American Journal of Pathology, Vol. 139, No. 3, September 1991

Alpha$_1$-antitrypsin Is Present Within the Primary Granules of Human Polymorphonuclear Leukocytes

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Elastase is a potent proteolytic enzyme found within human neutrophil primary granules. Its major inhibitor in the serum is α_1-antitrypsin, a protein that is synthesized by hepatocytes but which has recently also been shown to be synthesized by circulating neutrophils. The authors have therefore carried out an immunocytochemical study at the light microscopic and ultrastructural level to determine the intracellular localization of α_1-antitrypsin. Double labeling with colloidal gold showed that α_1-antitrypsin is localized at the same site as neutrophil elastase, i.e., within primary granules. Secondary granules (detected by labeling for lactoferrin) were unstained for α_1-antitrypsin. Elastase and its major inhibitor therefore coexist within the same granule population within human neutrophils. Some difference in their intraorganelle distribution existed at the ultrastructural level (in that elastase tended to be localized at the periphery of the granules whereas α_1-antitrypsin was usually diffusely present in the matrix of the granules), but further studies are required to determine whether the two molecules are already complexed with each other within the neutrophil. (Am J Pathol 1991, 139:623–628)

One of the major proteins present in human neutrophil leukocytes is the proteolytic enzyme elastase, which is synthesized in bone marrow myeloid precursors and stored in the mature neutrophil.[1] Release of elastase from neutrophils is believed to be a major etiologic factor in emphysematous lung disease,[2] and inherited deficiency of the major inhibitor for this enzyme (serum α_1-antitrypsin) predisposes patients to emphysema.[3]

It has been known for some time that α_1-antitrypsin is detectable within human hepatocytes and macrophages[4,5] but it has recently been shown that it is also detectable in human neutrophils and that it is synthesised by these cells, as demonstrated by the production of specific mRNA.[6] Both elastase and its major inhibitor are thus present within the same cell, but it is not clear whether these two molecules are present at the same or different sites. The authors report an ultrastructural study, based on immunolabeling with colloidal gold, aimed at localizing elastase and α_1-antitrypsin within the cytoplasm of the human neutrophil.

Materials and Methods

Antibody

The primary antibodies used for immunocytochemistry in this study are detailed in Table 1. Goat antibodies against mouse and rabbit IgG, labeled, respectively, with 5- or 15-nm gold particles, were obtained from Janssen Pharmaceuticals, Beerse, Belgium. Polyclonal mouse antiserum against human lactoferrin was produced by immunization of a mouse on five occasions at intervals of 10 days with 50 μg of purified human lactoferrin (kindly provided by Dr. J. Askaa). Serum was obtained 10 days later.

Cell Preparations

Buffy-coat smears from normal human peripheral blood were prepared on clean glass slides. For electron mi-

Supported by the Leukemia Research Fund, UK.

Accepted for publication May 2, 1991

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Table 1. *Antibodies Used for Ultrastructural Detection of Neutrophil Proteins*

Antibody	Source	Optimal dilution
Monoclonal mouse anti-elastase (NP57)	Authors' laboratory[7]	1/1
Polyclonal mouse anti-lactoferrin	Authors' laboratory	1/50
Polyclonal rabbit anti-α_1-antitrypsin	Dakopatts a/s, Copenhagen	1/5
Polyclonal rabbit anti-α_1-antitrypsin	Boehringer Mannheim	1/25

croscopy, neutrophils were isolated by the dextran-Radioselectan sedimentation technique[8] from normal human peripheral blood that had been anticoagulated with heparin. They were cryofixed and freeze substituted as previously published.[9,10] Briefly, the pellet was transferred onto filter paper for fast-freeze fixation. The filter paper was placed on a foam rubber block that was covered with a layer of mica on the specimen mount of a Reichert-Jung cryoblock.[9,10] Excess water was removed, and the sample was immediately slammed onto a polished copper block that was cooled under vacuum with helium to −260°C. The frozen sample was transferred to acetone at −85°C in the presence of a molecular sieve (Merck, 0.4nm) to absorb water. These conditions were maintained for 3 days at −85°C, followed by 2 hours at −30°C, and 30 minutes at room temperature. The specimen was then washed in acetone that contained increasing proportions of water, embedded in glycol methacrylate (GMA), and ultrathin sections were cut.

Immunocytochemical Labeling

Light Microscopy

Neutrophil smears were fixed in buffered formal acetone[11] and stained with polyclonal rabbit anti-α_1-antitrypsin (diluted between 1/50 and 1/1000), followed by alkaline phosphatase-conjugated anti-rabbit Ig (Dakopatts) diluted 1/20. The reaction was developed using a Fast Red substrate[11] and slides were counterstained with hematoxylin.

Electron Microscopy

Labeling was carried out as described previously.[12] Briefly, grids carrying GMA-embedded sections were etched for 10 minutes with 10% H_2O_2, incubated with 10% normal goat serum to block nonspecific binding of antibodies and then floated on primary antibodies for 2 hours. In double labeling experiments, the primary incubation was with a mixture of monoclonal mouse anti-elastase antibody and rabbit anti-α_1-antitrypsin, or polyclonal mouse anti-lactoferrin antibody and rabbit anti-α_1-antitrypsin. Different dilutions were tested from 1/5 to 1/150. After washing, grids were incubated with colloidal gold-labeled anti-mouse and anti-rabbit Ig antibodies, diluted 1/10, for 1 hour. Sections were washed, fixed for 1 minute in 2.5% glutaraldehyde, and counterstained with uranyl acetate and lead. Sections were examined in a Philips CM10 electron microscope. Platelets that contaminated the neutrophil preparation and contained alpha granules but neither neutrophil elastase nor α_1-antitrypsin, served as a negative control to exclude nonspecific labeling.

Results

Light microscopic examination of immunoalkaline phosphatase-stained cell smears confirmed that there was strong reactivity of human neutrophils with rabbit anti-α_1-antitrypsin over a range of serum dilutions (Figure 1). Staining was intracytoplasmic and granular in appearance.

Double ultrastructural labeling of sections of neutrophils showed reactivity for α_1-antitrypsin in many large granules, which corresponded in size and number to the primary granules, whereas lactoferrin, detected by 5-nm gold particles, was always observed in small and dense granules that were clearly distinct from the α_1-antitrypsin-containing granules (Figure 2). Since the fastfreeze fixation freeze-substitution technique involves no chemical fixatives, the matrix of large primary granules was pale and partially extracted. However, protein antigenicity was preserved, and there was labeling in all large pale granules, associated with islands of denser material in the matrix. The great majority of secondary granules were labeled for lactoferrin, and none of these granules was labeled for α_1-antitrypsin. There was minimal background labeling with gold particles over the cytoplasm outside the granules, and over the nucleus.

Elastase was colocalized with α_1-antitrypsin in the same granule population (Figure 3), confirming the presence of α_1-antitrypsin in the primary granules. As previously shown,[13] there was a tendency for a localization of elastase at the periphery of the matrix of some primary granules (Figure 3), whereas α_1-antitrypsin showed no such preferential localization. Even when labeling for elastase was present at the centre of the matrix, one could see regularly aligned gold particles at the periphery of granules. No labeling of the small dense secondary

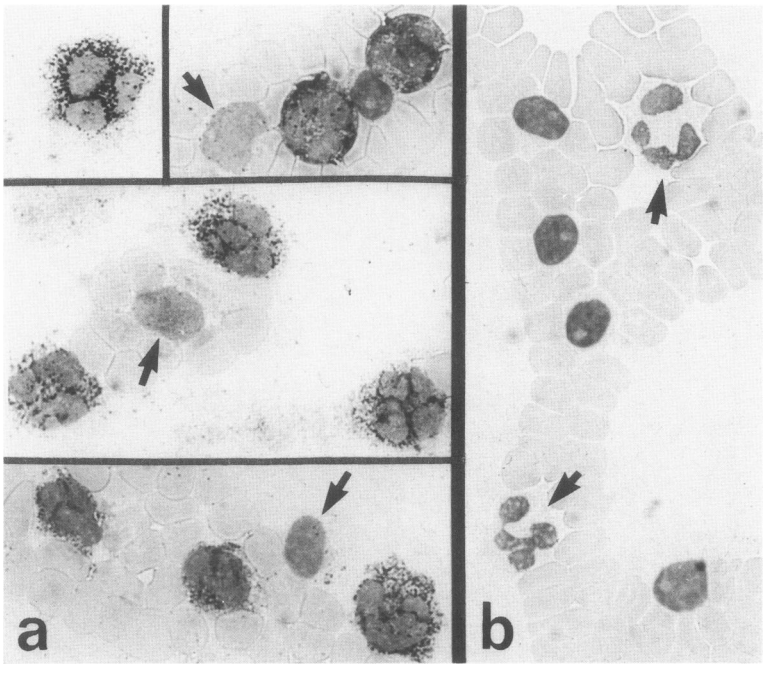

Figure 1. a: *Peripheral blood smear stained for α_1-antitrypsin showing granular positivity in the cytoplasm of neutrophils. Unstained lymphoid cells are arrowed. (Immunoalkaline phosphatase, hematoxylin counterstain).* **b**: *Negative control in which the primary antibody was omitted. Unstained granulocytes are arrowed. (Immunoalkaline phosphatase, hematoxylin counterstain).*

granules for elastase or α_1-antitrypsin was seen in these experiments.

A small number (approximately 1%) of pale large primary granules were labeled only for α_1-antitrypsin, and not for elastase, and approximately the same number showed the reverse pattern.

The granules of platelets were constantly unreactive with all antibodies tested.

Discussion

The localization of α_1-antitrypsin within primary granules of human neutrophils was reported. For this study, we chose to use cryofixation to improve the preservation of the ultrastructure and of the antigenic reactivity, rather than more widely used cell-processing procedures.

We have shown previously[13] that elastase can be detected on ultrathin frozen sections using monoclonal antibody NP57. The cryosectioning technique preserves granule morphology and antigenicity; however, possibly due to tight packaging of proteins, the antigenic sites appeared not always accessible to the antibody, since 30% of dense primary granules were negative for both myeloperoxidase and elastase. We were unable to label neutrophils with the same antibody in sections that had been fixed by aldehydes and embedded on glycol methacrylate, a method that has been used with success to localize many intragranular proteins.[12,14] It is well known that fixatives and embedding media can alter antigenic epitopes and can reduce antibody reactivity in an unpredictable fashion. However, cryofixation and drying had been shown to facilitate immunocytochemical localization at the ultrastructural level in neutrophils of cytochrome b[15] and several other intragranular proteins.[16] In preliminary experiments on cryofixed neutrophils, we were able to visualize elastase (with antibody NP57) in virtually all primary granules (data not shown), and we therefore chose the same methodology for the localization of α_1-antitrypsin.

In double-labeling experiments, it was observed that α_1-antitrypsin is present in large pale granules distinct from the small dense granules that contain LF, and we never observed a granule labeled for both proteins. In contrast, we found strict colocalization of reactivity for elastase and α_1-antitrypsin in primary granules. Few granules were labeled for α_1-antitrypsin or elastase alone. Labeling for elastase tended to be localized at the periphery of the granules as previously reported,[13] whereas α_1-antitrypsin is usually visualized within the matrix of the granules. In a previous report on the localization of elastase using the same monoclonal antibody, but in which the immunolabeling was done on ultrathin frozen sections rather than on freeze-substituted neutrophils, only approximately 70% of large primary granules were labeled for elastase.[13] In contrast, with the fast-freeze substitution method, the great majority of primary granules, although their content appeared more or less extracted, reacted for both elastase and α_1-antitrypsin.

The presence of α_1-antitrypsin in the same granule

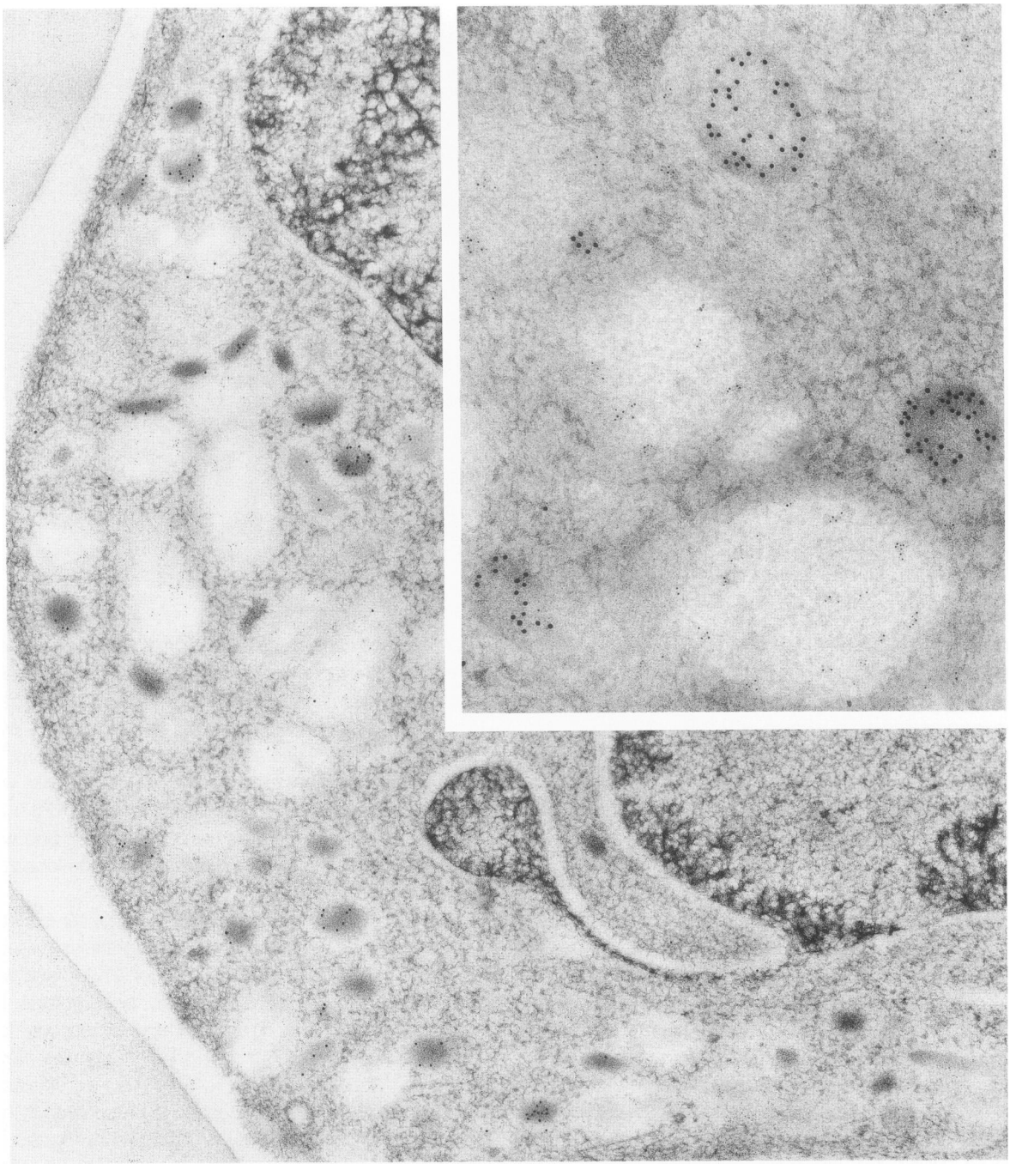

Figure 2. *Neutrophil polymorphonuclear leucocyte double labeled for α_1-antitrypsin (Boehringer antibody—small particles) and lactoferrin (large particles). The matrix of all large pale primary granules is labeled by small gold particles, whereas the majority of small dense granules are labeled by large particles indicating the presence of lactoferrin (×24,000). The inset shows at high magnification the clear distinction between primary granules, labeled for α_1-antitrypsin by small particles, and small dense secondary granules, labeled for lactoferrin (×34,500).*

population as elastase raises the question of how elastase can function if it is already in the presence of its inhibitor before release from neutrophils. This work gives some suggestion that the two molecules might possibly be differentially localized at different sites within primary granules. However, biosynthetic studies are required to ascertain whether the two molecules are already complexed to each other within neutrophils or whether they can only unite after release from the cell. This might be answered by internally labeling neutrophil α_1-antitrypsin

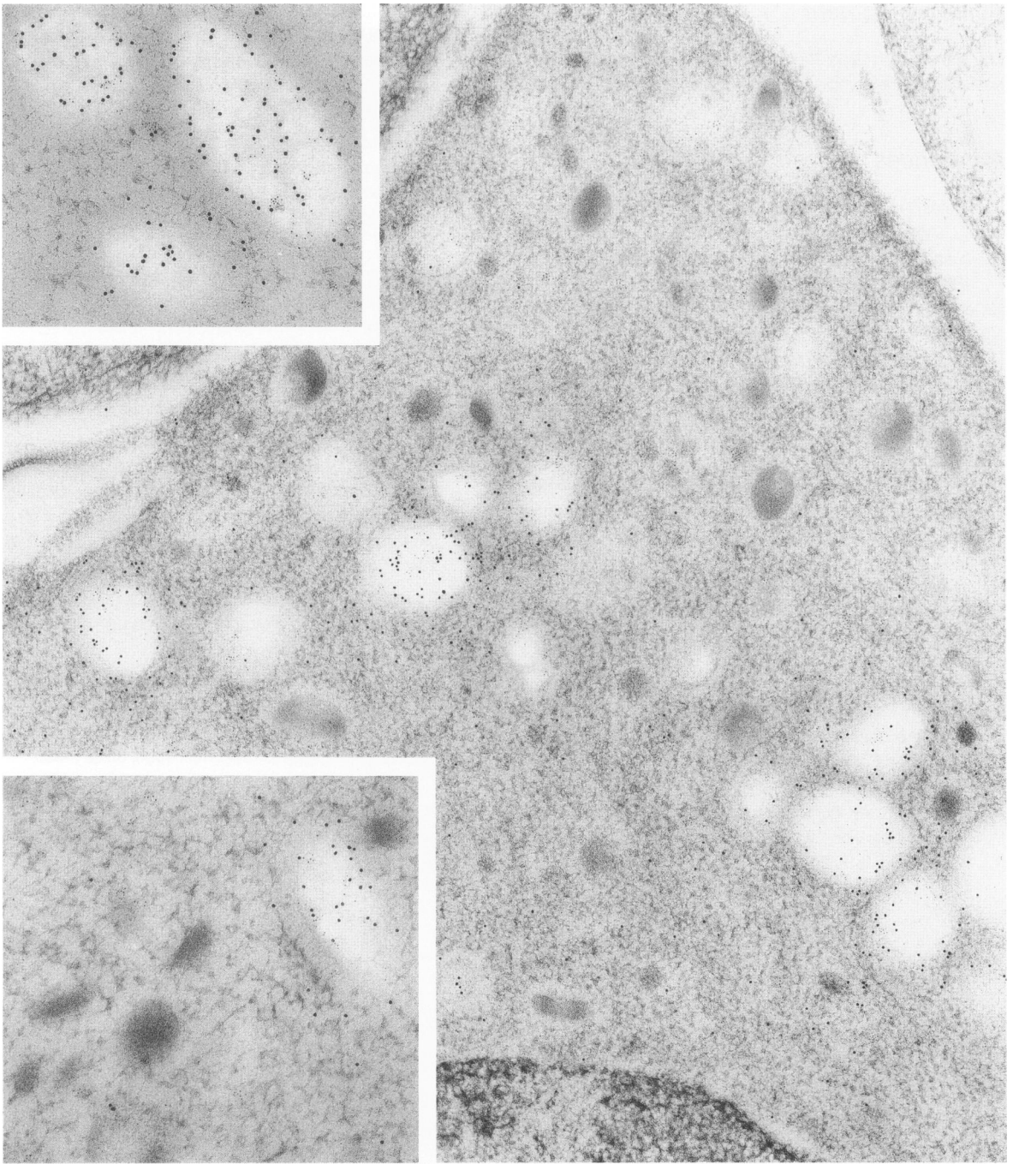

Figure 3. *General view of a neutrophil polymorphonuclear leucocyte double labeled for α_1-antitrypsin (Dakopatts antibody—small particles) and elastase (monoclonal antibody NP57—large particles). The large pale primary granules are colabeled with particles of both sizes, indicating that they contain both elastase and α_1-antitrypsin. Note that small dense secondary granules are unlabeled (×24,000). The upper inset shows the enlargement of three primary granules, two of which show double labeling. The large particles corresponding to elastase tend to be aligned along the periphery of the granule (×34,500). The lower inset shows the absence of labeling in secondary granules (×34,500).*

(e.g., with ^{35}S methionine) and then passing a cell lysate over an affinity column of anti-elastase. We have already shown that this technique can be used to immunopurify neutrophil elastase.[7] Electrophoresis and autoradiographic analysis of material eluted from the column should show whether radiolabeled α_1-antitrypsin was copurified with elastase (indicating complex formation within the neutrophil).

Acknowledgment

The authors thank Danielle Gaches for her expert technical assistance with the cryofixation and freeze substitution technique.

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