Ultrastructural Localization of Lactoferrin and Myeloperoxidase in Human Neutrophils by Immunogold

By Elisabeth Cramer, Katherine B. Pryzwansky, Jean-Luc Villeval, Ugo Testa, and Janine Breton-Gorius

Colloidal gold was used as a marker for immunoelectron microscopy to localize lactoferrin (LF) and myeloperoxidase (MPO) in human peripheral blood neutrophils. Cells were reacted with monospecific antibodies against LF or MPO and then with gold-labeled antiglobulin. MPO cytochemistry was also associated with immunologic detection of LF. Immunologic labeling of thin sections after embedding in glycol methacrylate gave good ultrastructural morphology and specific localization of both proteins. MPO was detected in the large azurophil granules, whereas LF was consistently localized in the matrix of another population of morphologically distinct granules, smaller and more numerous than azurophil granules. When cytochemical detection of MPO was coupled with immunologic detection of LF, LF was observed in the population of MPO-negative granules, which were identified as specific. This was confirmed on cells that were permeabilized with saponin and stained for LF and MPO before embedding. No other neutrophil organelles displayed labeling for LF; other blood cells also were unreactive for LF. In the bone marrow, myeloblast and promyelocyte granulations were not stained and LF-containing granules appeared at the myelocyte stage. In conclusion, we confirm previous biochemical and light microscopic studies by ultrastructural demonstration of LF and MPO in two categories of granules, the specific and azurophil granules, respectively. The method described in this article avoids disruption caused by cell fractionation procedures. In the future, other intragranular proteins can be localized by a similar approach.

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HUMAN peripheral blood neutrophils contain two main populations of cytoplasmic granules that are formed in the bone marrow at different stages of maturation.1,2 The azurophil granules are packaged in promyelocytes and are identified by light and electron microscopy by their peroxidase activity1,3; specific granules are formed later, during the myelocyte stage, and have been categorized by electron microscopy by an absence of peroxidase activity. Biochemical analysis of purified granules separated by density centrifugation also distinguishes two main granule classes4,5: (1) a fast-moving population of large granules that contain myeloperoxidase (MPO) and lysosomal enzymes and (2) a slower-sedimenting population of granules that lack MPO and lysosomal enzymes but contain lactoferrin (LF). The presence of LF in specific granules has been confirmed by immunocytochemistry performed by light microscopy.7,9 Using antibodies against MPO and LF, it has been shown that MPO is present in promyelocytes. LF is not present in promyelocytes and is observed in myelocytes in which, as described by electron microscopy, specific granulations appear.

An ultrastructural report10 has presented contrary data suggesting that LF is localized in a subpopulation of azurophil MPO-containing granules. This report has clouded previous biochemical and immunologic data. Therefore, we investigated the localization of LF and MPO by an immunogold staining technique at the ultrastructural level. The advantages of this method are that the integrity of the cell is preserved and the artifacts resulting from cell fractionation techniques are avoided. Colloidal gold is used as a marker because of its resolving power, electron density, and ability to combine with cytochemistry.11,12 MPO and LF were localized in two types of granules as selective markers for azurophil and specific granules, respectively. These data confirm biochemical studies on granules separated by gradient centrifugation.

MATERIALS AND METHODS

Cells

Neutrophils were isolated by dextran-Radioselectan sedimentation from heparinized human venous blood as previously described.1,1 The three blood samples were examined. One volume of blood was layered over 1 vol of a mixture comprising 24 vol of 9% dextran T 500 (Pharmacia, Uppsala, Sweden) in 0.9% NaCl and 10 vol of 38% Radioselectan (Sherring Lab, Lys-lez-Iannoy, France). After sedimentation of red blood cells for 40 minutes at 22 °C, the leukocyte-rich supernatant was removed and centrifuged at 300 g for eight minutes. The cell pellet was resuspended and washed three times in Hanks’ balanced salts solution, fixed in 1% glutaraldehyde in 0.1 mol/L IRIS HCI buffer, pH 7.2, for one hour. Cells were postfixed one hour in 1% glutaraldehyde at 4 °C for 30 minutes and processed for peroxidase cytochemistry14 in a medium containing 5 mg 3’-3’ diaminobenzidine (Sigma Chemical Co, St Louis) and 100 µL 1% H2O2 in 10 mL 0.05 mol/L TRIS HCl buffer, pH 7.2, for one hour. Cells were posixed for one hour in 1% glutaraldehyde at 22 °C and washed three times in phosphate buffer. Cells were then embedded in glycol methacrylate (GMA) according to the method of Leduc and Bernhard.15 The final embedding medium was composed of seven parts 97% GMA (Rohm and Haas, Darmstadt, West Germany) in 3% distilled water and three parts nondestabilized butyl methacrylate containing 1%

From INSERM U 91, Hôpital Henri Mondor, Creteil, France, and the Department of Pathology, University of North Carolina, Chapel Hill.

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Address reprint requests to Dr. E. Cramer, INSERM U 91, Hôpital Henri Mondor, 94010 Creteil, France.

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benzoyl peroxide (Merck, Darmstadt, West Germany). The mixture was partially polymerized by heat before use. Polymerization of the embedded cells was achieved at 4 °C under ultraviolet light from a type-A 405 lamp (PW Allen and Co., London, England) containing Philips 6-watt fluorescent tubes, color type D5. Thin sections were collected on nickel grids coated with formvar.

Bone marrow cells were obtained from two normal bone marrow graft donors. Heparinized sera were enriched in immature granulocytes by Ficoll-metrizoate density gradient centrifugation (density, 1.077 g/cm^3) and further treated like peripheral blood neutrophils.

Neutrophil monolayers were prepared by immersing formvar-coated cover glasses at a 45° angle in heparinized blood for 30 minutes. The adherent cells were placed in 35-mm Petri dishes in Gey's balanced salt solution.

**Antiserum**

Antisera against human MPO were developed in rabbits. MPO was purified from human peripheral blood neutrophils as previously reported and was 98% purified as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IgG fraction was obtained by chromatography on DEAE-AffiBlue gel (Biorad, Richmond, Calif). Rabbit antiserum was absorbed several times with human lymphocytes and red blood cells to remove heterophilic antibodies. Antiserum specificity and titer (1:50) were evaluated by Ouchterlony immunodiffusion technique.

Two antibodies to LF were used. Rabbit antiserum raised against human LF was purchased from Cappel Laboratory Inc (Downington, Pa, lot No. 17066) and used at 1:120 dilution. In addition, F(ab)2, affinity-purified antibody to LF was used. Specificity for these antisera has been reported.

Goat anti-rabbit IgG coupled to 5 nm colloidal gold (Janssen Pharmaceutica, Beerse, Belgium) diluted 1:10 or 20 nm colloidal gold (EY Lab, San Mateo, Calif) diluted 1:2 was used as the electron marker. The immunogold was diluted in 0.02 mol/L TRIS-buffered saline (TBS), pH 8.2, containing 1% bovine serum albumin.

**Postembedding Immunocytochemical Staining of MPO**

Immunocytochemical labeling of the sections was performed identical to that for LF. Samples reacted and not reacted for peroxidase cytochemistry were used; the sections were stained with anti-MPO at a 1:50 dilution.

**Quantitative Analysis of Labeling on the Subcellular Compartments**

The intensity of labeling with gold particles in subcellular compartments was quantitatively evaluated. Five micrograms from two samples incubated with the same concentration of antibodies were taken at a primary magnification of 10,000× and enlarged three times. They were put under a transparent paper with lattice according to the method of Weibel et al. The surface area of azurophil granules, specific granules, nucleus, mitochondria, and cytoplasm was examined. The labeling density was expressed as the number of gold particles per μm².

**Preembedding Immunocytochemistry of LF**

Monolayers of neutrophils were permeabilized with 1 mL each of 0.05% saponin, 0.05% paraformaldehyde, and 0.06% glutaraldehyde in a microtubule stabilizing buffer (60 mmol/L Pipes, 10 mmol/L EGTA, 2 mmol/L MgCl₂ in Gey's balanced salts, pH 7.0). The permeabilizing fixative was added at 37 °C, and the cells were immediately placed on ice for three minutes. Cells were washed three times in the buffer, stained with antibody to LF, and incubated overnight at 22 °C. The next day, cells were washed with 50 mmol/L Na₂HPO₄ containing 0.3 mg/mL polystyrene glycol (mol wt 20,000), stained with 20 nm goat anti-rabbit IgG gold complex (EY Lab), and incubated at room temperature for one hour. Cells were washed and then fixed with 1.25% glutaraldehyde for ten minutes, washed in TBS, reacted or not reacted for peroxidase cytochemistry, postfixed in 1% OsO₄ for two minutes, dehydrated in ethanol, and embedded in Epon. Sections were cut parallel to the plane of cell attachment.

**Controls**

To demonstrate the specificity of the staining, the following controls were performed: (1) replacement of the antisera with normal rabbit serum and (2) immunoabosorption of the anti-LF by affinity chromatography. The effluent was applied to the sections at the appropriate dilution.

**RESULTS**

**Postembedding Immunocytochemistry**

Glutaraldehyde fixation of cells embedded in GMA without osmication resulted in an overall good preservation of cellular organelles, with the exception of the occasional loss of material from the azurophil granules.
ules, which made them look lighter than the other granules. This particular trend of azurophils to be extracted has been pointed out in earlier electron microscopic studies. Specific granules, which are smaller and more numerous than azurophils, appeared better preserved and more electron dense than the azurophils (Fig 1A). Mitochondria, endoplasmic reticulum, perinuclear cisternae, and nuclei were easily recognized using the procedure.

Myeloperoxidase

MPO localization in neutrophils by immunogold was consistently observed in the population of large granules. These granules were identified as azurophils according to their number, size, and reaction to MPO (Fig 1B). Gold particles were numerous over the non-extracted part of the granules and were found in both spherical and ellipsoid granules. However, owing to extraction of material from the granule, we could not identify the crystalloid structure inside any of the labeled granules. A few gold particles were sometimes present on the remaining granules, cytoplasm, and nucleus; this corresponded to background staining of the preparation. Table 1 summarizes labeling intensity of each cellular compartment. Eosinophil, lymphocyte, and platelet granules were not labeled. Sections of neutrophils that were first reacted for MPO cytochemistry were then stained by anti-MPO and immunogold; gold labels were observed in MPO-positive granules over the benzidine precipitate (Fig 3C). We thus concluded that benzidine reaction product does not mask MPO antigenic sites. When anti-MPO was replaced with normal rabbit serum, followed by antibody coupled to gold, no staining of neutrophils was observed (Fig 1A).

Lactoferrin

Peripheral blood neutrophils. LF localization by immunogold was observed in all blood neutrophils over the matrix of specific granules, which were smaller and denser than azurophils and often dumbbell-shaped (Fig 1C). The ratio of LF-labeled specific granules to azurophil granules was approximately 3:1. The two main forms of specific granules, spherical and dumbbell-shaped, were equally and intensely labeled. Azurophil granules were not labeled, including the zone of the granule-containing dense material. No other neutrophil organelles were labeled (Fig 2), including the plasma membrane, nucleus, perinuclear cisternae, mitochondria, and endoplasmic reticulum; background staining was lower than for anti-MPO staining. Similarly, eosinophil, monocyte, lymphocyte, and platelet granules were not stained. Table 1 summarizes the labeling intensity for LF of the different cellular compartments.

Neutrophils that were first reacted for MPO cytochemically and then incubated with anti-LF and immunogold (Fig 3A and B) were labeled exclusively in MPO-negative granules; some granules were dumbbell-shaped and resembled specific granules. There was insignificant labeling of LF in the large MPO-positive granules, although we have shown that by simultaneous enzymatic and immunologic detection of MPO, antigenic sites were not masked by benzidine reaction product and that gold particles can be visualized over electron-dense structures (Fig 3C). When antiserum was replaced by normal rabbit serum (Fig 1A), no labeling was observed. In addition, antiserum absorbed by affinity chromatography demonstrated no labeling of specific granules in neutrophils.

Bone marrow neutrophils. Immunogold staining of LF performed on bone marrow neutrophils showed that myeloblasts and promyelocytes granules were all devoid of LF (Fig 4A). Perinuclear cisternae, endoplasmic reticulum, and Golgi complex were not stained.

At the myelocyte stage, a new population of smaller and intensely labeled granules appeared beside the large azurophil granules (Fig 4B). Occasionally spherical granules with concentric rings in their matrixes were stained for LF. The perinuclear cisternae and endoplasmic reticulum were not labeled. Gold particles were sometimes more numerous in the Golgi area, but it was not possible to determine whether this was specific labeling of the Golgi complex.

Preembedding Staining of LF

Because Parmley et al., using an immunocytochemical technique performed on permeabilized cells, claimed that LF was present in azurophil granules, we also used a preembedding technique with a similar principle to compare results. Optimal concentration of saponin and exposure of neutrophils to the permeabilizing fixation was first determined by immunofluorescence.

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Table 1. Labeling Density of Subcellular Compartments*

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Nucleus</th>
<th>Specific Granules</th>
<th>Azurophil Granules</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>31.71 ± 16.50</td>
<td>50.29 ± 27.46</td>
<td>494.00 ± 125.91</td>
<td>33.65 ± 24.25</td>
<td>40.24 ± 28.37</td>
</tr>
<tr>
<td>LF</td>
<td>3.84 ± 3.75</td>
<td>833.40 ± 102.12</td>
<td>21.44 ± 18.72</td>
<td>2.42 ± 2.21</td>
<td>9.83 ± 6.27</td>
</tr>
</tbody>
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MPO, myeloperoxidase; LF, lactoferrin.

*Gold particles per μm² ± SEM
Fig 2. Postembedding procedure for LF localization in peripheral blood neutrophils. Lower magnification of a neutrophil stained for LF as in Fig 1C. Specific granules (s) are intensely labeled. Azurophil granules (a), nucleus (n), perinuclear cisternae (arrow), plasma membrane (pm), and mitochondria (m) do not display specific staining (original magnification ×25,140).

cence microscopy (not shown). A granular cytoplasmic staining of neutrophils after permeabilization was considered optimal. It should be noted that longer exposure or higher concentrations of saponin (optimal three-minute exposure to 0.05% saponin) caused diffuse staining of the cytoplasm, nuclear staining, or no staining. Higher concentration of fixatives in the permeabilization solution as well as incubating the cells at 4 °C suppressed permeabilization.

The ultrastructure of saponin-treated neutrophils was fair (Fig 5A); cell shape was unaltered, and the nucleus and mitochondria were well preserved. The plasma membrane and perinuclear cisternae were not recognizable. When immunologic staining of LF was performed on such treated cells, a population of large, well-limited, although partially extracted granules was identified, resembling azurophils in their size and number, and no labeling for LF was observed on their matrix. Numerous small granules were intensely labeled by gold; their size and number led us to conclude that they correspond to specific granules. The membrane of specific granules seemed to be more sensitive to permeabilization than the membrane of azurophil granules. The nucleus and mitochondria were never labeled. The other blood cell granules (eosinophils, monocytes, lymphocytes, and platelets) were not labeled. When cells immunologically stained for LF were then reacted for peroxidase cytochemistry, no gold labels were seen in MPO-positive azurophil granules, but they were observed in the small MPO-negative specific granules (Fig 5B).

DISCUSSION

We have confirmed by immunoelectron microscopy that LF and MPO are markers for the specific and azurophil granules, respectively. LF was localized in the specific granules of neutrophils using two staining techniques (preembedding and postembedding) and two sources of antibody. MPO was observed in azurophil granules by both cytochemical and immunocytochemical methods. Although the immunoreactivity of LF was satisfactory in the preembedding technique,
the ultrastructure of the granules was poor, despite careful adjustment of saponin concentration to ensure membrane permeabilization and granule preservation.

Postembedding staining was better than preembedding staining because the ultrastructure was adequately preserved. When Epon was used as an embedding medium, no labeling of LF was obtained. Embedding in GMA retained LF and MPO antigenicity. Unlike Epon, GMA is a hydroxysoluble embedding medium that avoids the use of organic solvents and allows polymerization at 4 °C. GMA has been used with success for demonstration of other antigens.22,23

We chose colloidal gold as a labeling agent because of its electron density and size, which permit good visibility of the immunoreactive site. Also, nonspecific background staining is minimal. These properties make gold a more convenient marker than ferritin. In addition, the high resolution of gold makes it a better tool than immunoperoxidase for high-resolution detection of intracellular antigens.24 Moreover, as shown in this study, gold permits cytochemistry and immunocytochemistry to be performed on the same sample, and simultaneous labeling of different cell antigens using different-size gold particles.25 Finally, immunocytochemical methods using colloidal gold as a marker have been successfully applied to the ultrastructural localization of numerous intracytoplasmic proteins.11,19,26

Our results concerning localization of LF and MPO are in agreement with previous biochemical studies on subcellular fractionation of human neutrophils4 and immunocytochemical studies at the optical level.7 9 Also, in support of the coincident localization of LF in specific granules, patients have been described with absence of eosinophils. Also, future use of monoclonal antibodies against peroxidase may identify poorly differentiated cells according not only to the presence and localization of a peroxidatic activity, but to its nature as well (platelet peroxidase,35 macrophage peroxidase,36,37 peroxidase from hairy cells,38 or fibroblasts39). The subcellular localization of other granular proteins in the azurophil granules of human neutrophils confirms numerous cytochemical and biologic works. This new approach of enzyme localization, however, will permit identification of the cell lineage, according to the different immunologic reactivity of distinct apoproteins with the same cytochemical peroxidase activity (for example, eosinophil vs neutrophil). Also, future use of monoclonal antibodies against peroxidase may identify poorly differentiated cells according not only to the presence and localization of a peroxidatic activity, but to its nature as well (platelet peroxidase,35 macrophage peroxidase,36,37 peroxidase from hairy cells,38 or fibroblasts39).

We did not observe LF associated with nuclear lobes or perinuclear cisternae, as found by others using immunofluorescence,31 and we agree that this finding corresponds to a fixation artifact.32 To isolate neutrophils, a dextran-radioselactan sedimentation technique13 was used in which neutrophils are not in contact with macromolecular medium in order to avoid possible degranulation. Using these conditions, no staining of the plasma membrane for LF was observed, in contrast to that of activated neutrophils.18

We have also confirmed our results by studying LF in bone marrow granulocyte precursors; myeloblasts and promyelocytes, although labeled by anti-MPO (not shown) were not stained by anti-LF, which stained more mature granulocytic cells (myelocytes). Moreover, we have used phorbol myristate acetate, a potent cocarcinogen which induces the selective exocytosis of specific granules in neutrophils33 and we have observed a drastic decrease in the number of labeled intracytoplasmic granules and localization of LF in vacuoles.34

The immunocytochemical localization of MPO in the azurophil granules of human neutrophils confirms numerous cytochemical and biologic works. This new approach of enzyme localization, however, will permit identification of the cell lineage, according to the different immunologic reactivity of distinct apoproteins with the same cytochemical peroxidase activity (for example, eosinophil vs neutrophil). Also, future use of monoclonal antibodies against peroxidase may identify poorly differentiated cells according not only to the presence and localization of a peroxidatic activity, but to its nature as well (platelet peroxidase,35 macrophage peroxidase,36,37 peroxidase from hairy cells,38 or fibroblasts39). The subcellular localization of other granular proteins in the azurophil granules of human neutrophils confirms numerous cytochemical and biologic works. This new approach of enzyme localization, however, will permit identification of the cell lineage, according to the different immunologic reactivity of distinct apoproteins with the same cytochemical peroxidase activity (for example, eosinophil vs neutrophil). Also, future use of monoclonal antibodies against peroxidase may identify poorly differentiated cells according not only to the presence and localization of a peroxidatic activity, but to its nature as well (platelet peroxidase,35 macrophage peroxidase,36,37 peroxidase from hairy cells,38 or fibroblasts39).
proteins can be studied by the same technique: we have already been able to show lysozyme in azurophil granules in a study that is being completed.

Finally, both biochemical and ultrastructural analyses are important in studying the various populations of neutrophil granules. However, the advantage of this method over biochemical analyses of separated granules is that the integrity of the cell is preserved and the risk of artifact is decreased. Ultrastructural studies also have the advantage of analyzing the granules in relationship to other organelles, without disruption caused by cell fractionation procedure.

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Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold

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