granules contain the small gold label for lactoferrin within their matrices and the large gold label for LAR on their membranes (arrowheads).  $\times 69,000$ .

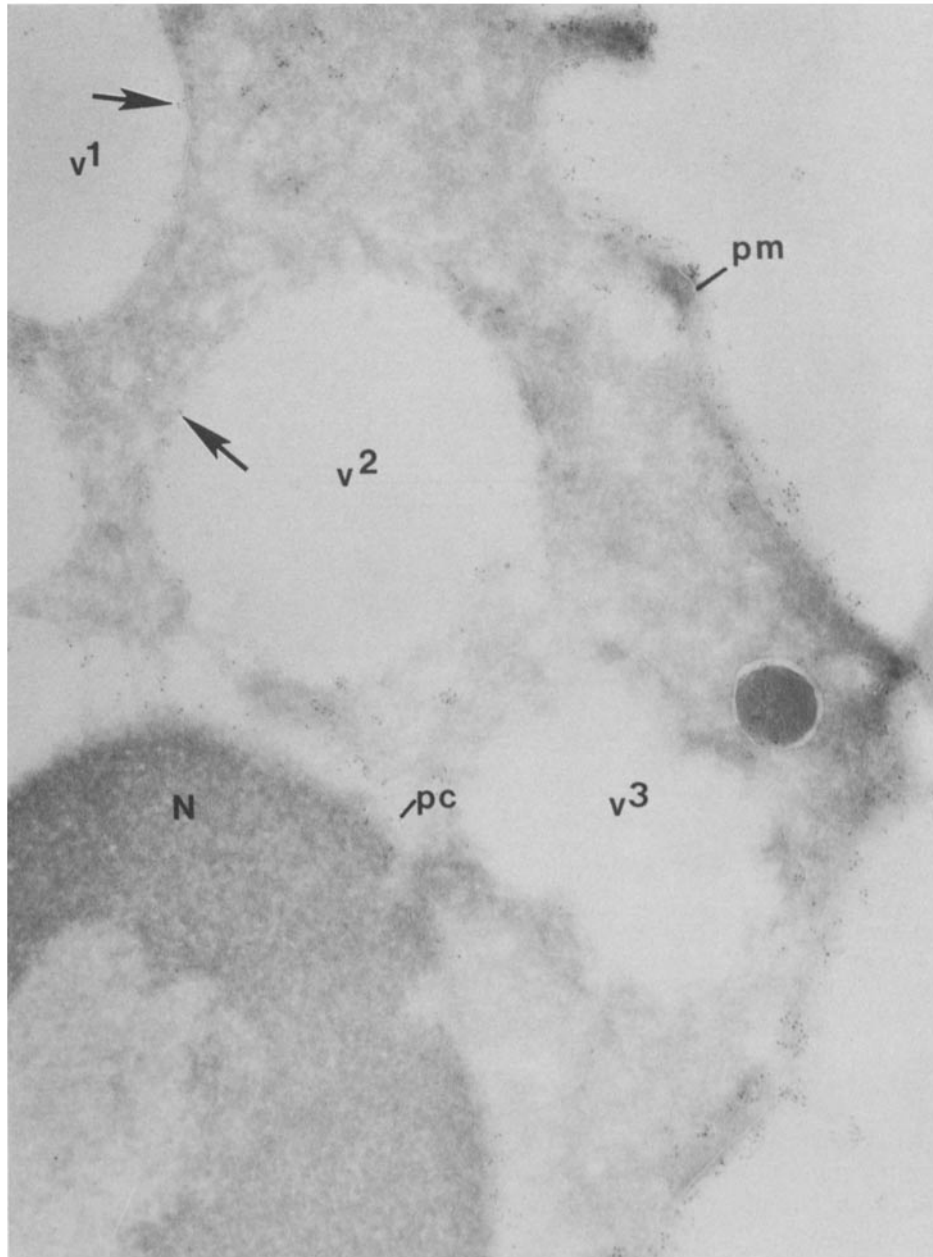
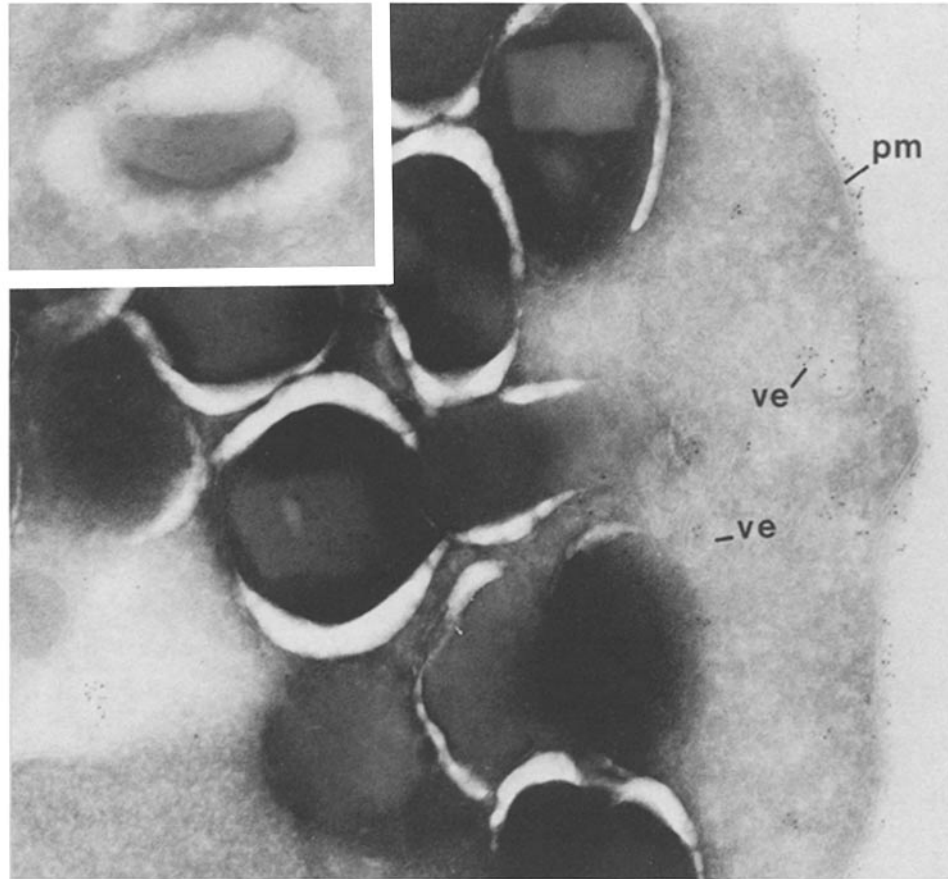


FIGURE 3. Polymorphonuclear neutrophilic leukocytes stimulated with PMA and then fixed and immunolabeled for LAR as in Fig. 1. After PMA stimulation, which causes partial degranulation and the formation of many large intracellular vacuoles, immunolabel for LAR is markedly greater than on the plasma membrane (*pm*) of the resting cell in Fig. 1 *a*. Label is also present (*arrows*) in some of the vacuoles (*V*<sup>1</sup> and *V*<sup>2</sup>) and in the perinuclear cisterna (*pc*). These cells were not incubated for peroxidase.  $\times 35,000$ .



**FIGURE 4.** Portion of an eosinophil stained for peroxidase and then immunolabeled for LAR. Heavy labeling of the plasma membrane (*pm*) can be seen as well as label in small vesicles (*ve*). The characteristic crystalline granules of the eosinophil are very dense with peroxidase reaction product. Even in specimens not reacted for peroxidase (*inset*), no gold label was detected in eosinophil granules.  $\times 58,000$ ; *inset*,  $\times 44,000$ .

### Discussion

We have used a rabbit polyclonal antiserum raised against p150,95 to demonstrate that an intracellular pool of LAR resides in the peroxidase-negative specific granules of neutrophils. Because the antiserum recognizes both the  $\alpha$  and the  $\beta$  subunits of p150,95, and since Mac-1 and LFA-1 have the same  $\beta$  subunit as p150,95, the antibody might have labeled the intracellular pools of these adhesion proteins as well. Stimulated mobilization to the cell surface of these proteins suggests that the latent pool in neutrophils contains more Mac-1 than p150,95, and little or no LFA-1, and thus, Mac-1 may have been the major intracellular LAR labeled in these studies. The present data are sufficient to state that the intracellular pool of the family of leukocyte adhesion proteins is found mainly in peroxidase-negative specific granules, and that LAR expression on the plasma membrane is increased after PMA stimulation. These findings are consistent with previous subcellular fractionation studies showing cosedimenta-

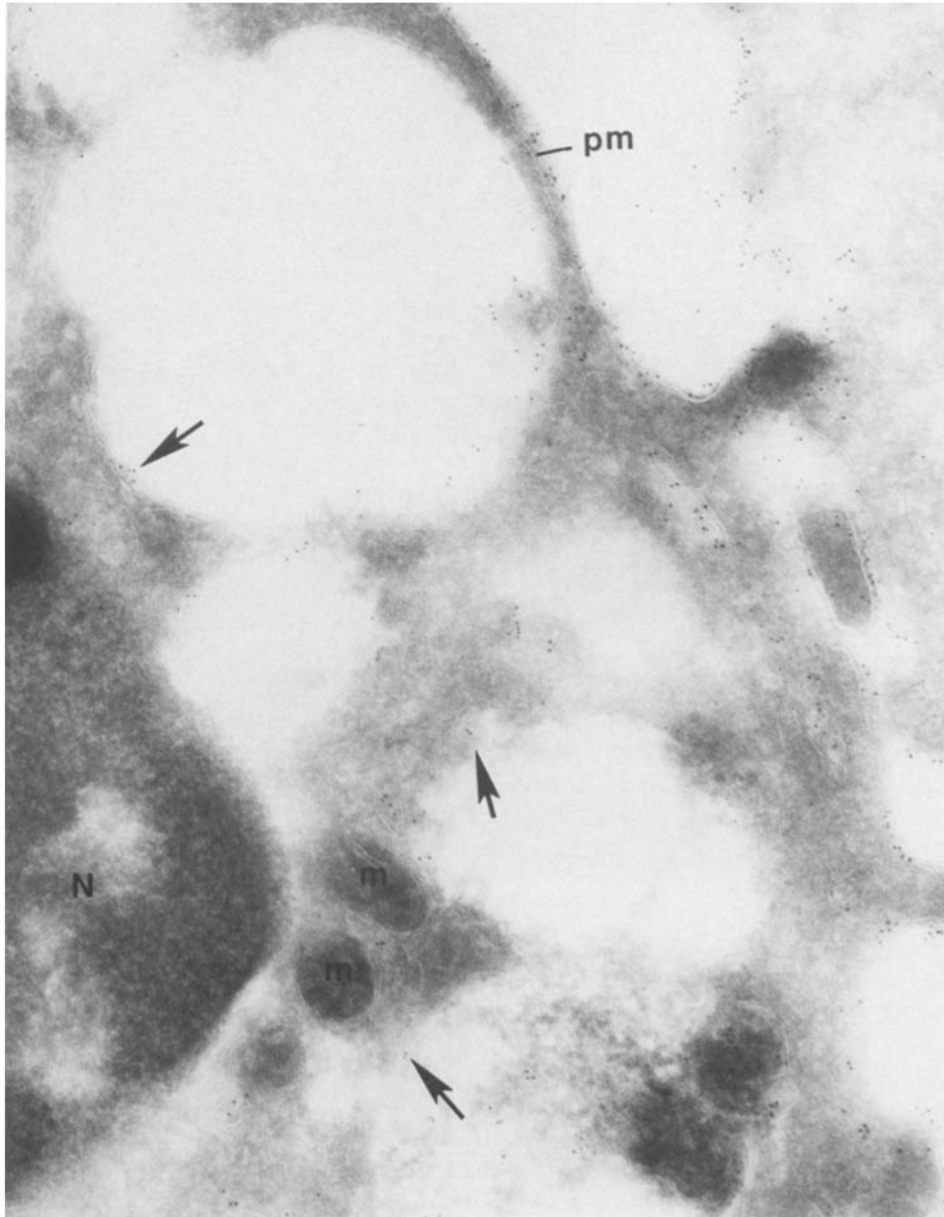


FIGURE 5. Portion of a basophilic granulocyte reacted for peroxidase and immunolabeled for LAR. Note the heavy label on the plasma membrane (*pm*) as well as in some partially extracted basophil granules (*arrows*). *N*, nucleus, *m*, mitochondria.  $\times 45,000$ .

tion of Mac-1 (Mo1) with vitamin B<sub>12</sub>-binding proteins, a specific granule marker (8). The detection of LAR on eosinophil and basophil plasma membranes has not been previously described, and deserves further investigation.

Leukocyte adhesion receptors are transmembrane glycoproteins. The p150,95  $\alpha$  and  $\beta$  subunits have extracellular domains of 1,081 and 677 amino acids, and



much smaller cytoplasmic domains of 30 and 46 amino acids, respectively (2, 23, 24). Most GAR-5 staining of the plasma membrane was localized to the exterior, and thus the antibody labels predominantly the extracellular domain. GAR-5 staining of peroxidase-negative granules was localized on the interior of the surrounding membrane. This suggests that the extracellular domain of LAR is oriented to the interior of the peroxidase-negative granules. Fusion of the granule membrane with the plasma membrane during exocytosis would result in expression on the exterior surface of the plasma membrane.

Thus far we have considered only the two major populations of granules in neutrophils, the peroxidase-positive azurophil (or primary) granules, and the peroxidase-negative specific (or secondary) granules. There may well be subpopulations of chemically distinct granules within the populations of primary and secondary granules, as broadly defined by cytochemical methods (25). In 1982, investigators detected a gelatinase-containing organelle in neutrophils that secretes its contents without secreting B<sub>12</sub> binding proteins or other markers of specific granules (26). A recent study suggested that 50% of intracellular Mo-1 (Mac-1) is localized in these labile, gelatinase-containing organelles (27). Another recently defined granule that contains latent alkaline phosphatase has been shown to be readily mobilized to the cell surface by concentrations of FMLP that stimulate increased Mac-1 and p150,95 surface expression (28). More work is necessary to clarify the relationship of these different organelles to the peroxidase-negative granules studied here. In work with Dr. Margaret Hibbs (29) using an affinity-purified antibody to gelatinase, we have colocalized gelatinase-positive granules within a population of lactoferrin-positive granules. It would be helpful if more attention were paid to lactoferrin in cell fractionation studies, so that the results obtained by cell fractionation could be better integrated with those obtained by immunocytochemical techniques. Unfortunately, there are not yet any antibody probes for the B<sub>12</sub>-binding proteins. It should be noted that, when demonstrated by immunocytochemical techniques, lactoferrin is present in only 80–90% of specific granules. It is not clear whether this reflects a limitation in the method of detection or an absence of lactoferrin in some granules. We found, similarly, that not all of the specific granules were labeled for LAR, but it was clear in the double-labeling experiment that most specific granules contained both.

The present studies, together with work on the extracellular matrix receptors such as the fibronectin receptor and platelet GPIIb-IIIa, which are structurally homologous to LAR (3), suggest several distinct mechanisms for regulating adhesiveness during differentiation and activation of hematopoietic cells. Our findings and the previous localization of Mac-1 by others (8, 30) suggest that Mac-1 and p150,95 are synthesized during the myelocyte stage of maturation, since that is when specific granules are formed (15). As already discussed, small amounts of Mac-1 and p150,95 are present on the plasma membranes of mature circulating PMN and monocytes. Chemoattractant agents can cause a significant upregulation of these proteins, i.e., stimulate partial mobilization to the cell surface of their intracellular granular pools, facilitating adherence of these leukocytes to endothelial cells and subsequent migration to sites of local inflammation (1). Qualitative changes, in addition to quantitative changes, may be

important in regulating adhesive function in LAR expression (31). On the other hand, the fibronectin receptor appears, and is functional, early in erythroid maturation: cells from proerythroblasts through reticulocytes bind to fibronectin-coated surfaces (32). Upon entering the circulation, reticulocytes quickly lose this receptor because of events occurring in the spleen, and mature circulating red blood cells do not bind to fibronectin-coated surfaces (33). Finally, GPIIb/IIIa, the adhesion receptor on platelets that binds fibrinogen, fibronectin, and von Willebrand's factor, does not function through downregulation or upregulation. This receptor appears very early in maturation on committed stem cells (34), is present at all stages of megakaryocyte development, and is present on platelets that are circulating in the presence of its ligands. It binds its ligands only after platelet activation with an agonist such as ADP or thrombin, which presumably causes conformational change in the receptor (35). As more adhesion molecules of this supergene family of adhesion proteins (3) and their ligands are discovered, it should be interesting to see whether they conform to the model of downregulation or disappearance of a functionally important receptor, upregulation from an intracellular pool allowing adhesion, activation causing the receptor to become competent to bind its ligand, or combinations of these models.

### Summary

Previous studies have suggested that the leukocyte adhesion proteins Mac-1 and p150,95 are stored in a latent intracellular pool in neutrophils, and cellular fractionation studies have shown that Mac-1 is localized primarily in the peroxidase-negative specific granules. To determine the subcellular location of leukocyte adhesion receptors (LAR), we used immunocytochemical techniques on frozen thin sections of human blood leukocytes that had been incubated for peroxidase to mark the peroxidase-positive azurophil granules. To enhance the sensitivity of detection, polyclonal antibodies against immunoaffinity-purified p150,95 were raised in rabbits and absorbed with leukocytes from a patient deficient in this protein. The antiserum reacted with p150,95 and two other antigens with the same  $\beta$  subunit, Mac-1 and lymphocyte function-associated antigen 1 (LFA-1). In neutrophils, we observed immunogold label for LAR predominantly on the membranes of peroxidase-negative granules, and in smaller amounts on the plasma and perinuclear membranes. In double-label experiments, there was colocalization of LAR with lactoferrin in some of the peroxidase-negative granules. We conclude that the latent pool of LAR resides in the membranes of peroxidase-negative granules. A significant increase in label on the plasma membrane of neutrophils stimulated with PMA is consistent with secretion of LAR to the exterior of the cell during degranulation. While LFA-1 appears very early in neutrophil maturation, it is becoming clear that Mac-1 and p150,95 are upregulated from an intracellular storage pool of peroxidase-negative granules that appear during the myelocyte stage of differentiation. Further studies are indicated to determine the significance of these proteins on the plasma membrane of two other granulocytes, eosinophils and basophils.

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