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Morphological heterogeneity of myeloperoxidase-positive granules in normal circulating neutrophils: an ultrastructural study by cryosection

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Summary. Ultrastructural localization of myeloperoxidase (MPO) in the granules of human circulating neutrophils was examined by cryosection. On careful comparison with the morphological characteristics of the granules by conventional transmission electron microscopic study, large MPO-positive granules were divided into five types by immunocryoultramicrotomy using monoclonal antibody. Double staining of MPO and lactoferrin (or lysozyme) was also performed. Lactoferrin was generally detected in MPO-negative granules. Lysozyme immunostaining was present in MPO-positive and -negative granules. These data may suggest different functions among large MPO-positive granules of human circulating neutrophils.

Key words: Myeloperoxidase, Lactoferrin, Lysozyme, Immunocryoultramicrotomy, Electron microscopy

Introduction

The granules of circulating normal human neutrophil are generally classified into two groups at light or electron microscopic level; azurophil (or primary) and specific (or secondary) granules (Breton-Gorius, 1966; Bainton and Farquhar, 1966). The former are characterized by the presence of myeloperoxidase (MPO) whose messenger-RNA is mainly detected at the promyelocytic stage and the absence of lactoferrin (Lf) (Fouret et al., 1989). On the other hand, the latter, which are smaller, contain Lf whose messenger-RNA is mainly detected at the metamyelocytic stage, but not MPO (Fouret et al., 1989). Lysozyme (Ly) is contained in both types of granules (Cramer and Breton-Gorius, 1987). Therefore, the detection of intragranular MPO, Lf or Ly is important in order to distinguish neutrophilic granules. By electron microscopy, primary granules are recognized to be heterogeneous in electron density, size and shape (Ackerman and Clark, 1971). In general, the ultrastructural localization of MPO has been performed employing a cytochemical technique (Graham and Karnovksy, 1966). However, using this technique, the homogeneous chemical product induced by the cytochemical reaction makes it difficult to identify intragranular structure. Moreover, it is not easy to precisely pinpoint which granules observed by conventional transmission electron microscopy are MPO positive (MPO^+). To overcome these disadvantages, an ultrastructural immunogold staining, by which the structure can still be observed even after positive reaction for detecting intragranular proteins, has been developed. Intragranular proteins are immunologically detected by several post-embedding immunogold staining methods, in which fixed cells are embedded in medium such as Lowicryl K4M (Valentino et al., 1985), LR White (Esaguy et al., 1989) or glycol methacrylate (Cramer et al., 1985). However, few monoclonal antibodies show good staining using these post-embedding techniques because of loss of antigenicity during fixation, embedding and staining. Using cryosections, this shortcoming has been overcome by a new technique known as immunocryoultramicrotomy by which the antigenicity is retained and the subcellular structure can be observed (Saito et al., 1992).

In this study, we observe the ultrastructural characteristics of MPO^+ granules of circulating normal human neutrophils by means of immunocryoultramicrotomy. The double stainings by MPO and Lf (MPO Ly, Lf and Ly) are also performed.

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Materials and methods

Preparation of cells for immunocryoultramicrotomy

Peripheral blood cells of the buffy coat from 8 normal volunteers were used for immunocryoultramicrotomy as previously reported (Saito et al., 1992). Briefly, the cells were fixed in a mixture of 0.5% glutaraldehyde, 2% paraformaldehyde and 0.32% picric acid in 0.05M phosphate buffer (pH 7.4) containing 1.5% sucrose for

30 min at 4 °C. After washing with 0.1M phosphate buffer (pH 7.4), the cells were suspended in a solution of human plasma and $CaCl_2$, followed by addition of thrombin. The cells were centrifuged and the pellet was incubated in 0.5% paraformaldehyde in 0.05M phosphate buffer (pH 7.4) containing 8% sucrose for 24 hours at 4 °C. The cells were then stored in 0.1M phosphate buffer (pH 7.4) containing 2.3M sucrose for a few days before cryosectioning.

Making of ultrathin frozen sections and immunostaining of MPO

Samples were cryosectioned and immunostained as previously reported (Saito et al., 1992). Briefly, small blocks of cell samples were mounted on a specimen carrier and rapidly frozen in liquid nitrogen. Ultrathin frozen sections were made using a cryosectioning system (ULTRACUT FC4E, Reichert-Jung) at -95 °C. The ultrathin frozen sections were carried onto a Formvar-carbon coated grid with a droplet of the solution containing sucrose, gelatin and glycerol. The grid was floated on 0.1% gltuaraldehyde in 0.05M phosphate buffer (pH 7.4) for 5 min at 20 °C to enhance the adherence of the sections to the surface and then on 0.01M phosphate-buffered saline (PBS) containing 0.02M glycine for 10 min to neutralize any residual aldehyde. After washing with PBS, the grid was preincubated in 1% normal goat serum and then allowed to react with appropriately diluted mouse IgG fraction specific for human myeloperoxidase (MPO, Dakopatts) for 12 hours at 4 °C. After washing with saline, the grid was incubated in 10 nm gold particle-labeled goat antiserum specific for mouse IgG (GAM G10, Amersham). The ultrathin sections were fixed in 2% glutaraldehyde in 0.05M phosphate buffer (pH 7.4) after washing with saline, and then rapidly dehydrated in single changes of 60% and 80% ethanol. Finally, the sections were embedded in Spurr epoxy resin (TAAB) and polymerization was achieved in 24 hours at 60 °C. After hardening, the sections were rinsed in 10% methanol for 10 minutes, and stained with both uranyl acetate and lead citrate. As negative control, the primary antibody was omitted in the procedure. To classify MPO^+ granules, 500 MPO^+ granules were examined.

Double staining of MPO and Lf (MPO and Ly) by cryosection

After the sections had been immunostained with MPO antiserum and GAM G10, these were floated on 1% bovine serum albumin in 0.05M phosphate buffer (pH 7.4) for 15 min to prevent nonspecific reaction. Then, appropriately diluted rabbit IgG fraction specific for human Lf (Cappel) or Ly (Dakopatts) was allowed to act with the ultrathin section for 90 min. After washing with saline, 20 nm gold particles-labeled goat antiserum specific for rabbit IgG (Biocell) was used as the secondary antibody. Following washing with saline, the sections were fixed in 2% glutaraldehyde in 0.05M phosphate buffer (pH 7.4), and embedded in Spurr (Polysciences) solution as described above. For negative control, the procedure was carried out without rabbit IgG fraction specific for human Lf (or Ly). Fifty neutrophils were examined.

Double staining of Lf and Ly by post-embedding method

Peripheral blood cells from 2 normal volunteers were used for double staining by post-embedding method. The buffy coat was fixed in a mixture of 1% glutaraldehyde and 1% paraformaldehyde in 0.05M phosphate buffer (pH 7.4) containing 1.5% sucrose for 30 min at 4 °C. After washing with 0.1M phosphate buffer (pH 7.4), the cells were dehydrated and embedded by Lowicryl K4M (Polysciences). Ultrathin section was made and double staining of Lf and Ly was performed according to the double staining method as previously reported (Saito, 1990). Briefly, ultrathin sections were mounted on nickel grids. The rabbit IgG fractions specific for human Lf (Cappel) and Ly (Dakopatts) were used as specific antisera for the staining. Firstly, the sections were rinsed in the antiserum for Lf followed by 15 nm gold particle-labeled goat antiserum specific for rabbit IgG (Amersham). Secondly, after rinsing in appropriately diluted goat antiserum specific for rabbit IgG (Amersham) to imbibe the antiserum for Lf, the sections were reacted in antiserum for Ly. This was followed by reaction of 5 nm gold particle-labeled goat antiserum specific for rabbit IgG (Amersham). Controls were performed as normal rabbit serum (NRS) was used in place of the each anti-serum or as 5 nm gold particle-labeled goat antiserum specific for rabbit IgG was reacted at the moment after the sections were reacted with goat antiserum specific for rabbit IgG.

Conventional transmission electron microscopy

Circulating normal human neutrophils were obtained from 4 normal volunteers and fixed in 2% glutaraldehyde in 0.05M phosphate buffer (pH 7.4) for 30 min, followed by postfixation in 2% osmium tetroxide in 0.05M phosphate buffer (pH 7.4) for 1 hour at 4 °C. The specimens were dehydrated in increasing concentrations of ethanol and embedded in Epok 812 (Oken Shoji). Ultrathin sections stained with uranyl acetate and lead citrate were observed using a Hitachi H-800 electron microscope at 75 kV.

Results

In cryosections, some cells were seen in a field by electron microscopy (Fig. 1). Ultrastructural localization of MPO was demonstrated by 10 nm gold particles. The shape and intragranular structure of MPO^+ granules were heterogeneous. On careful comparison of the granular characteristics with conventional transmission electron microscopy, MPO^+ granules were hetero-

geneous. On careful comparison of the granular characteristics with conventional transmission electron microscopy, MPO^+ granules, which were 0.3~0.8 μm in diameter and could be identified the intragranular structure, were classified into five subtypes: i) spherical granules with little amorphous material at the periphery (type 1, 45.2% of MPO^+ granules, Figs. 2a, 3a). This type was mostly seen at the peripheral area of the cytoplasm; ii) those with a halo containing completely dense material (type 2, 3.8% of MPO^+ granules, Figs. 2b, 3b); iii) those which contained partially amorphous material (type 3, 3.8% of all MPO^+ granules, Figs. 2c, 3c); iv) oval granules with less central contents (type 4, 12.2% of MPO^+ granules, Figs. 2d, 3d); and v) elongated granules (type 5, 6.0% of MPO^+ granules, Figs. 2e, 3e). In addition, some large structures were also positive for MPO, but could not be considered as one type of MPO^+ granules because of morphological irregularity (Figs. 2f, 3f). In addition to these MPO^+ granules, smaller MPO^+ granular structures were observed. However, because those MPO^+ ones could not be recognized as being tangential sections of small MPO^+ granules as previously described (Pryzwansky and Breton-Gorius, 1985; Parmley et al., 1987) or as the fragments of 5 subtypes of granules, they were excluded from the classification for MPO^+ granules in this study.

In sections double stained for MPO and Lf, Lf was generally detected in MPO-negative (MPO^-) granules (Fig. 4) which were generally smaller than MPO^+ granules. Gold particles indicating Lf were also observed in a small number of type 1, type 2 and type 4 MPO^+ granules (Fig. 5). Gold particles indicating Lf were not observed in type 3 and type 5 MPO^+ granules. Both MPO^+ and Lf^+ granules were approximately 3.5% of the 500 large MPO^+ granules examined, weak as the positivity of Lf was. Granules which were neither positive for MPO nor Lf were also observed.

Ly was detected in some of type 1, type 2 and type 3 MPO^+ granules as well as in MPO^- granules (Fig. 6). Ly was not localized in either type 4 or type 5 granules. By post-embedding method, the double stainability of Lf and Ly was observed in type 1- (Fig. 7) and type 2- (not shown, see Saito et al., 1988) like granules.

No gold labeling was seen in organelles other than the granules. In controls, no labelings of 10 nm particles were observed on the sections without the antibody against MPO. No 20 nm gold particles were observed on the sections without the antibody against Lf (or Ly) by cryosections. No 15 nm gold particles (or 5 nm ones) were observed by post-embedding method without the antibody against Lf (or Ly).

Discussion

Using cryosections, large MPO^+ granules of circulating neutrophils, which the intragranular structures were identified in detail, could be classified into 5 types based on the morphological characteristics of granular structures.

Although it is possible that these 5 types of granules represent several transverse sections of a single granule, no transitional structures among these 5 MPO^+ granules

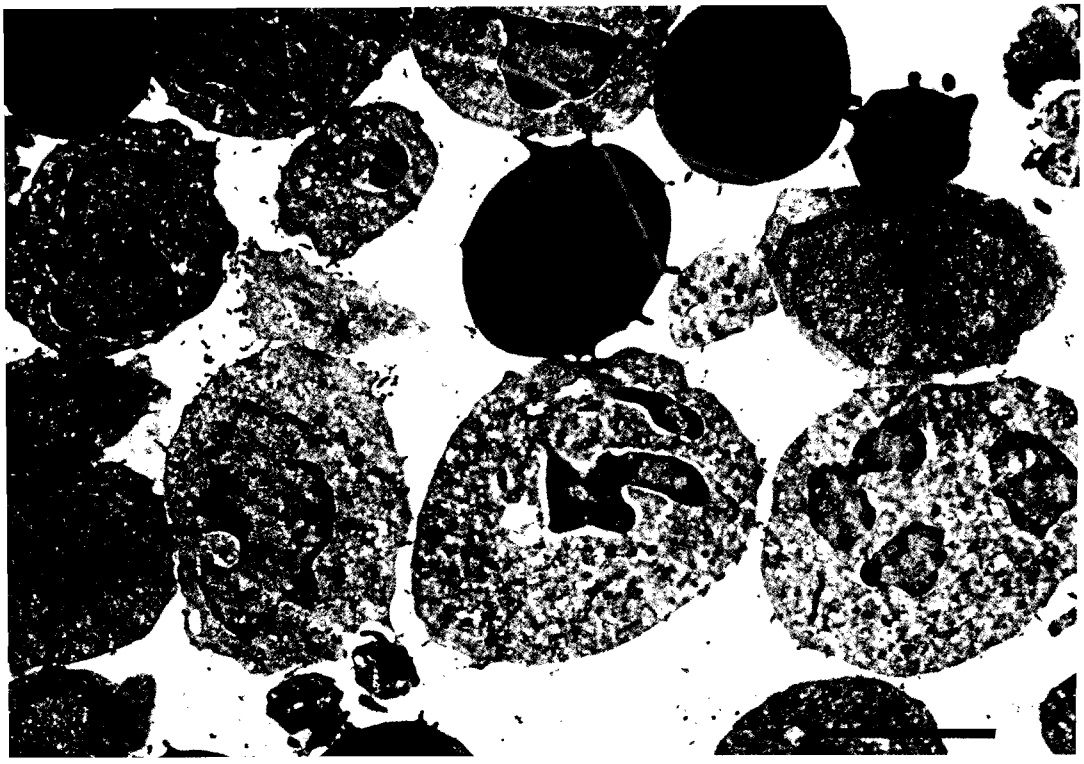

Fig. 1. Cryosection of the buffy coat of peripheral blood. Some neutrophils can be observed in a field. Bar: 5 μm.

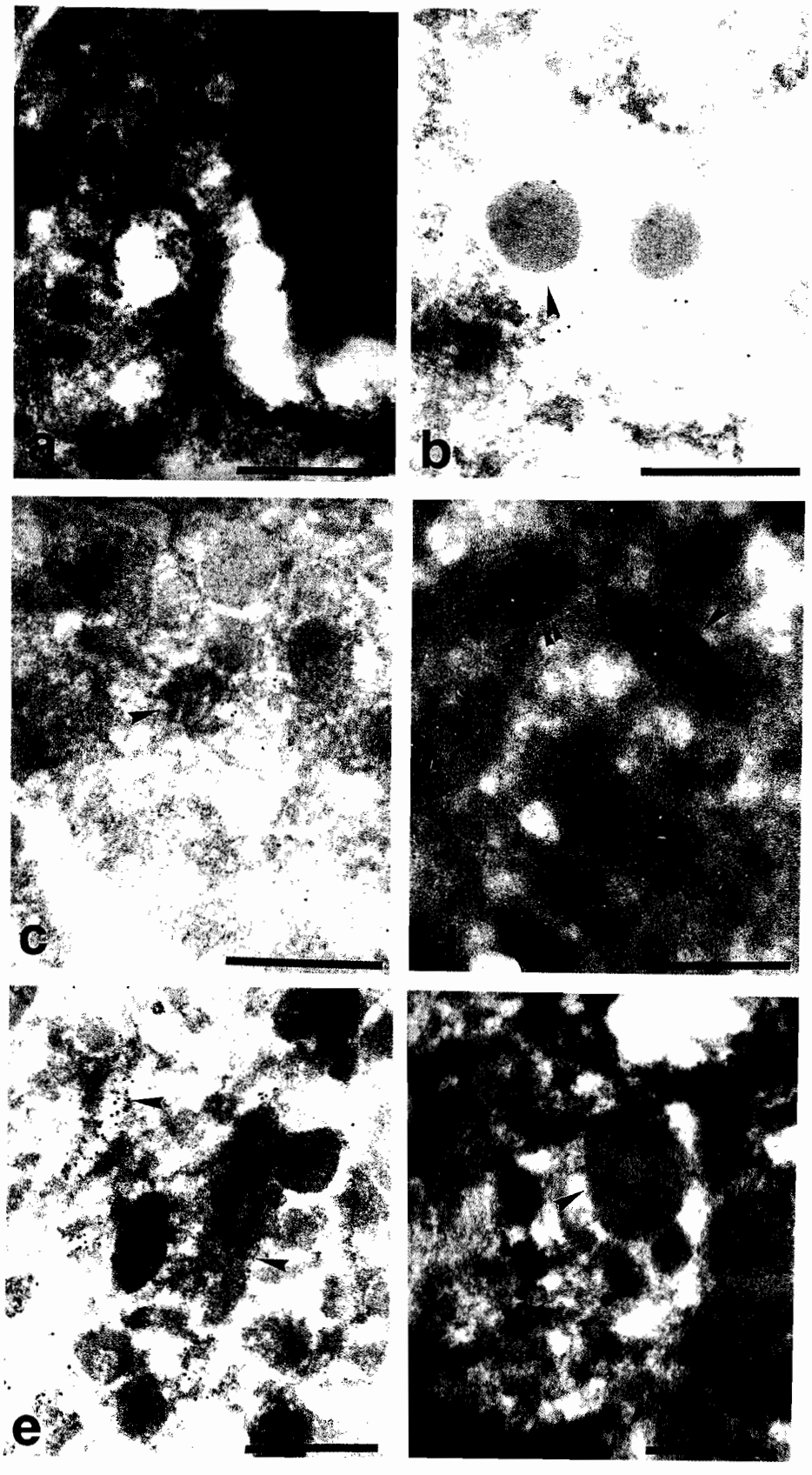

Fig. 2. Immunostaining with monoclonal antibody against MPO in cryosection. The gold particles are observed in several types of granules. Bar: 0.5 μm. **a.** Type 1 MPO$^+$ granule (arrowhead) with peripheral amorphous material. **b.** Type 2 MPO$^+$ granule with electron dense content (arrowhead). **c.** Type 3 MPO$^+$ granule with amorphous material in granule (arrowhead). **d.** Type 4 MPO$^+$ oval granules (arrowhead). A cross section (double arrowhead). **e.** Type 5 MPO$^+$ elongated granules (arrowheads). **f.** An irregularly-shaped structure positive for MPO (arrowhead).

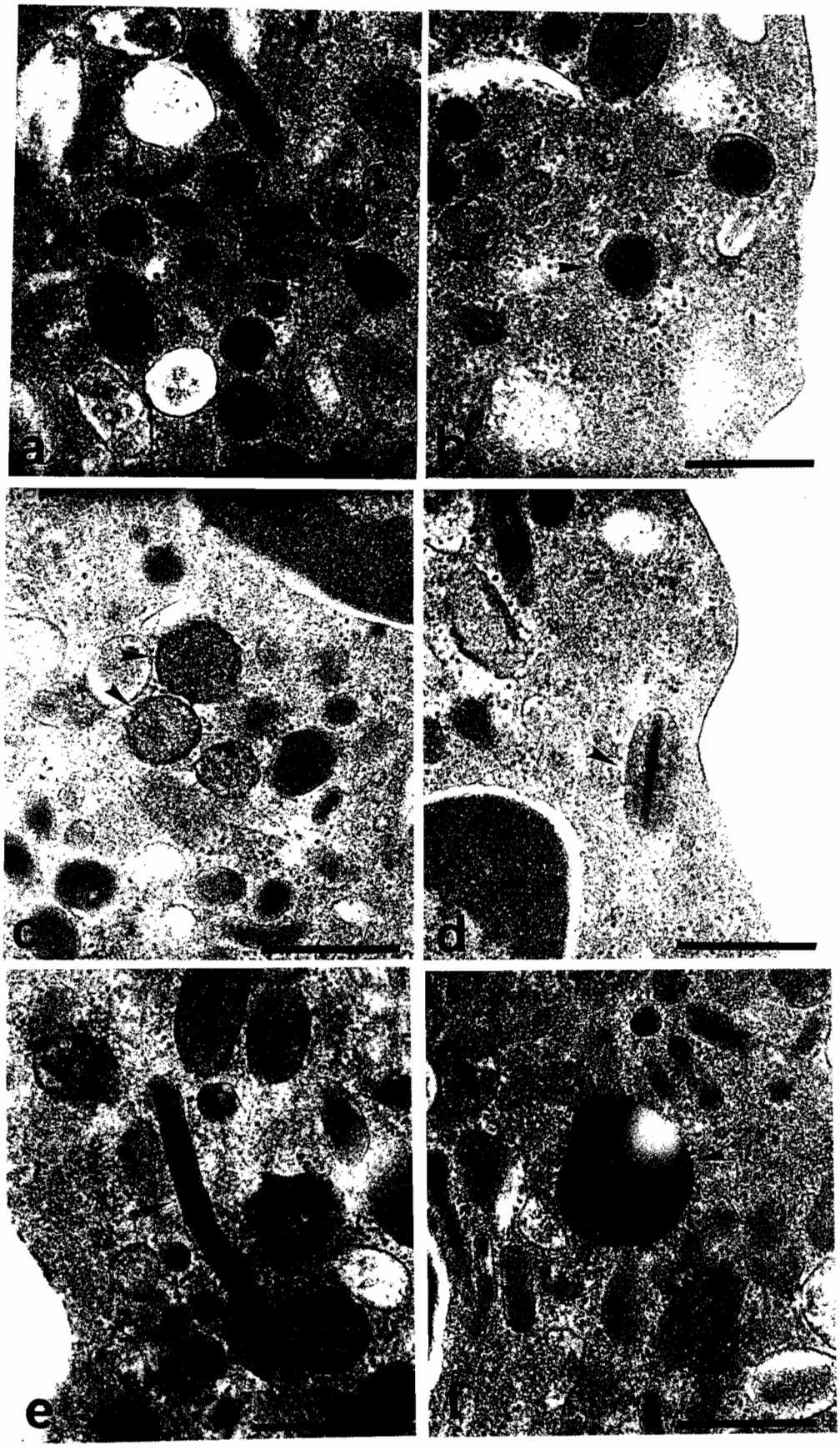

Fig. 3. Each type of MPO$^+$ granules observed by conventional transmission electron microscopy in comparison with the characteristics by immunocryoultramicrotomy. Bar: 0.5 μm. **a.** Type 1 granule shows a vacuolar-like structure (arrowheads). **b.** Type 2 granule with a homogeneous matrix of high electron density and a peripheral clear halo (arrowheads). **c.** Type 3 granules containing material of low electron density (arrowheads). **d.** Type 4 granule showing the rugby ball-like structure (arrowhead). **e.** Type 5 granule has an elongated form thickened at the ends (arrowhead). **f.** This structure is probably a phagosome or a lipid droplet (arrowhead).

were observed by conventional transmission electron microscopy. Some investigators reported that the morphological differences among MPO^+ granules were due to differences in cell maturation (Bainton et al., 1971; Brederoo et al., 1983). However, all subtypes of MPO^+ granules were observed in normal circulating neutrophils at the last stage of cell maturation in which messenger-RNA of MPO cannot be detected (Fouret et al., 1989). Therefore, it can be interfered that the categories of granular structures observed in this study are the transverse faces of different types of granules. There is a small MPO^+ granule indicated by Breton-Gorius and Reyes (1976), Parmely et al. (1987) or Egesten et al. (1994). Though such small MPO^+ granule-like structure was also observed in these cells, those were excluded from the subclassification of MPO^+ granules because of the vague structure. We have already reported that another two subpopulations than typical large MPO^+ granule exist in promyelocytic stage (Saito et al., 1995). Therefore, it was described that large MPO^+ granules of immature and mature neutrophils were divided into 7 types by electron microscopy. In addition to a small MPO^+ granule existing, the neutrophilic MPO^+ granules would be morphologically subclassified into eight kinds at least. Rice et al. (1986) divided neutrophils into 13 MPO^+ granule fractions by a density centrifugation system. A neutrophil also contains three distinct forms of MPO (Pember et al., 1982) and their insistences will line the morphological heterogeneity of MPO^+ granules in this study.

Brederoo et al. (1983) reported that type 1- and type 2-like granules are the same kind of granules in different developmental stages. However, these two MPO^+ granules were regarded to be of different types because both granules existed in the last maturating stage and because no transitional form was observed between both granules. The structure of type 4 MPO^+ granule resembled that of the nucleated granule reported by Brederoo et al. (1983), the Fibrillar Granule in our previous report (Saito et al., 1995), and the internal crystal-containing granule reported by Breton-Gorius and Reyes (1976). Both the nucleated granule (Brederoo et al., 1983) and the Fibrillar Granule (Saito et al., 1995) were observed in immature neutrophils (mainly in promyelocytes). The type 4 MPO^+ granules observed in circulating neutrophils were rather similar to the internal crystal-containing granules in which MPO activity lacked in the central area. The type 4 granule was positive for MPO except the central area, and this was consistent with the result of MPO cytochemical staining by Breton-Gorius (1976). Azakami and Eguchi (1993) reported that an elongated granule similar to type 5 MPO^+ was observed in rat neutrophils induced by recombinant human granulocyte colony-stimulating factor (G-CSF). A type 5 granule might be associated with cytokines for myelopoiesis such as G-CSF. Secretory vesicle in human neutrophils, which is an important strage site of surface membrane-bound receptors (Borregaad et al., 1987), was shown to be MPO^-. The large irregular MPO^+ structure (Figs. 2f, 3f) is considered not as a secretory vesicle but as a phagosome or a lipid droplet. Hence, large MPO^+ granules were exhibited to be classified into five types in the last maturating stage of neutrophils.

Several proteins exist in MPO^+ granules. For example, elastase seemed to be in the type 1, type 2 or

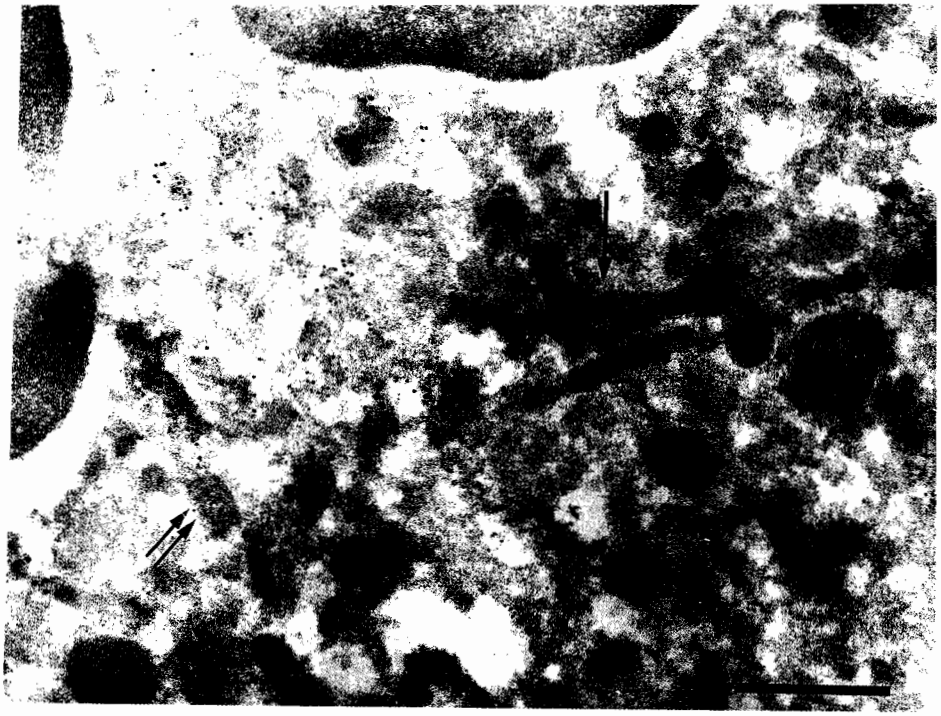

Fig. 4. Double staining of MPO and Lf in cryosection. MPO (double arrow) is generally detected in granules different from those which are Lf^+ ones (arrow). MPO, 10 nm gold particles; Lf, 20 nm gold particles. Bar: 0.5 μm.

type 3 MPO^+ granules that were classified in this study (Cramer et al., 1989; Egesten et al., 1994), cathepsin G in the type 1, type 2, type 3 and type 4 (Egesten et al., 1994), and CD 68 (Saito et al., 1991) and α_1-antitrypsin (Mason et al., 1991) were generally detected in the type 1 MPO^+ granule. CD68 was also reported to be detected in some activated T lymphocytes and natural killer cells (Hameed et al., 1994). The difference of the proteins in MPO^+ granules might be responsible for their function because the heterogeneity of MPO itself, granular morphology or intragranular proteins was recognized.

Lf is generally detected in MPO^- granules termed secondary granules (Cramer et al., 1985) and also detected in granules which are morphologically similar to a type 1 granule (Saito et al., 1993). In this study, gold particles indicating Lf were also observed in type 1, type 2 and type 4 MPO^+ granules did not exhibit true intracellular localization of Lf, it could be supposed that a slight amount of Lf had escaped into MPO^+granules during the processing. However, the labeling of Lf in MPO^+ granules by the double staining might be regarded as specific immunolabeling because of the following: (1) the concentration (x 10,000) of the antibody against Lf used was too low for making non-specific reaction; (2) gold particles were not observed in the extracellular area; (3) gold particles were not observed in the control containing no antibody against Lf; (4) gold particles indicating Lf were observed in the constant types of MPO^+ granules; and (5) the cells had been faintly fixed to prevent the relocation of intra-granular Lf as soon as ultrathin frozen sections were made. Biochemical analysis of centrifuged granular proteins of neutrophils (Spitznage et al., 1974; West et al., 1974; Wright et al., 1977; Wilde et al., 1990) and the different appearance of messenger-RNA of MPO and Lf in immature neutrophils (Tobler et al., 1988; Fouret et

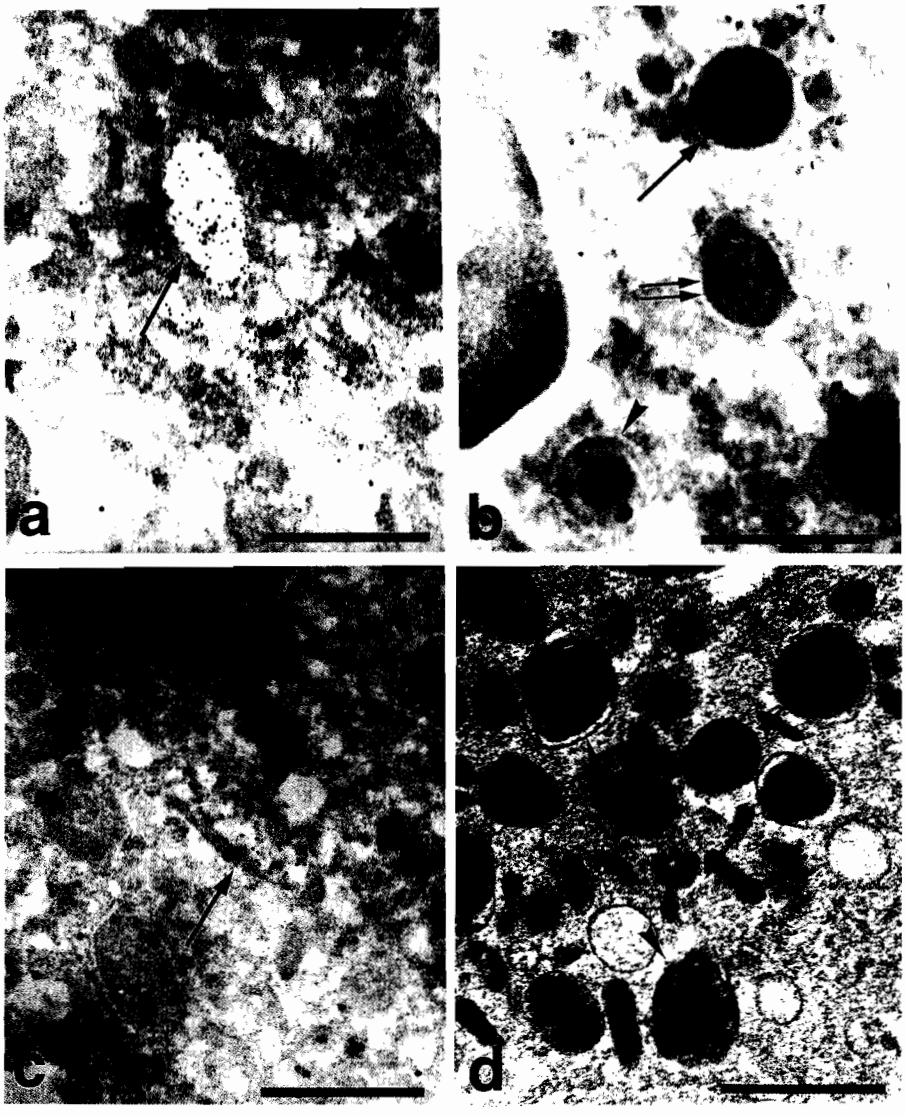

Fig. 5. Double staining of MPO and Lf in cryosection. Lf is faintly detected in type 1, type 2 and type 4 MPO^+ granules (arrows in Figs. a-c). This type 1 granule is deformed by the electron beam. Type 3 MPO^+ and Lf- granule (double arrow) is also seen (Fig. b). A granule (arrowhead) in Fig. b, indicated by the arrowhead of Fig. d by conventional transmission electron microscopy, contains neither MPO nor Lf. MPO, 10 nm gold particles; Lf, 20 nm gold particles. Bar: 0.5 μm.

al., 1989) have given support to the hypothesis that Lf is one of the good markers for the MPO^- granules (Bainton and Farquhar, 1966; Cramer et al., 1985). However, the biochemical studies also reported that a small quantity of MPO and Lf co-existed in the same sedimented fraction (Spitznagel et al., 1974; Rice et al., 1986). Lf was also reported to be in some MPO^+ granules (Parmely et al., 1982). Both Lf and Ly were shown to be in type 1- and type 2- like granules (Saito et al., 1988). Ly was stained in both MPO^+ and MPO^- granules (Cramer and Breton-Grous et al., 1987; Saito et al., 1987, 1988). Hence, not only Ly but also other proteins will be contained in both MPO^+ and MPO^- granules. The difference in localization of MPO from Lf seems to be a settled problem worldwide. However, the hypothesis that a small amount of Lf exists in some MPO^+ granules should not be ignored. For the one-to-one correspondence between granules and intragranular proteins, the granules should be examined not as subcellular fractions but a single cell.

An MPO^- Lf^- granule was also observed in mature neutrophils. The MPO detection by immunogold staining method is not always consistent with results by cytochemical study (unpublished observation). The presence of an MPO^- Lf^- granule must be ascertained using another method.

In conclusion, by using immunocryoultramicrotomy,

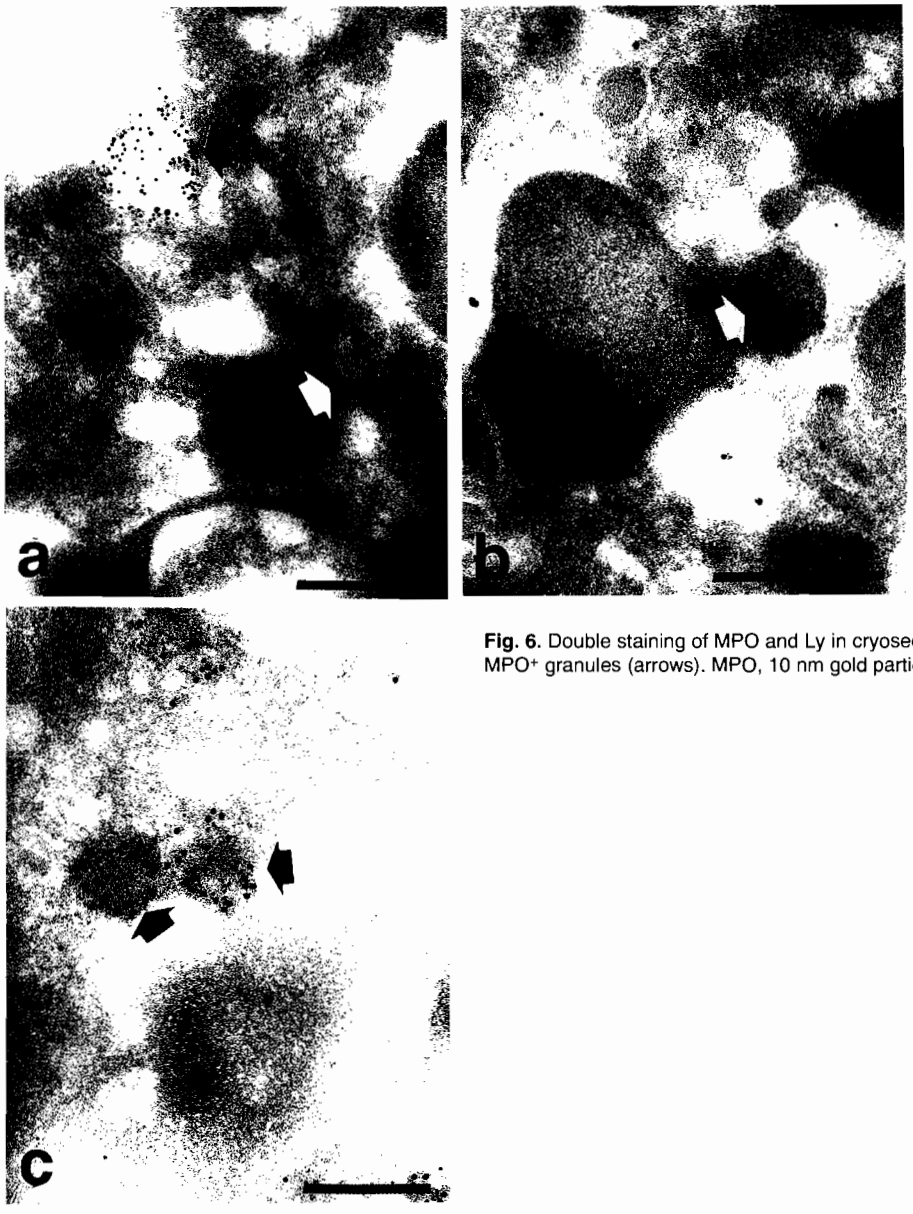

Fig. 6. Double staining of MPO and Ly in cryosection. Ly is located in the type 1, type 2 and type 3 MPO^+ granules (arrows). MPO, 10 nm gold particles; Ly, 20 nm gold particles. Bar: 0.05 μm.

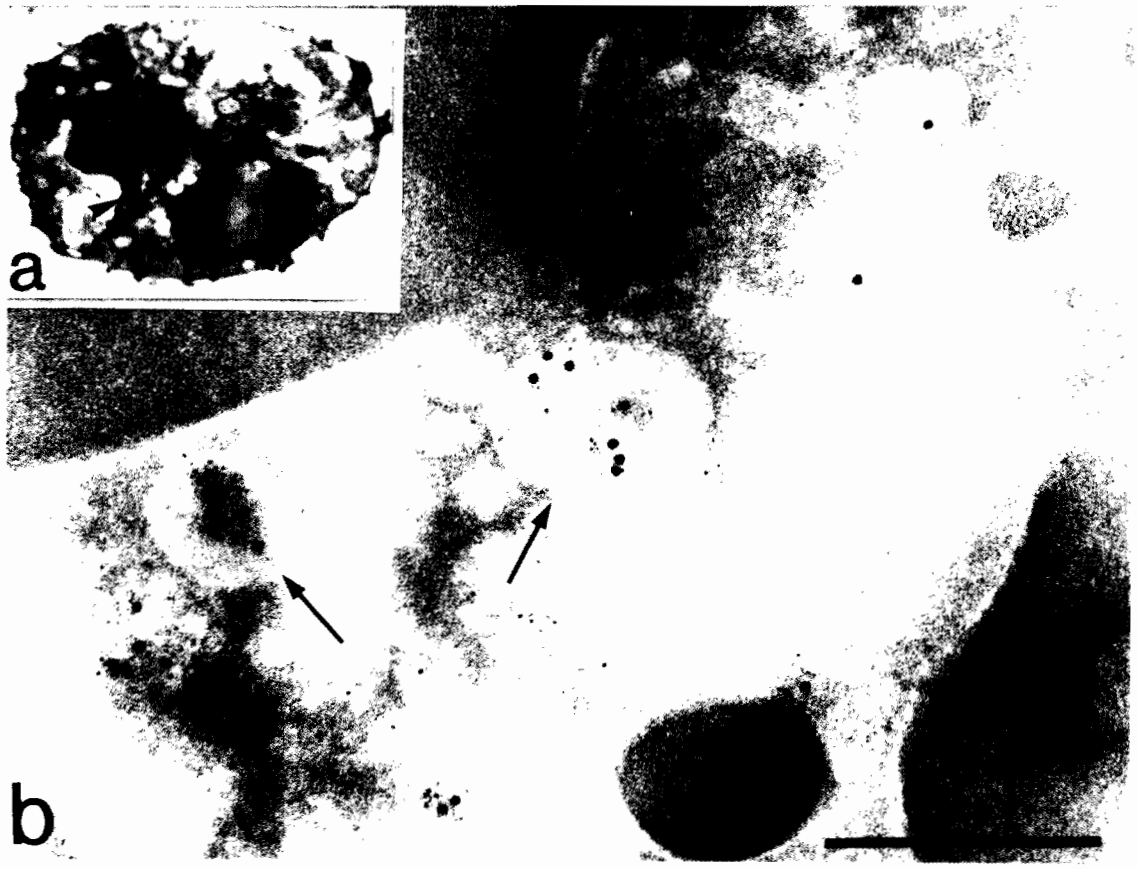

Fig. 7. Double staining of Lf and Ly by post-embedding method. **a.** A circulating neutrophil. **b.** Higher magnification of the arrowhead in Fig. 7a. Double stainability is observed in type 1-like granules (arrows). Lf, 15 nm gold particles; Ly, 5 nm gold particles. Bar: 0.5 μm.

large MPO^+ granules were divided into 5 types. It was considered that some proteins are contained in both MPO^+ and MPO^- granules and that elucidation of the role of neutrophil granules may further shed light on the cell's function.

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