American Journal of Pathology, Vol. 134, No. 6, June 1989

Colocalization of Elastase and Myeloperoxidase in Human Blood and Bone Marrow Neutrophils Using a Monoclonal Antibody and Immunogold

Elisabeth M. Cramer,*§ Julian E. Beesley,† Karen A. F. Pulford,‡ Janine Breton-Gorius,§ and David Y. Mason‡

From the Département d'angio-hématologie and INSERM U 150, Hôpital Lariboisière, Paris, France, the Wellcome Research Laboratories, Beckenham, United Kingdom,† the Nuffield Department of Pathology, John Radcliffe Hospital, Oxford, United Kingdom,‡ and the INSERM U.91 Hôpital Henri Mondor, Créteil, France§*

The authors have localized elastase in human blood and bone marrow neutrophils by immunoelectron microscopy using a monoclonal anti-human elastase antibody (NP 57) and compared its distribution with myeloperoxidase (MPO) and lactoferrin (LF), which mark primary and secondary neutrophil granule, respectively. Human bone marrow and blood polymorphonuclear leukocytes (PMN), either unstimulated or after phagocytosis of latex microbeads, were fixed in 4% paraformaldehyde. Ultrathin frozen sections were immunolabeled with NP 57, followed by an immunogold probe. In bone marrow granulocyte precursors elastase appeared simultaneously in the immature first granules of myeloblasts with MPO. As these granules became denser with maturation, labeling for both enzymes became weaker and sometimes negative (possibly due to masking of immunoreactivity). The ellipsoidal primary granules were strongly labeled by NP57. LF positive granules appeared later, at the myelocyte stage, and contained neither MPO nor elastase. In mature neutrophils, immunolabeling for elastase was found together with MPO in the large electron-dense primary granules and in a different granule population from the LF-positive secondary granules. Double labeling with two different-sized gold particles was used to compare the kinetics of degranulation of secondary and primary granules. The observation and the analysis of single phagosome content was made possible by this new technique. In conclusion, immunoelectron microscopy was used to show elastase in the primary granules of neutrophils, where it appears simultaneously with MPO. This technique has also allowed comparison of the kinetics of degranulation of both types of granules, and could be applied to different experimental and pathologic conditions. (Am J Pathol 1989, 134: 1275–1284)

Elastase is a powerful proteolytic enzyme of mature neutrophils,[1] the functions of which include the killing of microorganisms as well as the initiation of tissue injury in pathologic conditions such as emphysema, glomerulonephritis, and rheumatoid arthritis.[2–5]

Although numerous subpopulations of granules are being described iteratively,[6–9] neutrophil granules can be divided into two main types—primary or azurophilic, and secondary or specific—that have different content and function and are produced at different stages of the maturation of bone marrow precursors.[10] The isolation of pure populations of azurophilic and specific granules has made it possible to analyze their enzyme and other protein content.[11–13] This also has been achieved and comparable data been acquired by immunofluorescent staining of neutrophils.[14–16] In electron microscopy, primary granules can be recognized by cytochemical detection of myeloperoxidase (MPO), whereas lactoferrin (LF) has been shown to be a specific marker of secondary granules that lack MPO.[17]

Elastase was shown, mainly by biochemical assays, to be associated to the primary granule fraction.[11–13] Moreover, a recent ultrastructural report[18] using a combination of cytochemical and immunogold staining techniques reported that elastase was associated with primary granules but that only 20 to 25% of MPO-positive

Accepted for publication March 15, 1989.

Address reprint requests to Dr. E. Cramer, Département d'angio-hématologie, Hôpital Lariboisière, 2 rue Ambroise Paré, 75010 Paris, France.

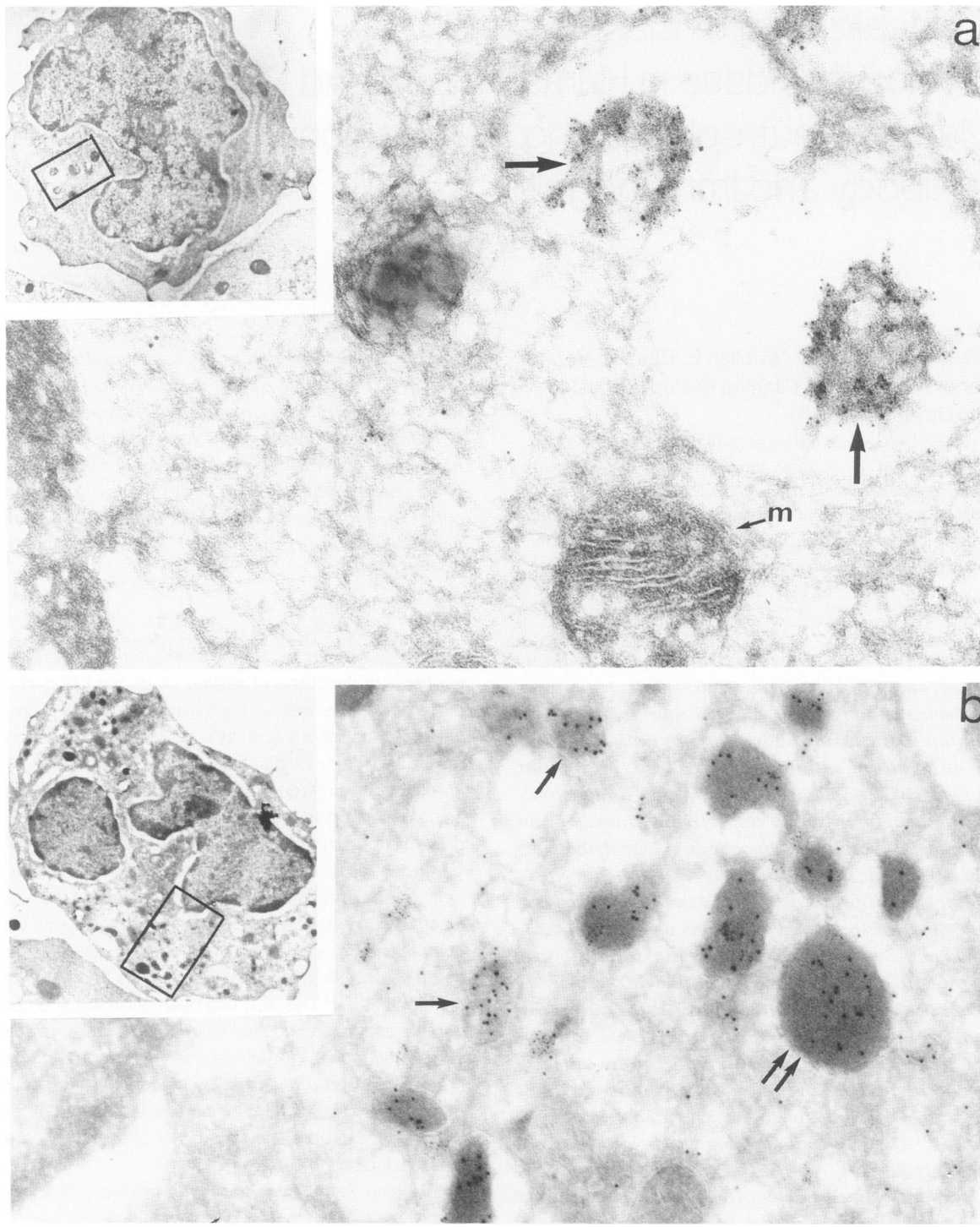

Figure 1a: *A myeloblast double labeled for MPO (5 nm gold particles) and elastase (10 nm gold particles). Both enzymes are found in the matrix of immature granules (arrows). m, mitochondria (×75,200).* **b.** *A promyelocyte labeled by the anti-elastase antibody NP57. All the primary granules are labeled (arrow) including the ones whose matrix electron density increases with maturation (double arrows) (×63,700).*

primary granules contained elastase. In this study, however, Damiano et al,[18] investigated MPO and elastase distribution using two different techniques, MPO by its enzymatic reactivity and elastase by its immunologic reactivity.

The aim of the present study was to investigate elastase subcellular localization in maturing neutrophils and to compare its distribution to MPO, assessing the distribution of both molecules by the same immunocytochemical

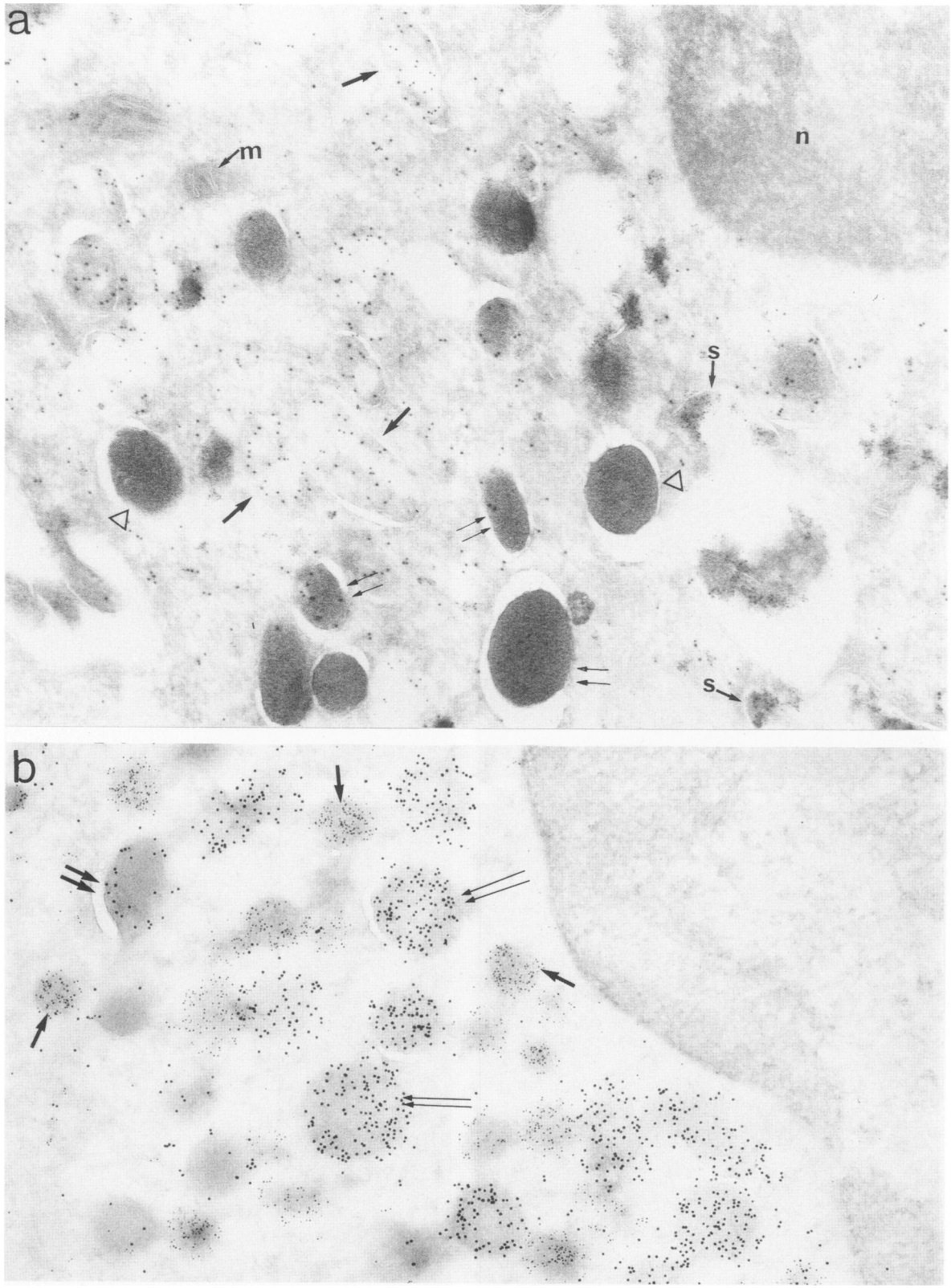

Figure 2a. *Part of a myelocyte immunolabeled for elastase (10 nm gold particles) and LF (5 nm). The gold probes of larger size heavily label the newly formed ellipsoid granules from which the crystal has been extracted (single arrows), and some denser, more mature primary granules (double arrows). Some of the dense spherical granules are negative for elastase, however (arrowheads). Some small specific granules (s) containing LF appear in the centriolar region (m, mitochondria; n, nucleus) (×54,000).* **b:** *Part of a metamyelocyte double-labeled for LF (5 nm gold particles) and elastase (10 nm gold particles). Both proteins are found in different granule populations, LF in specific granules (single arrows) and elastase either in the electron-dense primary granules (where labeling is sometimes restricted to their periphery) (double thick arrows) or less dense primary granules (double thin arrows) (×46,000).*

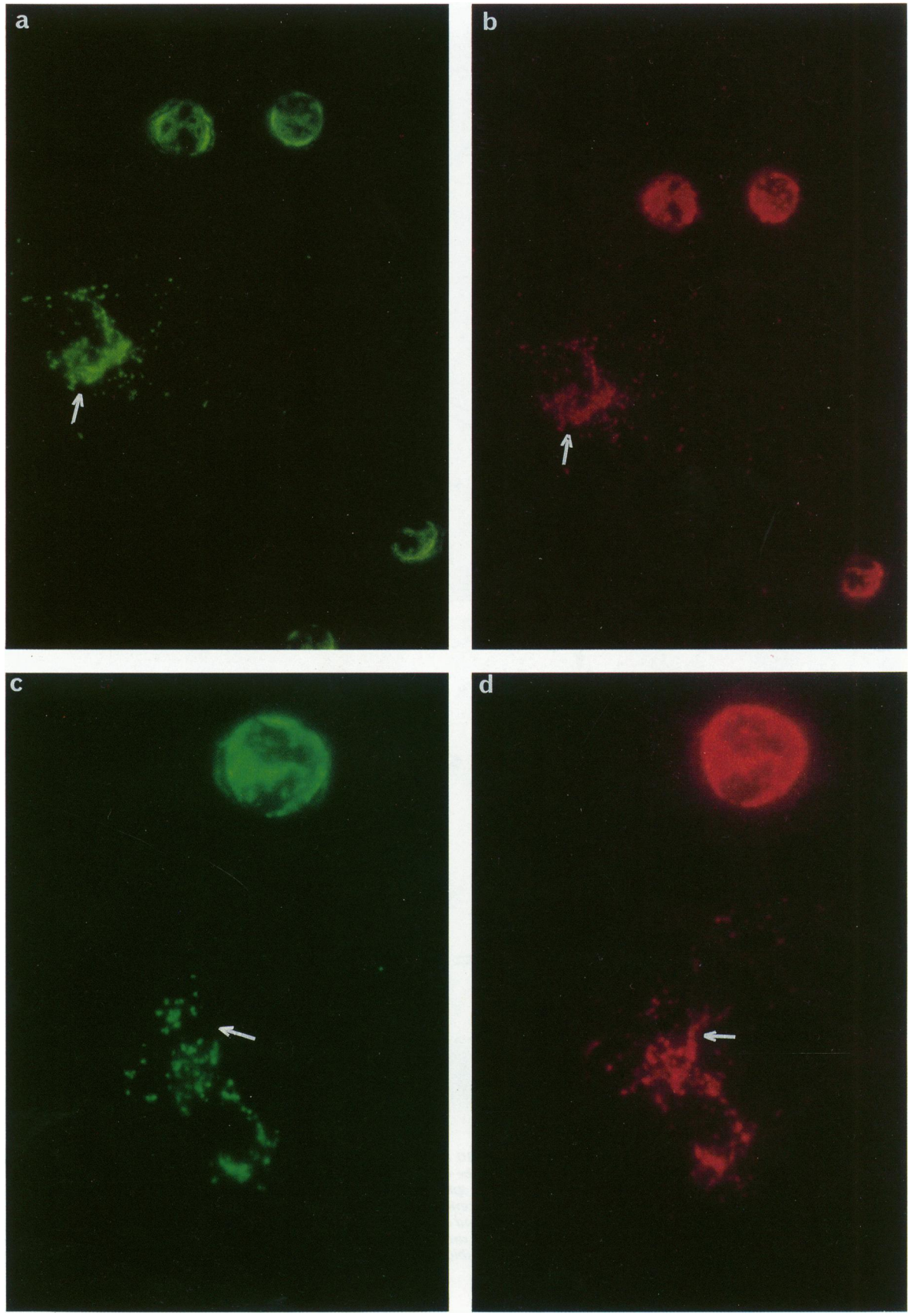
a
b
c
d

Figure 3. *Double immunofluorescence labeling of a burst disrupted PMN (arrows) in a cell smear for either elastase and MPO, or for elastase and LF. The localization of elastase-positive granules (labeled with fluorescein)* **(a)** *is identical to that of MPO-positive granules (rhodamine)* **(b)**. *Immunolabeling for elastase-positive granules (fluorescein)* **(c)** *and LF positive granules (rhodamine)* **(d)** *shows different distribution patterns (arrows).*

technique. The recent production of a mònoclonal antibody against human elastase,[19] combined with an excellent specificity of the technique, made it possible. Double-labeling experiments were performed, using gold particles of different size to demonstrate elastase simultaneously with LF or MPO. The simultaneous localization of these markers by the same technique has allowed a comparison of their distribution pattern among themselves and in relation to other cellular organelles in maturing neutrophils.

Elastase exerts its function through neutrophil degranulation. This leads to the differential release of granules[20] and to liberation of their contents, which may be selective. The kinetics of fusion of the different granule populations is still debated.[21] In this study, the use of different-sized gold particles labeling elastase and LF, the respective markers of both granule types, allowed the comparison of their kinetics of redistribution after phagocytosis of foreign particles by blood polymorphonuclear neutrophils (PMN). This double-labeling method is a powerful technique that also could be applied to the study of the fate of these and other granule components in different pathologic situations, and therefore contribute to our knowledge of their function.

Material and Methods

Cells

Bone marrow was obtained from a normal bone marrow graft donor. Heparinized samples enriched in immature neutrophils were prepared from Ficoll-Hypaque light density cells.[22] Normal human blood was harvested on lithium heparinate (10 U/ml) and neutrophils and monocytes were isolated by the dextran-radioselectan sedimentation technique.[23] Three samples from normal individuals were studied.

For phagocytosis experiments, latex beads 0.65 μ diameter (Polysciences, Inc., St. Goar, RFA) were dialyzed against distilled water, opsonized with normal human serum before use, and added to preheated Hank's balanced salt solution (HBSS) containing the cells and 20% human serum. The neutrophil:bead ratio was approximately 1:200. The suspension was placed in a 37 C incubator and agitated. The reaction was stopped after 30 seconds by adding a large excess of cold fixative and the cells were treated further for immunolabeling as described below.

Antibodies

Monoclonal Antibody (NP57) Against Elastase

The production and characterization of this antibody after immunisation with a granule preparation from human blood neutrophils has been described elsewhere.[19]

Polyclonal Antibodies

Rabbit polyclonal antibody against human LF was purchased from Cappel Laboratories, Downingtown, PA. Rabbit polyclonal antibody against human MPO was produced in one of the author's laboratory and monospecificity of both polyclonal antibodies was assessed previously.[17]

Immunogold probes (5 and 10 nm) directed against either mouse or rabbit IgG were purchased from Janssen Pharmaceutica, Beerse, Belgium. Fluorescent probes were Tetramethylrhodamine B isothiocyanate (TRITC) goat anti-rabbit Ig (Dakopatts, Denmark), and Fluorescein isothiocyanate (FITC) goat anti-mouse (Stago, Asnières, France).

Immunolabeling

Immunofluorescence

Buffy coat smears of normal peripheral blood were fixed in methanol:formalin for 60 seconds, washed in phosphate-buffered saline (PBS) and then incubated in a mixture of the monoclonal anti-elastase antibody NP57 (undiluted supernatant) and rabbit anti-LF (final concentration, 1:200). After 30 minutes the smears were washed and incubated with a mixture of TRITC goat anti-rabbit Ig and FITC goat anti-mouse Ig, with both antibodies used at a final concentration of 1:30. Smears were then mounted in polyvinyl alcohol for immunofluorescent microscopy.

Immunoelectron Microscopy

Cells were fixed for 1 hour with freshly prepared 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. They were embedded in 10% gelatin, then refixed. After cryoprotection in 2.3 M sucrose for 1 hour they were rapidly frozen in liquid nitrogen slush. Ultrathin sections were cut using an LKB Cryo Nova and mounted on 400

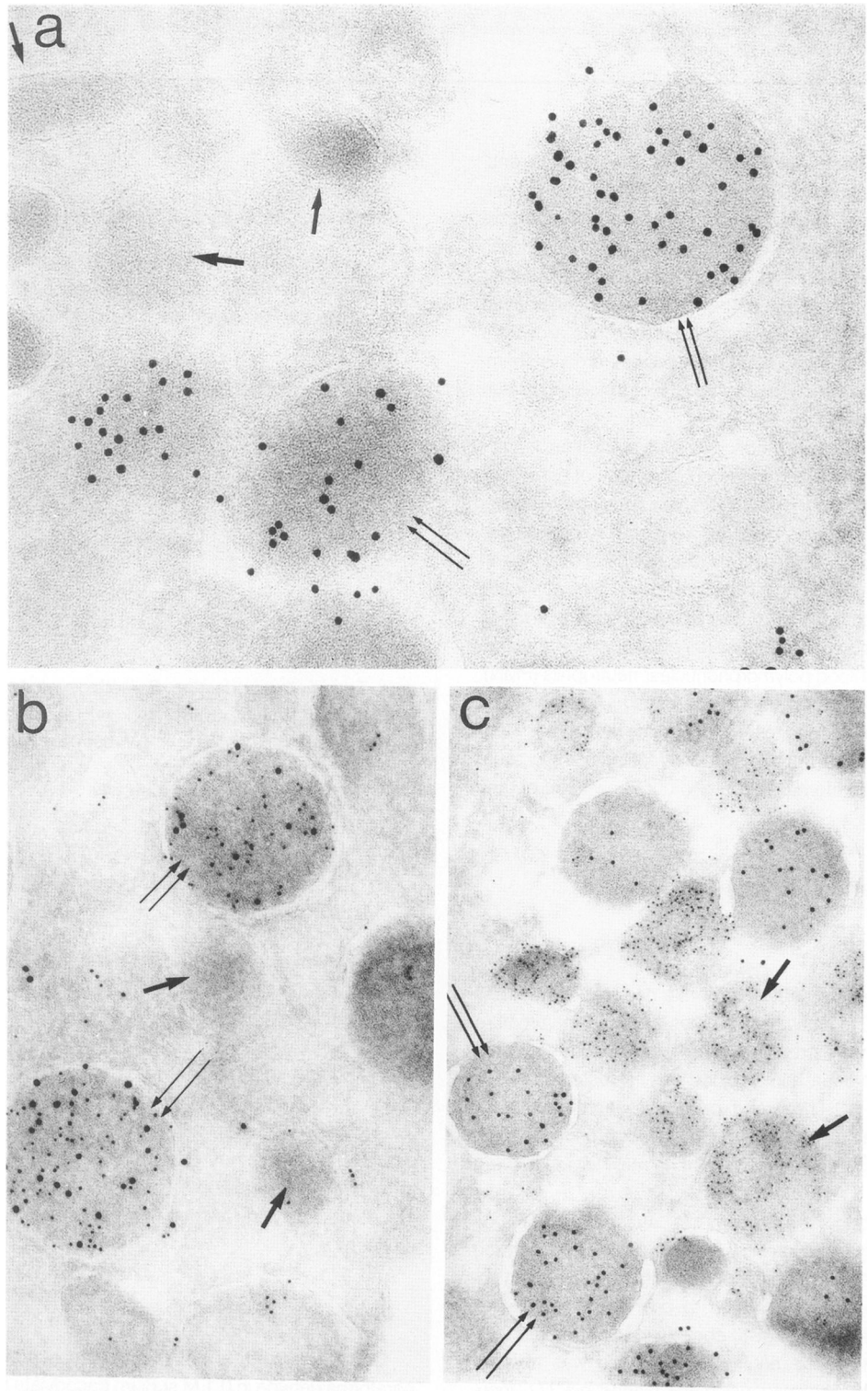

Figure 4. *Mature blood PMN stained for granule constituents.* **a:** *Labeling for elastase shows the gold label restricted to the large electron dense granules (double arrows), whereas the smaller secondary granules are negative (single arrows) (×110,000).* **b:** *Double labeling for MPO (small particles) and elastase (large particles) shows that both enzymes are colocalized in the same primary granules (double arrows). The small specific MPO-negative granules contain no elastase (single arrows) (×85,000).* **c:** *Double labeling for LF (small particles) and elastase (large particles) shows that the two proteins are located in the secondary (single arrows) and primary (double arrows) granules, respectively (×52,000).*

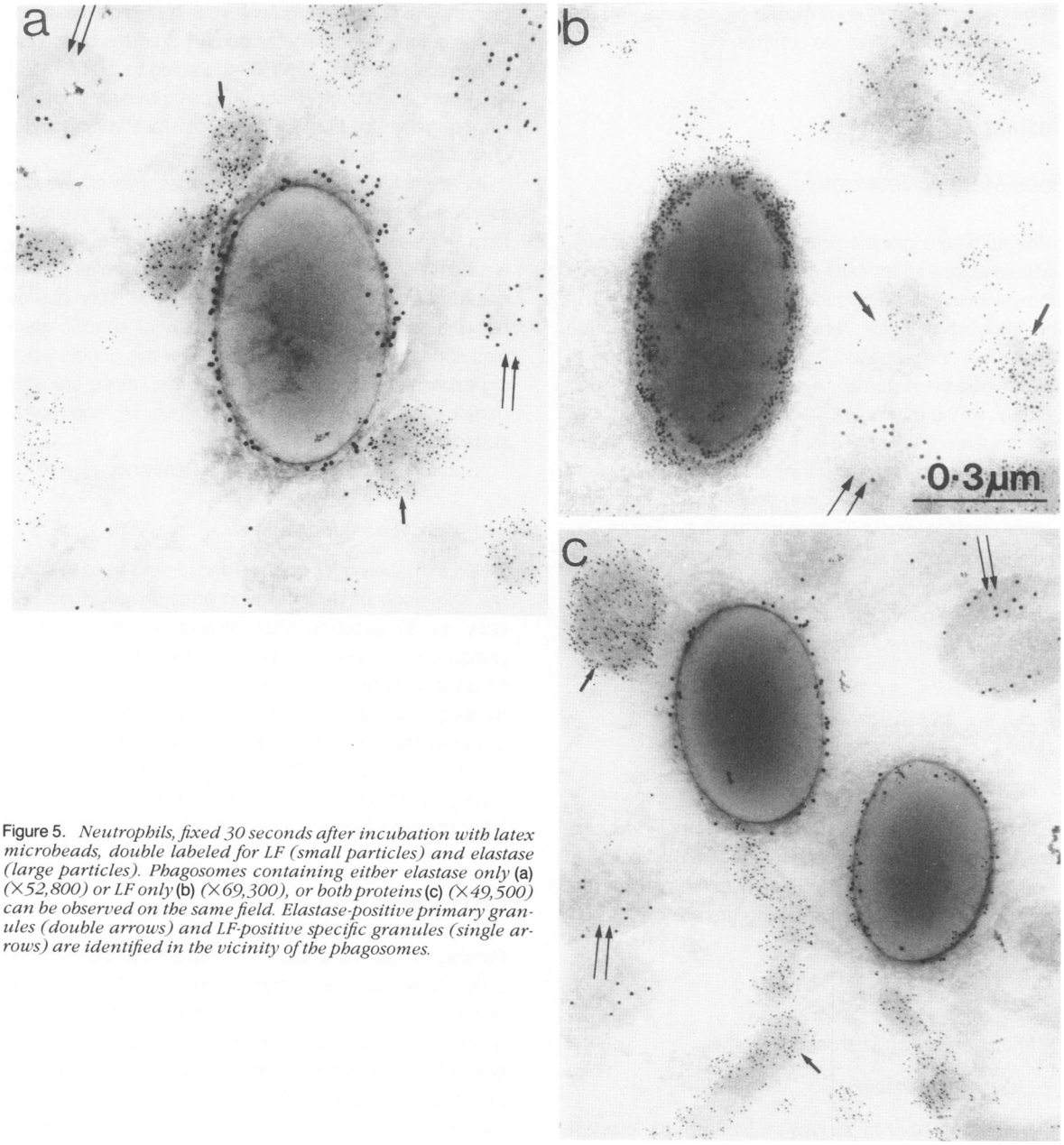

Figure 5. *Neutrophils, fixed 30 seconds after incubation with latex microbeads, double labeled for LF (small particles) and elastase (large particles). Phagosomes containing either elastase only* (a) *(×52,800) or LF only* (b) *(×69,300), or both proteins* (c) *(×49,500) can be observed on the same field. Elastase-positive primary granules (double arrows) and LF-positive specific granules (single arrows) are identified in the vicinity of the phagosomes.*

mesh butvar carbon-coated grids. They were immunolabeled by floating on droplets of 1% gelatin in PBS, followed by the primary antibody and the gold probe. All incubations were performed in the presence of 1% bovine serum albumin, which contributed with gelatin to block the nonspecific binding of primary antibody. The sections were stabilized with neutral uranyl acetate, then stained with 2% aqueous uranyl acetate before they were embedded in methyl cellulose.

In double labeling experiments the monoclonal (mouse) and the polyclonal (rabbit) antibodies were mixed. Sections were floated on the mixture and the reactions were revealed by gold probes of different specificities and size, mixed together, and used at a 10^{-1} dilution.

For each cell stage or experimental condition a minimum of 10 different cell equatorial sections were observed.

Controls

In the above reactions for fluorescence and electron microscopy, the polyclonal antibodies were replaced by either non immune rabbit serum or a polyclonal anti-von Willebrand factor antibodies (Dakopatts, Denmark) and

the monoclonal NP 57 by a mouse monoclonal anti-von Willebrand factor antibody as controls.

Results

Bone Marrow Neutrophils

In the immature granulocytes containing few granules, elastase appeared as early as MPO (Figure 1a). In early promyelocytes, all granulations were labeled (Figure 1b). The ellipsoid granules, which appeared less electron dense than the spherical granules and the crystal of which[28] had been partially extracted, also were labeled strongly. At the myelocyte stage, LF containing granulations appeared, which did not contain elastase. At this maturation stage, about one third of the spherical primary granules with a highly electron-dense matrix displayed neither elastase nor MPO immunoreactivity (Figure 2a). The metamyelocyte stage was characterized by the presence of two clear-cut populations of granules, elastase-positive, LF-negative primary granules, and LF-positive, elastase-negative secondary granules (Figure 2b).

Blood Neutrophils

Immunofluorescence

On blood smears, all neutrophils were labeled for elastase, MPO, and LF in an indistinct pattern. When cells had burst on smears, however, there was a similarity between MPO and elastase-containing granule distribution, whereas the pattern of staining for LF was not superimposable (Figure 3).

Immunoelectron Microscopy

Elastase Localization

Mature neutrophils displayed labeling for elastase within large granules, which corresponded in size and number and according to previous descriptions[5,28] to the primary granules (Figure 4a). Only 70% of the dense primary granules were labeled, however, and of these, about 10% were labeled only on the periphery of their matrix.

Double-Labeling Experiments

Two different-sized gold particles were used to localize simultaneously elastase (5 nm gold particles) and MPO (10 nm gold particles) according to Tapia et al (Figure 4b).[29] A strict colocalization of both proteins was observed, directly confirming the presence of elastase in the primary, MPO-positive granules. Some large electron-dense granules, identified as primary granules by the cytochemical detection of MPO, according to Graham and Karnowsky, and by the technique described previously,[30] were negative for both MPO and elastase immunocytochemical detection.

LF detected by small gold particles was always observed in a granule population that was clearly distinct from the elastase-containing granules (Figure 4c): they were smaller, less electron dense, about twice as numerous, and previously identified by a similar technique as being secondary granules.[17,27] Virtually all specific granules appeared to label for LF. However this double labeling experiment confirmed that the large, dense granules negative for elastase did not contain LF and corresponded to primary granules.

Control experiments were consistently negative.

Phagocytosing Neutrophils

Double labeling for elastase and LF was used to study the sequence of degranulation after phagocytosis. As early as 30 seconds after initiation of phagocytosis, phagosomes containing either the elastase marker only (large gold particles) (Figure 5a) or the LF marker only (small gold particles) (Figure 5b) or both proteins (Figure 5c) were seen. The results were the same after 5 and 10 minutes of phagocytosis except that more gold particles were present within the phagosomes, and less granules left in the cytoplasm.

Discussion

Previous biochemical data[11-13,24] have indicated that elastase is associated with primary granules. One previous ultrastructural enzymatic cytochemical study, however, reported elastase activity as being ubiquitous in its distribution in the neutrophil, being found both in primary and secondary granules.[25] This result is probably caused by the nonspecificity of the elastase cytochemical procedure. The present study avoided this problem by using a specific monoclonal antibody to elastase to probe for its site of localization. Use of this reagent in conjunction with antibodies to other markers has made it possible for us to localize this enzyme to primary granules and to show that it is restricted to this class of heterogeneous granules. A recent immunoelectron microscopic study also showed the association of elastase with MPO-positive granules, but only part of these granules displayed elastase.[18] When we used an immunogold technique to localize MPO as well, the same results were obtained for both MPO and elastase, ie, labeling of only part of the primary granules. Although a discrepancy has been observed between cellular MPO and elastase content in acute myeloblastic leu-

kemia, with some cases expressing MPO in the absence of elastase,[19] we consistently observed the simultaneous appearance of both enzymes in the immature granules of normal bone marrow myeloblasts and never saw one without the other. Elastase labeling of all primary granules was present up to the promyelocyte stage, but in more mature cells some of the dense spherical primary granules were negative. The matrix of these granules had become more electron-dense with maturation, shown on standard electron microscopy stain. Double-labeling experiments showed a parallel absence of MPO labeling in these granules, and elastase-negative primary granules, although MPO cytochemical reactivity was preserved in the same conditions.[17,30] A possible explanation is that the content of these dense granules is very tightly packed, and that the antigenic site is therefore inaccessible to the antibody whereas the enzymatic activity is still detectable. This can be compared with the well-known enzymatic latency of lysosomes[26] or the lack of immunoreactivity of neutrophil secondary granules for lysozyme,[27] and might explain the absence of labeling in a subpopulation of primary granules in PMN.

The matrix of ellipsoid granules (which appear later than spherical granules[28]) was labeled consistently. The concentration of labeling at the periphery of some granules resembles the pattern of labeling described by Rice et al[8] and Bainton and coworkers[10] for MPO. In our previous study, primary granules were partially extracted by the embedding technique;[17] we could thus observe the immunologic localization of MPO within the primary granules, but it was not possible to see a specific intragranular distribution pattern. In contrast, the cryosectioning technique used in this study preserves granule morphology better and allows visualization of its whole matrix. Thus, it seems that within the mature granule itself, the distribution of proteins is not homogeneous. This may lead to a selective release of the different components present in an individual granule during intra- or extracellular degranulation. However, the different subpopulations of primary granules[2-4] could not be distinguished based on their elastase content.

The double-labeling experiments possess two advantages. First, they directly show the strict colocalization of the immunologic reactivity of MPO and elastase in the primary granules, as well as the different localization of LF and elastase within the secondary and primary granules, respectively. Second, during phagocytosis, the use of gold particles of different size to label the two main types of granules allows their simultaneous vizualization, and thus a comparison of the kinetics of their degranulation. Although latex particles are not physiologic stimuli, we used the same technical conditions as Segal and coworkers[21] to compare our results of degranulation kinetics in PMN. Under these technical conditions, both types of granules were observed emptying their content simultaneously, shortly after the initiation of phagocytosis.

Double-labeling experiments also allow the semi-quantitative analysis of the content in a specific protein within a single phagosome. Because it is hypothesized that release of granule components could be selective, this technique provides a good tool to perform such a study after different physiologic or pathologic stimulations.

Finally, it will be of interest to investigate why masking of some granule antigenic reactivity occurs during neutrophil maturation at the same time as the enzymatic reactivity is preserved, and whether this is due to physicochemical interaction with other granule components.

References

1. Ohlsson K, Olsson I: The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. Eur J Biochem 1974, 42:519–527
2. Crocker J, Jenkins R, Burnett D: Immunohistochemical demonstration of leukocyte elastase in human tissues. J Clin Pathol 1984, 37:1114–1118
3. Damiano VV, Tsang A, Abrams WR, Rosenbloom J, Kimbel P, Weinaum G: Immunolocalization of elastase in human emphysematous lungs. J Clin Invest 1986, 78:482–493
4. Janoff A: Elastase in tissue injury. Ann Rev Med 1985, 36: 207–216
5. Senior RM, Campbell EJ: Neutral proteinases from human inflammatory cells: A critical review of their role in extracellular matrix degradation. Clin Lab Med 1983, 3:645–666
6. Dewald B, Bretz U, Baggiolini M: Release of gelatinase from a novel secretory compartment of human neutrophils. J Clin Invest 1982, 70:518–525
7. Kinkade JM, Pember SO, Barnes KC, Shapira R, Spitznagel JK, Martin LE: Differential distribution of distinct forms of myeloperoxidase in different azurophilic granule subpopulations from human neutrophils. Biochem Biophys Res Comm 1983, 114:296–303
8. Rice WG, Ganz T, Kinkade JM, Selsted ME, Lehrer RI, Parmley RT: Defensin-rich dense granules of human neutrophils. Blood 1987, 70:757–765
9. Pryzwansky KB, Breton-Gorius J: Identification of a sub-population of primary granules in human neutrophils based upon maturation and distribution. J Lab Invest 1985, 53:664–671
10. Bainton DF, Ullyot J, Farquhar MG: The development of neutrophilic polymorphonuclear leukocytes in human bone marrow: Origin and content of azurophil and specific granules. J Exp Med 1971, 134:907–934
11. Bretz U, Baggiolini M: Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. J Cell Biol 1974, 63:251–269
12. Spitznagel JK, Dalldorf FG, Leffell MS, Folds JD, Welsh IRT, Cooney MH, Martin LE: Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab Invest 1974, 30:774–785

13. West BC, Rosenthal AS, Gelbna, Kimball HR: Separation and characterization of human neutrophil granules. Am J Pathol 1974, 77:41–61
14. Kramps JA, Van Der Valk P, Van Der Sandt MM, Lindeman J, Meijer CJ: Elastase as a marker for neutrophilic myeloid cells. J Histochem Cytochem 1984, 32:389–394
15. Pryzwansky KB, Martin LE, Spitznagel JK: Immunocytochemical localisation of myeloperoxidase, lactoferrin, lysozyme and neutral protease in human monocytes and neutrophilic granulocytes. J Reticulendothelial Soc 1978, 24:295–310
16. Pryzwansky KB, Rausch PG, Spitznagel JK, Herion JC: Immunocytochemical distinction between primary and secondary granule formation in developing human neutrophils: Correlations with Romanovsky stain. Blood 1979, 53:179–185
17. Cramer E, Pryzwanski KB, Villeval JL, Testa U, Breton-Gorius J: Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. Blood 1985, 65: 423–432
18. Damiano VV, Kucich U, Murer E, Laudenslager N, Weinbaum G: Ultrastructural quantitation of peroxidase- and elastase-containing granules in human neutrophils. Am J Pathol 1988, 131:235–245
19. Pulford KAF, Erber WN, Crick JA, Olsson I, Micklem KJ, Gatter KC, Mason DY: Use of monoclonal antibody against human neutrophil elastase in normal and leukaemic myeloid cells. J Clin Pathol 1988, 41:853–860
20. Bainton DF: Sequential degranulation of the two types of polymorphonuclear leukocytes granules during phagocytosis of microorganisms. J Cell Biol 1973, 58:249–264
21. Segal AW, Dorling J, Coade S: Kinetic of fusion of the cytoplasmic granules with phapocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. J Cell Biol 1980, 85:42–59
22. Boyum A: Isolation of lymphocytes, granulocytes and macrophages. Scan J Immunol 1976, 5:9–15
23. Perianin A, Labro MT, Hakim J: Chemokinetic activity of N-formyl-methionyl-leucyl-phenylalanine on human neutrophil, and its modulation by phenylbutazone. Biochem Pharmacol 1982, 31:3071–3076
24. Garcia RC, Peterson CGB, Segal AW, Venge P: Elastase in the different primary granules of the human neutrophils. Biochem Biophys Res Comm 1985, 132:1130–1136
25. Clark JM, Vaughan DW, Aiken BM, Kagan HM: Elastase-like enzymes in human neutrophils localized by ultrastructural cytochemistry. J Cell Biol 1980, 84:102–119
26. Ruth RC, Weglicki WB: Mechanism of loss of latency of lysosomal enzymes. Biochem J 1980, 186:243–256
27. Cramer EM, Breton-Gorius J: Ultrastructural localization of lysozyme in neutrophils. J Leukocyte Biol 1987, 41:242–247
28. Breton-Gorius J, Reyes F: Ultrastructure of human bone marrow cell maturation. Int Rev Cytol 1976, 46:251–321
29. Tapia FJ, Varndell IM, Probert L, De Mey J, Polak JM: Double immunogold staining method for the simultaneous ultrastructural localization of regulatory peptides. J Histochem Cytochem 1983, 31:977–81
30. Cramer E, Auclair C, Hakim I, Felui E, Boucherot J, Troube H, Bernard JF, Bergogne E, Boivin P: Metabolic activity of phagocytosing granulocytes in chronic granulocytic leukemia: Ultrastructural observation of a degranulation defect. Blood 1977, 50:93–106

Acknowledgment

The authors thank Dr. Rodolpho C. Garcia for helpful discussion, Mark P. Betts for expert technical assistance, and Jean-Marc Massé for photographic help. They also thank Mrs. Anne-Marie Dulac for typing the manuscript.