ELASTASE-LIKE ENZYMES IN HUMAN NEUTROPHILS LOCALIZED BY ULTRASTRUCTURAL CYTOCHEMISTRY

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ABSTRACT

The thiol ester *N-t*-Boc-L-alanine-*p*-nitrothiophenyl ester (Boc-Ala-SNp) was synthesized and applied as an ultrastructural cytochemical substrate for intracellular elastase-like enzymes. Mature human neutrophils incubated with Boc-Ala-SNp and gold ions generate an electron-dense reaction product, gold *p*-nitrothiophenolate, which is found in the nuclear membrane, Golgi complex, endoplasmic reticulum, mitochondria, and granules of these cells. Enzyme activity against Boc-Ala-SNp is also observed in developing monkey bone marrow neutrophils and in other blood cells. The intracellular neutrophil enzyme activity is elastase-like because it is characterized by a slightly alkaline pH optimum and is inactivated by exposure of the cells to general and specific active site inhibitors of neutrophil elastase. This substrate appears to have important potential for use in ultrastructural studies of intracellular elastase-like enzymes.

KEY WORDS ultrastructural cytochemistry · neutrophil · elastase-like enzymes

Elastolytic enzymes have been implicated in the pathogenesis of emphysema (20), glomerulonephritis (16), arthritis (8, 32), and atherosclerosis (30). A cytochemical substrate that can be used for the ultrastructural localization of elastase-like enzymes should aid considerably in further defining the role of these enzymes in normal and disease states. Although cytochemical substrates for elastase-like enzymes have been used for studies at the light microscope level (34), and elastase antibodies have been used for the extracellular localization of elastase in electron microscopy (29), elastase-like enzymes have not been described intracellularly in fine structural studies.

This report describes the synthesis and use of the substrate *N-t*-Boc-L-alanine-*p*-nitrothiophenyl ester (Boc-Ala-SNp) for the ultrastructural localization of intracellular elastase-like enzymes. The basic principle of this method has been applied to the detection of nonspecific esterases by Vatter et al. (43). The intracellular hydrolysis of Boc-Ala-SNp by enzymes of elastase-like specificity produces *p*-nitrothiophenolate (PNT) ion, which is immediately precipitated in the presence of gold ions at the enzyme site as an insoluble mercaptide. The heavy metal complex is readily identified in the electron microscope, thus localizing the subcellular sites of reactive enzyme. The present study reveals the association of elastase-like activity in human neutrophils not only with granules, as expected (28), but also with other membranous organelles within these cells.

MATERIALS AND METHODS

Synthesis of Boc-Ala-SNp

t-Boc-L-Alanine (3.70 g, 20 mmol; Schwartz/Mann Div., Bec-

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ton, Dickinson & Co., Orangeburg, N.Y.) was dissolved in 40 ml of dry methylene chloride, and the solution was flushed with dry nitrogen for 5 min. *p*-Nitrothiophenol (2.75 g, 19 mmol; Aldrich Chemical Co., Inc., Milwaukee, Wis.) recrystallized from ethyl ether was added to the reaction mixture. *N,N'*-Dicyclohexylcarbodiimide (4.24 g, 20 mmol; Schwartz/Mann) was added immediately after dissolution of the *p*-nitrothiophenol, and the mixture was flushed with nitrogen, stoppered, and stirred for 18 h at 25°C. Insoluble dicyclohexylurea was removed by filtration. The filtrate was evaporated *in vacuo*, yielding an orange colored oil and a crop of yellow crystals formed when the oil was heated gently on a steam bath. The crystals were separated by filtration and then recrystallized from dry methanol. The yield of crystalline product was 3.22 g (52% of theoretical). The product melted at 105–106°C. Elemental analysis indicated the percent composition to be as follows (theoretical values for $C_{14}H_{18}O_5N_2S$ are in parentheses): C, 52.18 (51.50); H, 5.75 (5.56); S, 9.15 (9.82). The product had infrared absorption bands at 3,380 (N-H); 2,847, 2,910, 2,960 (aliphatic C-H); 1,620 (C=O); and 1,500 and 1,340 cm^{-1} (NO_2). Thin-layer chromatography of the thiol ester on silica gel plates using acetone:benzene:acetic acid (50:50:1) as solvent yielded only one spot, as detected under ultraviolet light (R_f, 0.73). The spot was ninhydrin negative and became visibly yellow after spraying the plate with 0.1 N NaOH and heating to 80°C, which is consistent with the alkaline hydrolysis of the ester to form free PNT ion.

Isolation and Preparation of Cells

Monolayers of leukocytes on coverslips were prepared according to the method of Briggs et al. (14). Cells were fixed in a solution of 0.1 M sodium cacodylate, pH 7.4, containing 1% sucrose and 2% glutaraldehyde for 60 min at 4°C, followed by three 1-h changes of 0.1 M sodium cacodylate, pH 7.4, containing 1% sucrose. The cells were then infiltrated with 10% glycerol in 0.1 M cacodylate–1% sucrose for 10 min. The coverslips were frozen at −29°C and then thawed at room temperature. The cells were rinsed briefly in cacodylate-sucrose buffer and then exposed to Tris-maleate buffer containing 0.115 M Tris base and 0.045 M maleic acid, at a final pH of 7.6. Cells fixed in the presence of 0.01–0.02% Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa.), rather than by the freezing and thawing procedure, yielded the same distribution of reaction product upon subsequent treatment with Boc-Ala-SNp and gold. Although the distribution of reaction product was the same, the results obtained with Triton X-100 were considerably less consistent than those obtained with frozen and thawed cells. Cells used for phagocytosis studies were incubated for 20 min at 37°C in Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing 1 mg/ml glucose and ~5 × 10^9 polystyrene beads, 1.1 μm in diameter (Polysciences Inc., Warrington, Pa.), before fixation.

Fetal monkey bones (*Macaca mulatta*) were generously provided by Dr. G. Raviola of the Department of Anatomy, Boston University School of Medicine. Bone marrow was flushed from the humerus or femur bones under pressure and strained through fine-mesh steel gauze to remove bone fragments. Cells were washed in Hanks' balanced salt solution; erythrocytes were lysed in a hypotonic salt solution; and the remaining cells were fixed in suspension in a solution of 0.5% formaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate–1% sucrose for 10 min at 4°C. The cells were then washed in buffer and refrigerated overnight.

Incubation with Substrate

The human neutrophils on coverslips or monkey bone marrow cells in suspension were exposed to a solution composed of 0.79 ml of Tris-maleate buffer, pH 7.6, prepared as described above; 0.15 ml of 10 mg/ml sodium aurothiosulfate (Sanocrysin, Ferrosan International, Copenhagen, Denmark) in Tris-maleate buffer; and 0.06 ml of Boc-Ala-SNp (1.25 mg/ml in dimethyl sulfoxide [DMSO]) for 90–120 min at room temperature. In the inhibition studies, cells were treated with inhibitors for 1 h before and during the incubation with Boc-Ala-SNp and gold ions. The inhibitors used included L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) obtained from Sigma Chemical Co., St. Louis, Mo., and peptide chloromethyl ketone inhibitors of elastase and cathepsin G generously provided by Dr. James C. Powers, Georgia Institute of Technology, Atlanta, Ga. Phenylmethanesulfonyl fluoride (PMSF) was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y. Depending upon their solubilities, inhibitors were made up as concentrated stock solutions in either Tris-maleate buffer or 100% methanol. Cells were preincubated in a solution consisting of 0.05 ml of the inhibitor solution in 0.95 ml Tris-maleate buffer. Inhibitor at the same concentration was also included in the incubation solution with the substrate and gold. Control studies established that the presence of 5% methanol in the incubating solution did not affect the extent or distribution of reaction product in cells.

Preparation for Electron Microscopy

After incubation with gold and Boc-Ala-SNp, cells were washed with Tris-maleate buffer, pH 7.6, postfixed in 0.8% osmium tetroxide in 0.09 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature, and stained en bloc in a solution of 0.5% uranyl acetate for 20 min, with washing in 0.1 M sodium acetate for 3 min before and after staining. The cells were dehydrated in ethanol and propylene oxide and infiltrated with Epon according to Luft (31). BEEM capsules (Better Equipment for Electron Microscopy, Bronx, N.Y.) were inverted over coverslips and removed 24 h later by immersion in liquid nitrogen. Ultrathin sections were examined without further staining on an AEI-6B electron microscope.

Bone marrow cells were washed in Tris-maleate buffer, rinsed in 0.09 M sodium cacodylate, pH 7.4, and pelleted in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 10,000 *g* for 4 min. The pellets were postfixed, stained en bloc, dehydrated, infiltrated with Epon, and embedded in BEEM capsules.

Incubations with Esterase and Peroxidase Cytochemical Substrates

Human neutrophils were incubated with *p*-nitrophenylthiol acetate (PNTA), a cytochemical substrate used for the determination of nonspecific esterase (43). Cells were incubated in the same medium used with Boc-Ala-SNp, except that PNTA (3.5 mg/ml in DMSO) was substituted for Boc-Ala-SNp. Endogenous peroxidase activity was located according to the method of Graham and Karnovsky (22) at pH 7.6.

Spectrophotometric Enzyme Assays

Enzymes were assayed at 25°C in 1-cm pathlength cuvettes after the enzyme-dependent hydrolysis of oxygen or sulfur esters by measuring the change in optical density at 410 nm upon the release of PNT or PNT ions. The assay mixtures contained 60 μl of DMSO stock solutions of *N-t*-Boc-L-alanine-*p*-nitrophenyl ester (Sigma Chemical Co.), Boc-Ala-SNp, or PNTA diluted to

0.99 ml with Tris-maleate buffer, pH 7.6. The nonenzymatic rate of hydrolysis was recorded, and the enzyme was added to the solution in 10-μl amounts to initiate the reaction. Reaction rates were determined as the linear increase in absorbance at 410 nm after the addition of enzyme.

Enzymes or enzyme preparations analyzed in this fashion included a whole-cell neutrophil lysate, purified neutrophil enzymes, elastase and cathepsin G prepared by the methods of Baugh and Travis et al. (9, 42), and porcine pancreatic elastase purified by the method of Shotton (38). The neutrophil lysate, neutrophil elastase, and cathepsin G were provided by Ms. Dianne Dunn, and pancreatic elastase was provided by Dr. Phillip Stone, both of the Department of Biochemistry, Boston University School of Medicine. Chymotrypsin, trypsin, cathepsins C and D, carboxyl esterase, acylase I, sulfatase, acetylcholinesterase, cholesterol esterase, and acetyl esterase were obtained in the purest form available from Sigma Chemical Co. The whole-cell neutrophil lysate was supplemented with 1% Triton X-100 to solubilize membrane-bound enzymes.

RESULTS

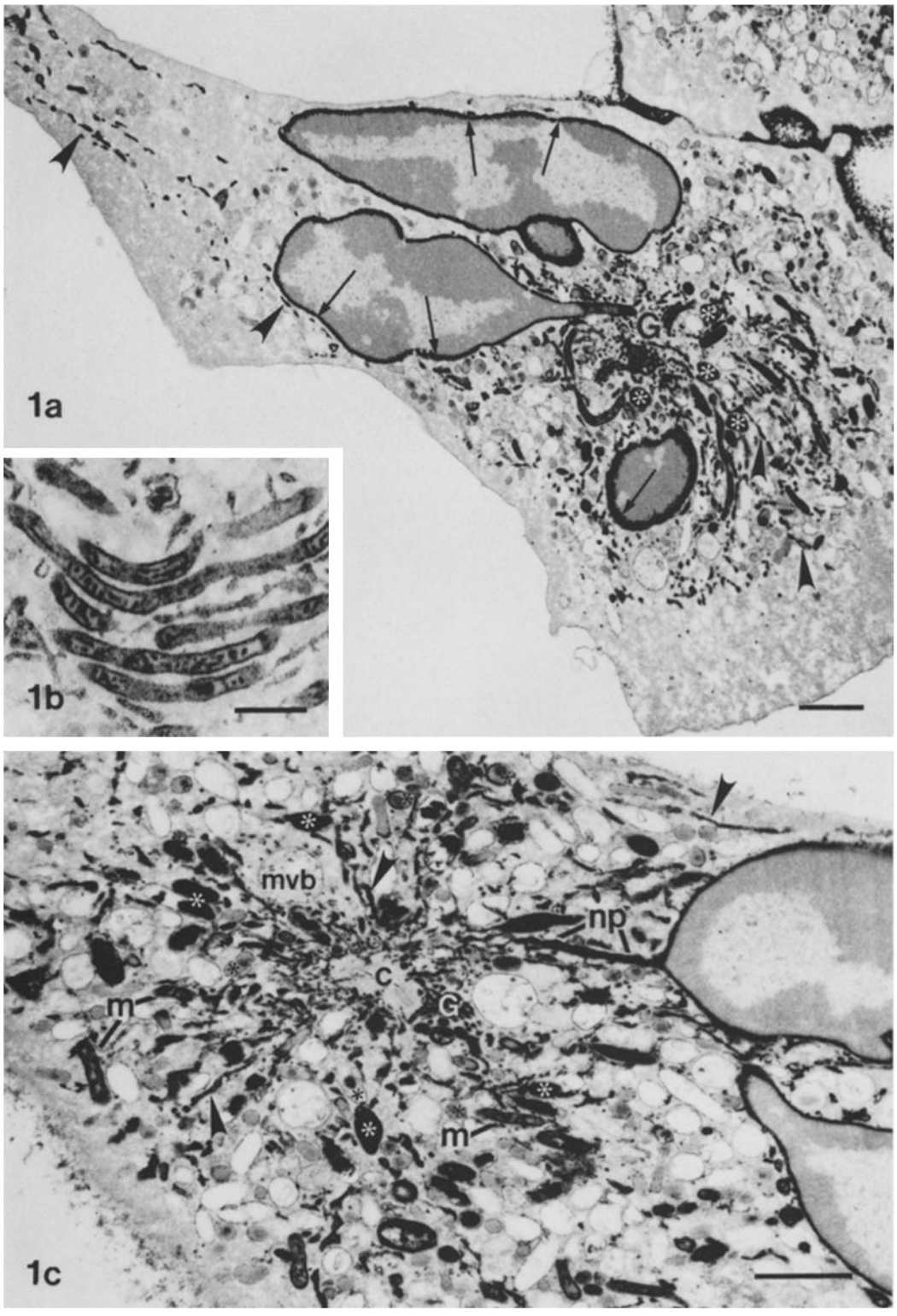
Localization of Reaction Product

The reaction product generated within the spread human neutrophils during the incubation with Boc-Ala-SNp and gold can be visualized by both light and electron microscopy. The insoluble complex observed by light microscopy is located around the nuclear lobes and in the central part of the cell cytoplasm encircled by the nuclear lobes (Fig. 6*b*). As seen in the electron microscope, electron-dense product is located within the nuclear envelope, the endoplasmic reticulum, the Golgi complex, some granules, and between the inner and outer mitochondrial membranes (Fig. 1 *a* and *b*). In fact, the reaction product accentuates organelles such as endoplasmic reticulum and mitochondria, which otherwise go largely unnoticed in mature neutrophils. The highest concentration of those organelles containing reaction product is in the centrosomal region of the spread cell (Fig. 1 *c*), where the organelles are organized around the centrioles. The Golgi cisternae are usually adjacent to the centrioles, with one stack of membranes associated with each centriole (Fig. 2 *a* and *b*). Reaction product, however, does not appear to delineate any GERL in this region. Granules, mitochondria, and endoplasmic reticulum radiate from the centrioles (Fig. 1 *c*). Multivesicular bodies with reaction product also are observed in this area of the cell (Fig. 1 *c*). Projections of the nuclear envelope containing reaction product often extend toward the Golgi complex in the centriolar area (Fig. 1 *c*). The endoplasmic reticulum in the centrosomal region is in close association with both the Golgi complex and the nuclear envelope (Fig. 1 *c*). In fact, the proximity of the nuclear envelope, Golgi complex, and endoplasmic reticulum suggests that these organelles may be interconnected.

Mitochondria and endoplasmic reticulum are also observed outside the centrosomal region of the spread cells distributed throughout the cytoplasm (Fig. 2 *c*). These two organelles are oriented parallel to the long axis of migratory cells, either extending from the centrioles toward the leading edge of the cell or skirting the nuclear lobes to extend well into the tail (Figs. 1 *a* and 2 *c*).

The neutrophil granules exhibit heterogeneity in response to incubation with Boc-Ala-SNp. Usually, only those granules associated with the centrosomal region contain large amounts of reaction product, and granules outside of this area are electron transparent (Fig. 1 *c*). There are varying distributions of reaction product within those granules that do react (Fig. 2 *d*), that is, the reaction product completely fills some of the granules, whereas in others it is confined to a central core that is immediately surrounded by either an electron-transparent or a slightly electron-dense matrix. The granules with reaction product may all be of the same type, with the different densities of reaction product possibly appearing because enzymes have been washed out to various degrees during the processing of the cells before incubation. Some support for this suggestion comes from

FIGURE 1 Distribution of reaction product in human neutrophils incubated with Boc-Ala-SNp. (*a*) In cells spread onto coverslips, reaction product is located in the nuclear envelope (arrows), Golgi complex (*G*), endoplasmic reticulum (arrowheads), granules (asterisks), and mitochondria. Most of the organelles with reaction product are concentrated in the center of the cell. There are fewer organelles in the peripheral areas. Bar, 1 μm. × 9,700. (*b*) Mitochondria contain reaction product between the inner and outer membranes. Bar, 0.5 μm. × 21,900. (*c*) Many organelles with reaction product are organized around the centrioles. The Golgi complex (*G*) is adjacent to the centriole (*c*) and the endoplasmic reticulum (arrowheads), granules (asterisks), and mitochondria (*m*) radiate from them. Nuclear projections (*np*) often extend into the centriolar area. Multivesicular bodies (*mvb*), which contain small amounts of reaction product, are also associated with this region of the spread cell. Bar, 1 μm. × 15,000.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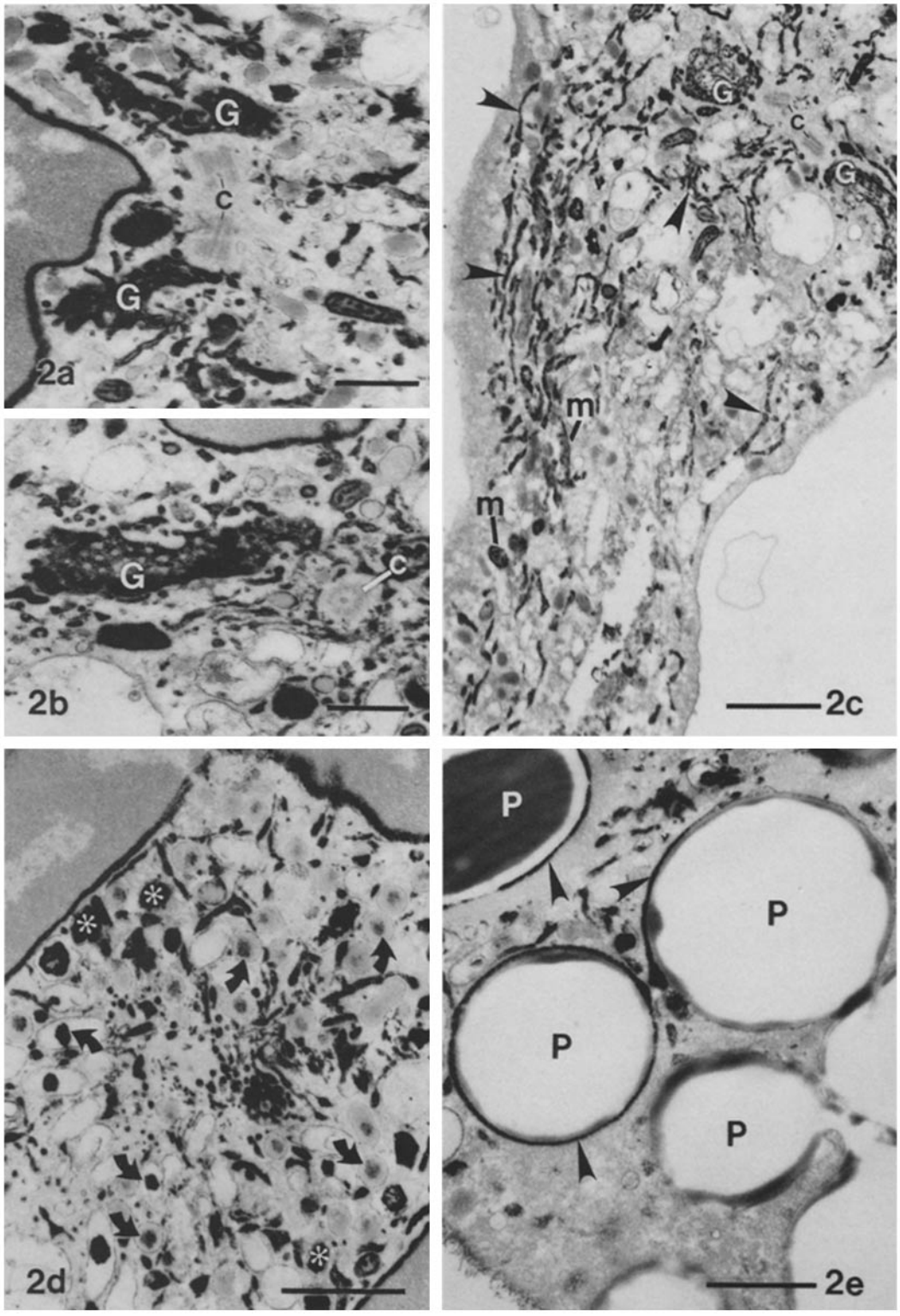
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previous observations that the morphology of neutrophil granules is quite sensitive to differences in the processing for electron microscopy (5). Many of the reactive granules are large, elongate, and resemble the azurophils described in other morphological studies (5, 13). Other reactive granules are more difficult to identify without further investigation.

Reaction product is observed in phagolysosomes when cells are stimulated to phagocytose polystyrene beads (Fig. 2*c*). Presumably, the enzyme activity appearing in phagolysosomes is derived from granules that have fused with phagocytic vacuoles, as has been shown in ultrastructural studies with other cytochemical substrates (4).

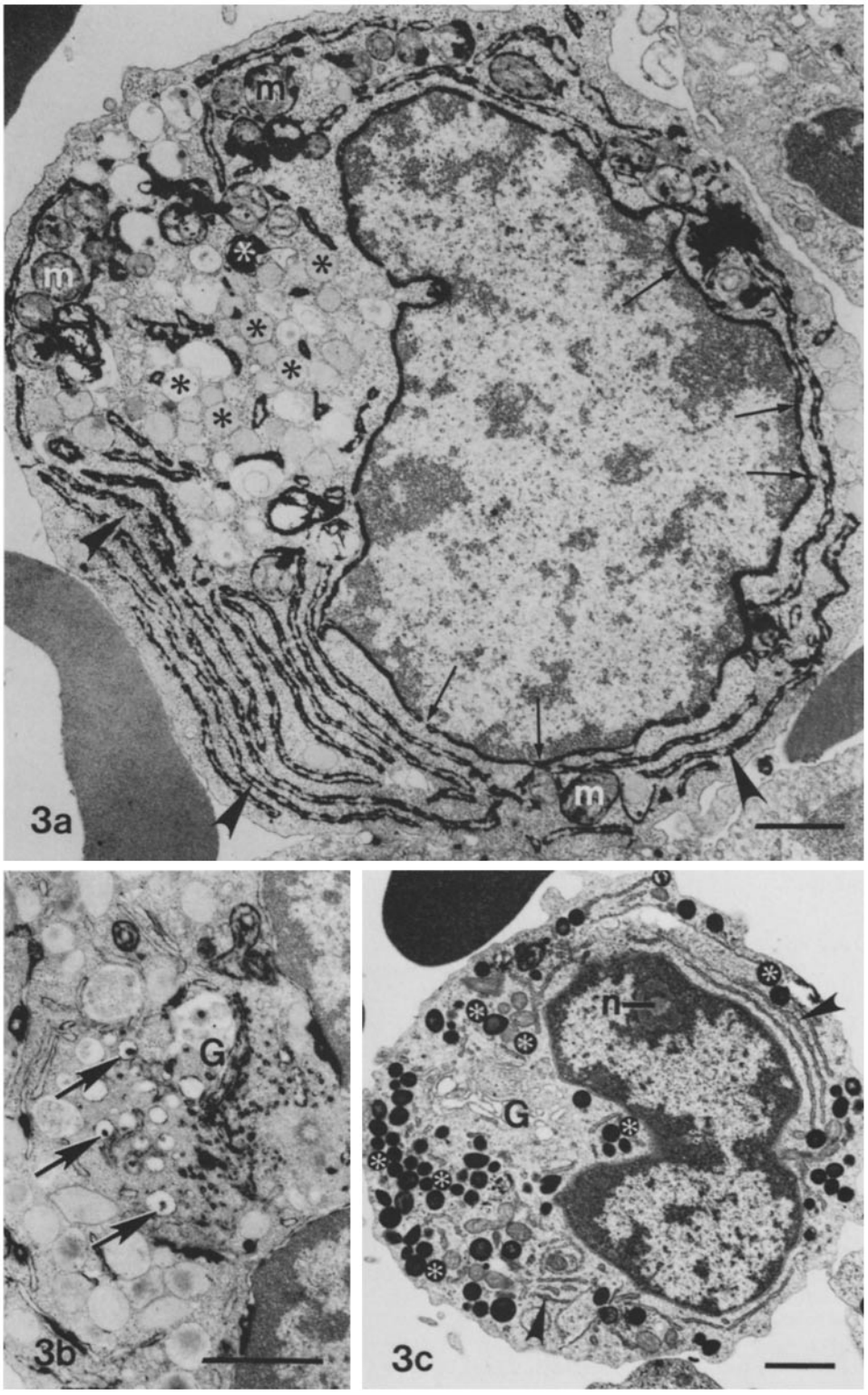
Reaction Product in Developing Neutrophils and Other Cell Types

Cells other than human peripheral neutrophils also contain enzymes active against Boc-Ala-SNp. Reaction product is observed in all stages of developing monkey bone marrow granulocytes. The morphology of the various stages of the developing monkey bone marrow neutrophils closely resembles that of the developing human bone marrow neutrophils, in which the stages are characterized, in part, by the granule population (5). Two major types of granules, azurophilic and specific, have been described in mature human neutrophils as being heterogeneous in both enzyme content and morphology (5, 13, 41, 45). These granules form sequentially in developing neutrophils, the azurophils being the first of the two types of granules to appear (5, 12).

Cells in the promyelocyte stage of development are characterized by the presence of azurophil granules and the absence of specific granules (5, 12). In this study, azurophilic granules were identified in monkey bone marrow cells by staining for peroxidase activity because this is a marker enzyme for the azurophils of human neutrophils (11, 18). Promyelocytes could thus be identified by their single population of peroxidase-positive granules, by their extensive, rough endoplasmic reticulum, and by their nuclei with diffuse chromatin and prominent nucleolus (5) (Fig. 3*c*). After incubation with Boc-Ala-SNp, the cells identified as promyelocytes contain electron-dense reaction product. The reactivity of the endoplasmic reticulum is striking (Fig. 3*a*). Reaction product is also observed in the nuclear envelope and in the Golgi complex and its associated immature granules (Fig. 3*a* and *b*). Electron-dense reaction product is also observed between the inner and outer membranes of mitochondria (Fig. 3*a*). Occasionally, mature granules also contain electron-dense product, but generally they do not react. The lack of reactivity of the granules in the monkey cells likely reflects the fact that these cells were not frozen and thawed before incubation with substrate. The granules of mature human neutrophils similarly treated do not react (Fig. 4*d*).

Neutrophilic myelocytes are identified by the presence of both peroxidase-positive and peroxidase-negative granules, by an indented nucleus that has a small amount of condensed chromatin, and by less prominent endoplasmic reticulum and fewer mitochondria than is characteristic of the promyelocyte stage (5) (Fig. 4*b*). Myelocytes also generate electron-dense product when incubated with Boc-Ala-SNp and gold (Fig. 4*a*). Again, the endoplasmic reticulum is reactive, although less so than in the promyelocyte stage, likely reflecting a decrease in the number of these membranes. The Golgi complex, mitochondria, and a few of the larger granules also contain reaction product. A similar distribution of product is seen in the metamyelocytes and mature neutrophils (Fig. 4*c*). Thus, enzyme activity against Boc-Ala-SNp is present throughout all stages of neutrophil development.

FIGURE 2 Distribution of enzymes active on Boc-Ala-SNp. (*a* and *b*) The Golgi complex contains reaction product throughout the cisternae (*G*), which are found adjacent to each centriole (*c*). The cisternae (2*b*) consist of interconnected tubules. (*a*) Bar, 0.5 μm. × 26,200. (*b*) Bar, 0.5 μm. × 25,000. (*c*) The endoplasmic reticulum (arrowheads) and mitochondria (*m*), which are not organized around the centrioles (*c*), are oriented parallel to the axis of the cell. *G*, Golgi complex. Bar, 1 μm. × 14,500. (*d*) Reactive granules in the cell center exhibit a variety of distributions of reaction product. Reaction product may fill the entire granule (asterisks), or it may be found as a core surrounded by a translucent or only slightly electron-dense area (arrows). Bar, 1 μm. × 18,750. (*e*) Reaction product is also observed in phagolysosomes (arrowheads) after neutrophils have been stimulated to phagocytose polystyrene beads (*P*) and granules have fused with the phagocytic vacuoles. Bar, 1 μm. × 17,000.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Other cells that have enzymatic activity against Boc-Ala-SNp were also noted in this study. The enzyme activity appears to be present in all blood cells. It is found in monkey bone marrow erythrocytes, human venous blood monocytes, lymphocytes, and eosinophils. As with the neutrophils, the activity is generally found in the nuclear envelope, endoplasmic reticulum, Golgi complex, and between the inner and outer mitochondrial membranes of these cells (Fig. 5).

Control Studies

Consistent with the chemistry of this reaction, reaction product is deposited only when Boc-Ala-SNp and gold are present in the incubation mixture. When either gold or substrate is omitted from the incubation solution, no reaction product is observed in the cells. Thus, electron-dense product does not seem to reflect the nonspecific adsorption of free gold ions to subcellular components. Also, gold and Boc-Ala-SNp must be added simultaneously. When they are added sequentially to the cells, no reaction product is generated, quite likely as a result of the diffusion of the PNT formed.

The contribution of gold ions to the electron density of the reaction product was assessed by the omission of osmium tetroxide and uranyl acetate from the preparation of cells for electron microscopy. The physical characteristics and density of the reaction product were not changed in cells prepared in the absence of these other heavy metals.

A variety of experiments were designed to test for the dependency of the generation of reaction product on intracellular enzyme activity and for the nonspecific adsorption of reaction product. Since Boc-Ala-SNp can slowly spontaneously hydrolyze at the slightly alkaline pH of the incubation medium, it is possible that some of the product seen in cells might be nonenzymatically generated. In testing this possibility, it was observed that cells heated at 90°C for 10 min before incubation with the substrate-metal ion mixture do not generate any visible reaction product, suggesting that heat-labile enzyme activity is the sole basis for the reaction seen. Furthermore, the density of reaction product in cells incubated at varying temperatures increases as the temperature of the incubation increases (Fig. 6). Room temperature was selected as the standard incubation temperature for the reaction. Similarly, there is a progressive increase in the density of the reaction product deposited as the incubation time increases. The reaction is maximal at 1.5–2 h at room temperature. In summary, the heat lability and the time and temperature dependency of the deposition of reaction product support the conclusion that the generation of reaction product is enzyme dependent.

To further assess whether reaction product is nonspecifically adsorbed to any of the subcellular compartments in which it is located, heat-inactivated neutrophils were incubated with substrate and gold in the presence of native pancreatic elastase or 1 mM L-histidine, both of which hydrolyze Boc-Ala-SNp. No intracellular electron-dense product was seen in these neutrophils although the substrate was hydrolyzed during the incubation with these agents. This result argues against nonspecific adsorption to any of the compartments in which reaction product is localized.

Nature and Specificity of the Intracellular Enzyme Activity in Mature Neutrophils

Experiments were carried out to characterize the leukocytic enzyme activity responsible for the hydrolysis of Boc-Ala-SNp by examining the pH optimum, substrate preference, and inhibitor profile of the reaction. The distribution and extent of reaction product were examined in neutrophils incubated with substrate and gold at varying pH values (Fig. 7). No reaction product is observed at

FIGURE 3 Monkey bone marrow promyelocytes. Cells are stained with uranyl acetate and lead citrate. (*a*) Promyelocytes incubated with Boc-Ala-SNp contain reaction product in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), and between the inner and outer membranes of mitochondria (*m*). Asterisks indicate granules. Bar, 1 μm. × 13,000. (*b*) The Golgi complex (*G*) of promyelocytes incubated with Boc-Ala-SNp also contains reaction product. The stacks of cisternae as well as vesicles and immature granules (arrows) are reactive. Bar 1 μm. × 17,800. (*c*) The promyelocyte stage of granulocytes was identified by its abundant endoplasmic reticulum (arrowheads), large Golgi complex (*G*), and nuclei with diffuse chromatin and prominent nucleolus (*n*). The most notable characteristic is the presence of azurophils (asterisks), which are stained for peroxidase. There are no peroxidase-negative specific granules. Bar, 1 μm. × 9,375.

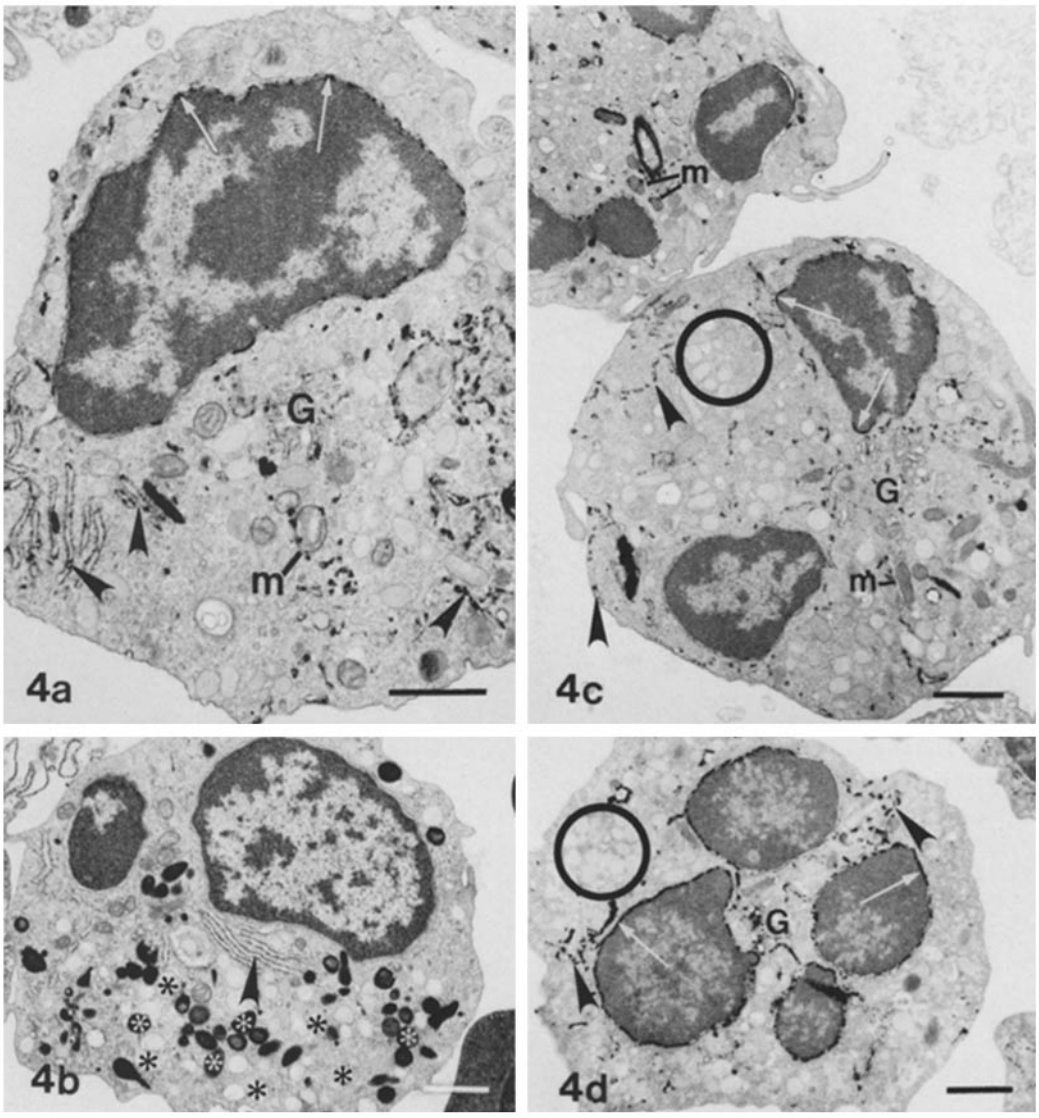


FIGURE 4 Myelocyte and mature stages of monkey bone marrow neutrophils. All cells were stained with uranyl acetate and lead citrate except that in *d*. (*a*) Myelocytes also exhibit enzyme activity against Boc-Ala-SNp. Reaction product is found in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), Golgi complex (*G*), and mitochondria (*m*). Bar, 1 μm. × 12,800. (*b*) Myelocytes were identified by the presence of two populations of granules: azurophils (white asterisks), which contain peroxidase, and specific granules (black asterisks), which are peroxidase negative. Also, the nuclear chromatin is more condensed, and there is less endoplasmic reticulum (arrowheads) than in the promyelocyte stage of development. Bar, 1 μm. × 8,400. (*c*) The mature monkey bone marrow neutrophil closely resembles the human neutrophil in *d*. The nucleus is lobed, and the chromatin is highly condensed. When incubated with Boc-Ala-SNp, the cells contain small amounts of reaction product in the nuclear envelope (arrows), Golgi complex (*G*), endoplasmic reticulum (arrowheads), and the mitochondria (*m*). The granules (encircled) do not contain reaction product. Bar, 1 μm. × 9,200. (*d*) This human venous blood neutrophil was prepared similarly to the monkey bone marrow cells (i.e., not frozen and thawed before incubation and incubated in suspension). The distribution of reaction product using Boc-Ala-SNp as substrate is similar to that observed in the monkey bone marrow mature neutrophils in *c*. Product is found in the Golgi complex (*G*), nuclear envelope (arrows), and the endoplasmic reticulum (arrowheads). The granules (encircled) in these preparations do not contain reaction product. Bar, 1 μm. × 7,500.

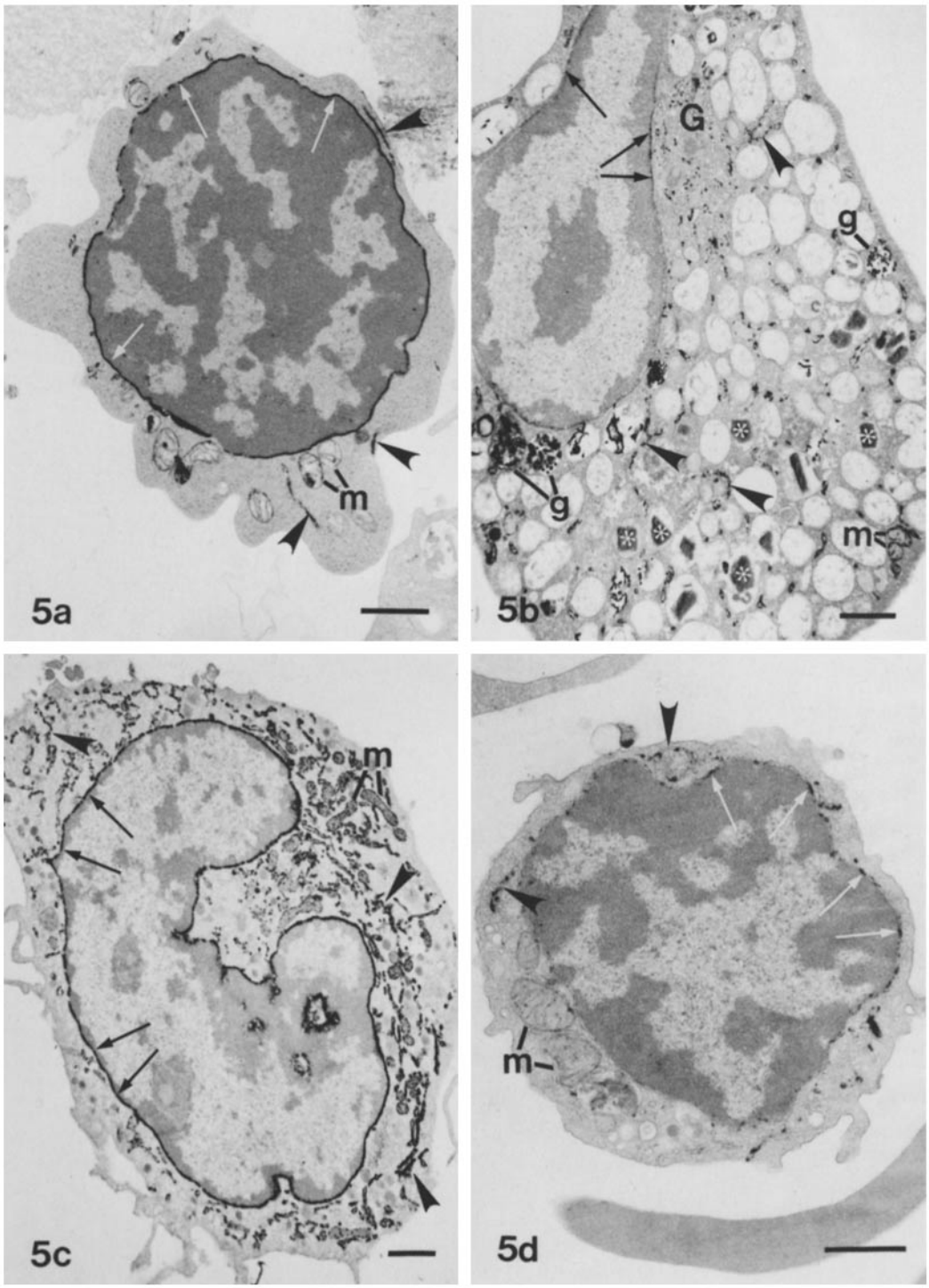
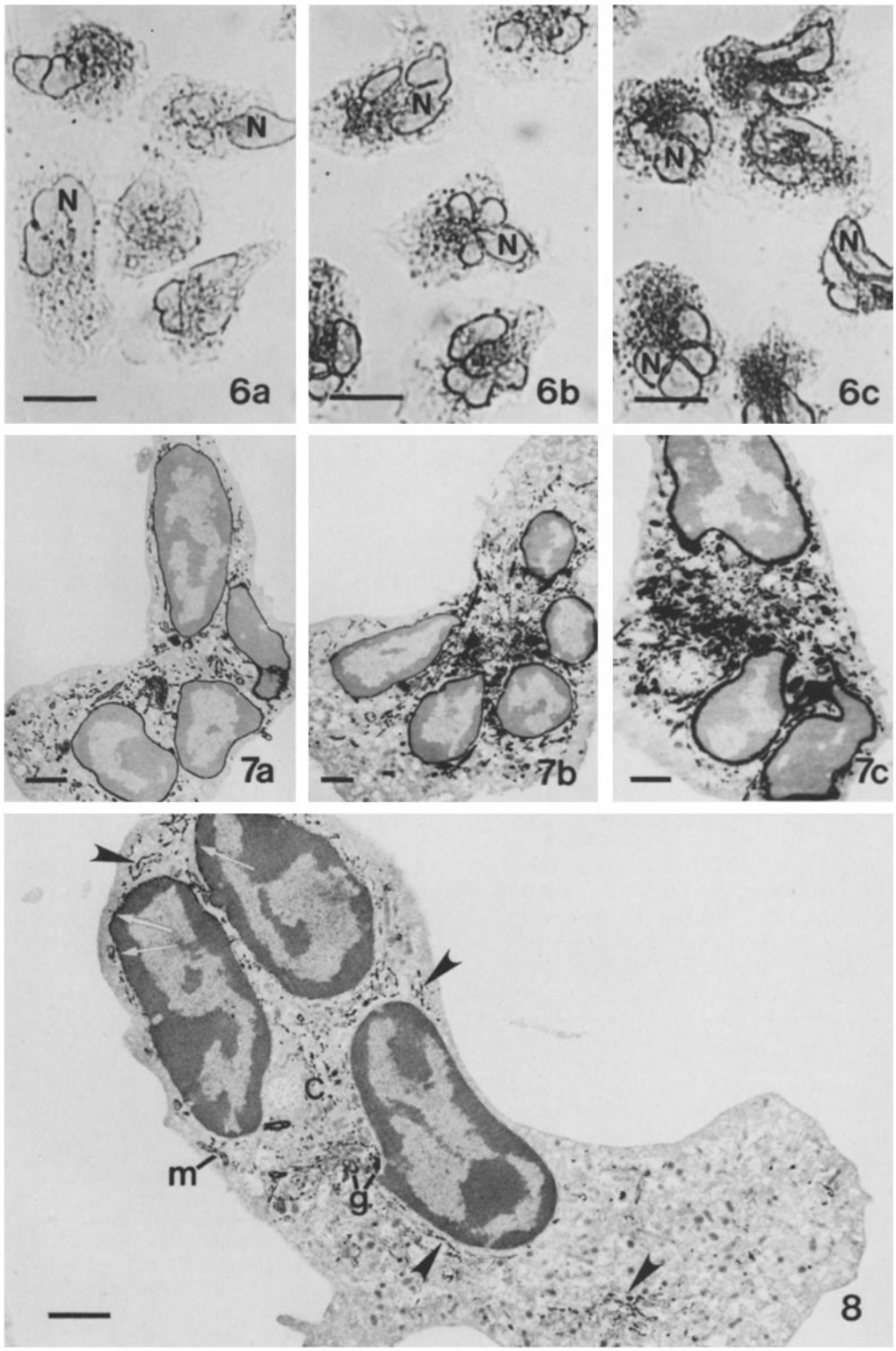


FIGURE 5 Other blood cells which generate reaction product upon incubation with Boc-Ala-SNp. (*a*) The monkey bone marrow erythroblast contains reaction product in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), and mitochondria (*m*). Stained with uranyl acetate and lead citrate. Bar, 1 μm. × 10,200. (*b*) Reaction product is also observed in the human venous blood eosinophils in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), Golgi complex (*G*), and mitochondria (*m*). The eosinophilic granules are damaged by the freezing procedure, and few granules contain characteristic crystalline cores (asterisks). Some of the granules (*g*) contain reaction product. Bar, 1 μm. × 8,100. (*c*) Human venous blood monocytes contain enzymes that generate reaction product from Boc-Ala-SNp in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), and mitochondria (*m*). Bar, 1 μm. × 6,700. (*d*) Reaction product is found in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), and mitochondria (*m*) of human venous blood lymphocytes. Bar, 1 μm. × 12,000.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pH 3.9 or 5.4, whereas all cells incubated at pH 6.4 contain small amounts of electron-dense product in the endoplasmic reticulum, nuclear envelope, Golgi complex, and granules. Maximal activity occurs in cells incubated at pH values between 7.4 and 8.1. Spontaneous base-catalyzed hydrolysis of Boc-Ala-SNp prevented the incubation of cells with substrate at pH values over 8.1. It does seem, however, that the optimal pH for this reaction is slightly alkaline. A pH of 7.6 was used routinely for the incubation of the cells.

Neutrophils were also incubated with the thiol acetate ester PNTA, which has been used to locate nonspecific esterase activity within cells (43). As shown in Fig. 8, the distribution of reaction product in neutrophils incubated with this substrate and gold is essentially the same as that resulting from hydrolysis of Boc-Ala-SNp, but the density of product is considerably lower than that seen with the alanine ester.

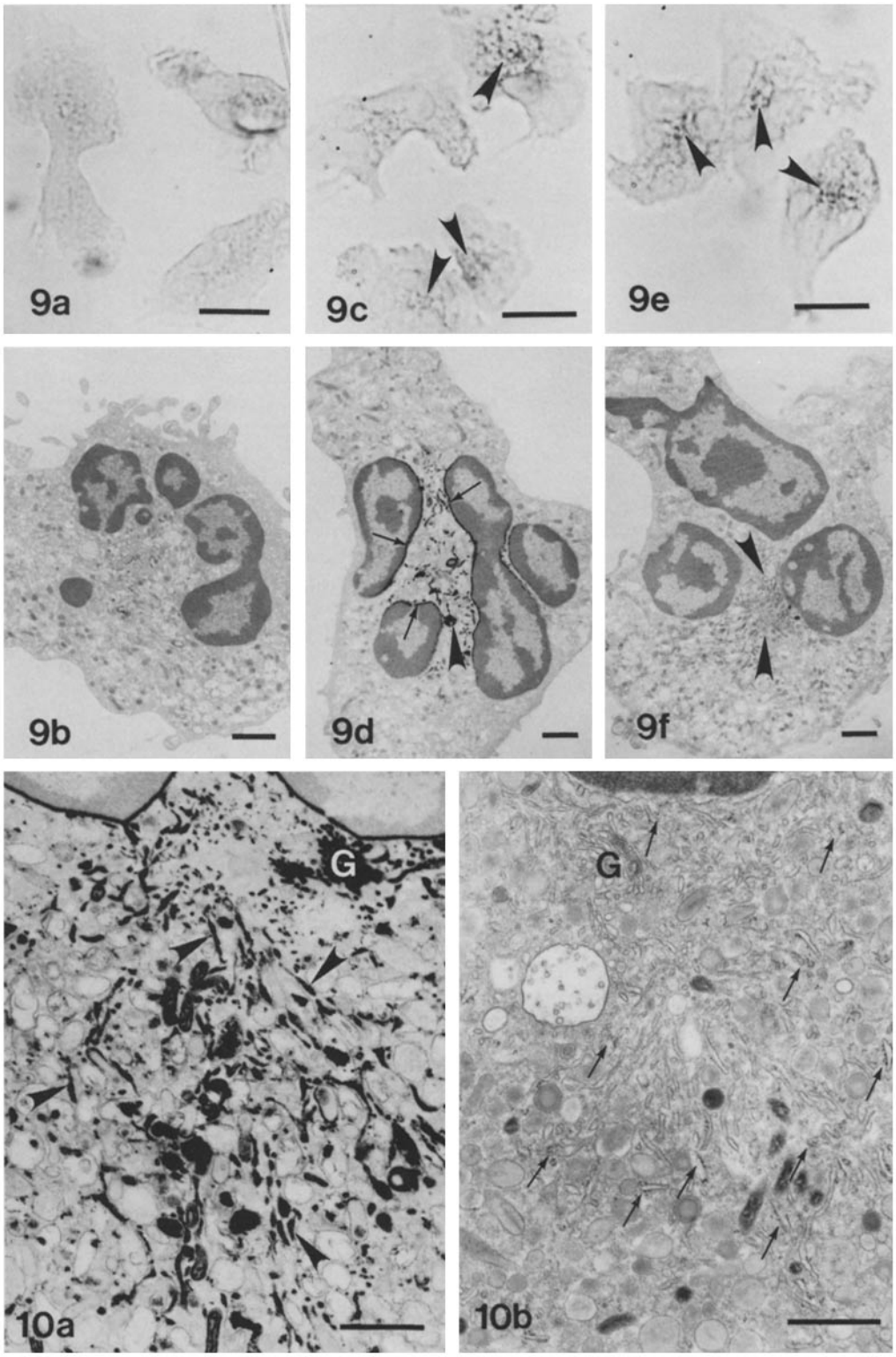
A variety of enzyme inhibitors were incubated with cells to further characterize the enzyme activity responsible for the hydrolysis of Boc-Ala-SNp. Neutrophils were incubated with PMSF, a sulfonyl fluoride that covalently modifies the reactive seryl residue at the active site of a variety of proteases and esterases (7, 19), before and during the exposure to Boc-Ala-SNp and gold. The generation of electron-dense product was prevented by the exposure of cells to 0.1 mM PMSF (Fig. 9 *a* and *b*). Because this reagent may also modify proteinases that have a functional cysteine residue at the active site (7, 46), other preparations of neutrophils were treated with 0.01–10 mM *N*-ethylmaleimide (NEM), an agent that covalently modifies the sulfhydryl groups of proteins (7). Preincubation of cells with NEM before incubation with the alanine ester and gold did not visibly inhibit the deposition of reaction product. Similarly, treatment of cells with 5 mM EDTA, an inhibitor of many metalloenzymes (7), did not inhibit or alter the deposition of reaction product.

Chloromethyl ketone (CMK) derivatives of amino acids and peptides covalently inactivate enzymes with affinities for the specific amino acid side chains contained in the inhibitor, including specific serine proteases such as neutrophil elastase (39). Neither inhibition of generation nor alteration of the distribution of the reaction product is observed in cells incubated with TLCK, a chloromethyl ketone inhibitor of enzymes having trypsin-like specificity (39). Similarly, there is no inhibition of the cytochemical reaction in cells treated with TPCK, an inhibitor of chymotrypsin enzymes (39). Treatment of neutrophils with Z-Gly-Leu-Phe-CMK (where Z indicates benzyloxycarbonyl), a tripeptide chloromethyl ketone that inhibits leukocytic cathepsin G (36), a chymotrypsin-like enzyme, neither inhibits enzyme activity nor alters the distribution of cytochemical product in neutrophils. Neutrophils were also exposed to chloromethyl ketone inhibitors of leukocytic elastase. Inhibition of enzyme activity by Ac-Ala-Ala-Pro-Val-CMK (35) is first observed at a 1-mM concentration of this compound. Formation of reaction product is largely prevented by treatment of cells with a 2-mM concentration of this inhibitor (Fig. 9 *c* and *d*). Similarly, exposure of cells to 1 mM MeO-Suc-Ala-Ala-Pro-Val-CMK, a more specific chloromethyl ketone inhibitor of neutrophil elastase (36), inhibits deposition of reaction product. Most cells examined by electron microscopy contained no reaction product. The occasional cells that did react contained a small amount of product, which was confined to the cell center (Fig. 9 *f*). In summary, as shown in Table I, reac-

FIGURE 6 Reaction product in neutrophils incubated with Boc-Ala-SNp at various temperatures. The product is observed surrounding the nuclear lobes (*N*) and in the cell center, which is surrounded by, or associated with, the nuclear lobes. These cells were incubated at 4° (*a*), 23° (*b*), and 37°C (*c*). As the temperature increases, the density of the reaction product also increases. Bar, 5 μm. × 2,200.

FIGURE 7 Effect of pH on the generation of reaction product. Neutrophils incubated at pH 6.4 (*a*), 7.4 (*b*), and 8.1 (*c*) generate reaction product, whereas those incubated at pH 3.1 and 5.4 do not (not shown). Neutrophils incubated at pH 7.4 and 8.1 contain the highest densities of reaction product. Bars, 1 μm. (*a*) × 5,500; (*b*) × 4,400; (*c*) × 6,000.

FIGURE 8 Neutrophil incubated with PNTA, a nonspecific esterase substrate. The reaction product generated from this substrate is located in the nuclear envelope (arrows), endoplasmic reticulum (arrowheads), mitochondria (*m*), and granules (*g*), although much less is generated than in cells incubated with Boc-Ala-SNp. *c*, cell center. Bar, 1 μm. × 8,000.


9a
9c
9e
9b
9d
9f
G
10a
G
10b

tion product derived from the intracellular hydrolysis of Boc-Ala-SNp is effectively inhibited only by inhibitors known to inactivate neutrophil elastase. The intracellular activity appears to be unaffected by inhibitors of tryptic, chymotryptic, and cathepsin G activity.

Spectrophotometric Assays

Colorimetric assays were performed with purified and partially purified enzyme preparations to complement and corroborate the results of the cytochemical studies. The conditions of these spectrophotometric assays were chosen to simulate those used in the cytochemical studies. The susceptibility of Boc-Ala-SNp to hydrolytic enzymes of differing substrate specificities was compared. As shown in Table II, human neutrophil elastase readily hydrolyzes both the sulfur and oxygen

TABLE I

Summary of the Effects of Inhibitors on the Hydrolysis of Boc-Ala-SNp by Intracellular Enzyme Activity

Inhibitor	Concentration	Effect
	mM	
NEM	0.01–10.0	−
EDTA	1.0–5.0	−
PMSF	0.01	−
	0.1–1.0	++
TPCK	0.01–1.0	−
TLCK	0.01–1.0	−
Z-Gly-Leu-Phe-CMK	0.01–1.0	−
Ac-Ala-Ala-Pro-Val-CMK	0.01–0.1	−
	1.0–2.0	+
MeO-Suc-Ala-Ala-Pro-Val-CMK	0.1	−
	1.0	++

Neutrophils were exposed to a variety of enzyme active site inhibitors in the concentrations shown above. The effects are expressed as no inhibition (−); partial inhibition (+); or complete inhibition (++) of the generation of reaction product in cells preincubated with inhibitor and then incubated with the inhibitor, gold ions, and Boc-Ala-SNp.

TABLE II

Hydrolysis of Boc-Ala-SNp, Boc-Ala-ONp, and PNTA by Various Enzymes

	ΔOD_{410} min^{-1} μg^{-1}		
Enzyme	Boc-Ala-SNp	Boc-Ala-ONp	PNTA
Human leukocytic elastase	0.0460	0.0970	0.0000
Pancreatic elastase	0.0560	0.0675	—
Human leukocytic cathepsin G	0.0075	0.0058	0.0000
Neutrophil lysate	0.0002	—	—
α-Chymotrypsin	0.0007	—	—
Trypsin	0.0001	—	—
Cathepsin D	0.0000	—	—
Acetylcholinesterase	0.0000	—	—
Acylase I	0.0001	—	—
Sulfatase	0.0000	—	—
Cathepsin C	0.0003	—	0.0022
Cholesterol esterase	0.0020	—	0.0400
Carboxyl esterase	0.0540	—	0.2920
Acetyl esterase	0.0018	—	0.0183

The enzymes listed above were assayed with Boc-Ala-SNp, Boc-Ala-ONp, or PNTA. Nonenzymatic hydrolysis was recorded as change in optial density (OD) at 410 nm and 25°C in an assay mixture containing 0.23 mM substrate in Tris-maleate buffer, pH 7.6. Enzyme was added in 10 μl amounts to initiate the reaction. −, not assayed.

FIGURE 9 Neutrophils incubated with enzyme inhibitors. (*a* and *b*) These neutrophils were preincubated and incubated with 0.1 mM PMSF, a serine protease inhibitor. Reaction product is not generated in these cells. (*a*) Bar, 5 μm. × 2,200. (*b*) Bar, 1 μm. × 5,900. (*c* and *d*) Neutrophils exposed to the elastase inhibitor Ac-Ala-Ala-Pro-Val-CMK in 2 mM concentration. There is some inhibition of enzyme activity (cf. 1 *a* and 7 *b*), but some cells contain reaction product in the cell center (arrowheads) and nuclear envelope (arrows). (*c*) Bar, 5 μm. × 2,200. (*d*) Bar, 1 μm. × 5,000. (*e* and *f*) Neutrophils exposed to a 1 mM concentration of the elastase inhibitor MeO-Suc-Ala-Ala-Pro-Val-CMK. Enzyme activity is largely inhibited by this chloromethyl ketone. In some cells there are small amounts of reaction product found in the cell center (arrowheads). (*e*) Bar, 5 μm. × 2,200. (*f*) Bar, 1 μm. × 4,600.

FIGURE 10 (*a*) The endoplasmic reticulum (arrowheads) contains reaction product in cells incubated with Boc-Ala-SNp. (*b*) It can be seen that ribosomes (arrows) are associated with the membranes of the endoplasmic reticulum when cells which had not been incubated with Boc-Ala-SNp are examined. *G*, Golgi complex. Bars, 1 μm. (*a*) × 12,500. (*b*) × 14,300.

esters of alanine-derived substrates, as does porcine pancreatic elastase. Cathepsin G, the chymotrypsin-like enzyme of neutrophil granules, also hydrolyzes both substrates, but at rates significantly lower than those of the elastase enzymes.

Pancreatic chymotrypsin and trypsin, cathepsins C and D, acylase, sulfatase, and acetylcholinesterase hydrolyze Boc-Ala-SNp either negligibly or not at all (Table II). A few esterases, however, do hydrolyze Boc-Ala-SNp to some extent. Although leukocyte elastase and cathepsin G show a marked preference for the alanine substrate Boc-Ala-SNp over the acetate ester PNTA, the esterases with slight activity against Boc-Ala-SNp more readily hydrolyze PNTA (Table II). Also, an analysis of the pH profile of the whole-cell lysate is consistent with the intracellular study, which showed that the neutrophil activity against Boc-Ala-SNp is greatest at slightly alkaline pH.

DISCUSSION

Elastolytic enzymes of pancreas and neutrophils are often assayed by the spectrophotometric detection of *p*-nitrophenolate ion released upon hydrolysis of Boc-Ala-ONp (27, 44). Substitution of sulfur for oxygen in the ester linkage of this substrate yields a new elastase substrate which has the clear potential, as shown in this study, to serve as an ultrastructural probe of elastase-like enzymes in human neutrophils and other cells. It should be noted that neutrophil elastase will hydrolyze an NH_2-terminus-blocked alanyl ester, although it prefers valine over alanine as the carbonyl donor at the susceptible bond in tetrapeptide model substrates (47). Although we used an alanine derivative in this study, we have also synthesized and used Boc-Val-SNp as a cytochemical substrate. We have concluded, however, that this compound is too insoluble to be of use as an elastase substrate for spectrophotometric assays or ultrastructural cytochemistry.

The advantages of the use of thiol esters as cytochemical probes of proteases are clear. Primary among them is the simplicity of the reaction. In contrast to certain other cytochemical procedures for locating proteases (40), an insoluble electron-dense precipitate is formed directly by the capture of the hydrolytic product of enzyme action by gold ions. Furthermore, this substrate can readily be used in spectrophotometric enzyme assays to compare the relative activities of tissue extracts or highly purified enzymes on the substrate.

In light of the substrate dependency, heat lability, time and temperature dependency, pH profile, and inhibitor profiles of the activity observed, it seems clear that the hydrolysis of this substrate in neutrophils is enzyme dependent. In addition, there is no nonspecific adsorption of either gold or the PNT-gold complex in any of the compartments in which reaction product is located.

In this study we attempted to characterize the intracellular neutrophil enzyme activity on Boc-Ala-SNp. The substrate is an NH_2- and C-terminus-blocked derivative of an amino acid, and presumably, therefore, reactive only with endoproteases. It is, however, an ester, and the possibility exists that other, esterolytic enzymes might contribute to the generation of reaction product. Although some commerically prepared peptidases and esterases were not active on Boc-Ala-SNp, a number of esterases, including cholesterol esterase, carboxylesterase, and acetyl esterase, did exhibit activity on this substrate in spectrophotometric assays. The purity of the enzymes was not established. Furthermore, the rates of hydrolysis of these enzymes against the nonspecific ester PNTA were considerably greater than their hydrolytic rates against Boc-Ala-SNp. This contrasts with the intracellular enzyme activity of neutrophils and the activity of neutrophil proteases in spectrophotometric assays under similar conditions, in which the alanine ester is preferred over the acetate ester. Taken together with the other criteria examined, these results strongly suggest that intracellular proteolytic activity against Boc-Ala-SNp is being observed. This is supported by studies that show that neutrophil enzymes that exhibit esterase activity are also proteolytic (6, 33, 37).

The characterization of the intracellular activity is consistent with the conclusion that the activity is elastase-like. This conclusion develops, in part, from the neutral to slightly alkaline pH preferred by the neutrophil enzymes and the inhibition of the cytochemical reaction by PMSF, an inhibitor of serine proteases, and by chloromethyl ketones, which are highly selective for neutrophil elastase. The apparent resistance of the intracellular reaction to EDTA, NEM, and chloromethyl ketones specific for chymotrypsin, trypsin, and cathepsin G offers additional support for this conclusion. The spectrophotometric studies revealed that purified neutrophil cathepsin G hydrolyzes the alanine ester less readily but significantly. It is possible that cathepsin G may contribute somewhat to the cytochemical reactions, although Z-Gly-

Leu-Phe-CMK, a potent inhibitor of this enzyme (36), did not inhibit the generation of intracellular reaction product. It is possible that the cells are impermeable to this inhibitor, although this seems unlikely in view of their permeability to other, similar peptidyl chloromethyl ketones. It is also possible that the relatively poor solubility of this inhibitor in the incubating solution resulted in limited availability of the inhibitor at the enzyme sites.

Function of Enzyme Activity

Previous studies have indicated that mitochondria and endoplasmic reticulum are sparse or lacking in mature, peripheral neutrophils (1, 25). However, these structures have been clearly demonstrated in this study. Their visualization is considerably enhanced by the presence of electron-dense reaction product. The ultrastructure of spread neutrophils has been described elsewhere (10), but the organization of the endoplasmic reticulum and mitochondria in the centrosomal region and the association of the nuclear envelope, endoplasmic reticulum, and Golgi complex has not been previously reported. Thus, this procedure facilitates the visualization of intracellular organelles and their interactions with one another.

The intracellular localization of reaction product suggests possible roles for the enzyme or enzymes that are active on Boc-Ala-SNp. The observation of activity in the mitochondria is consistent with studies by Aoki and co-workers (2, 3), in which an elastase-like enzyme has been purified from granulocyte mitochondria. This enzyme has been found in association with the inner membranes of the mitochondria of immature granulocytes and immature erythrocytes in the bone marrow of a variety of animals and is active on alanine esters blocked at the NH_2 terminus. Although the enzyme is inactive on insoluble elastin, it is inhibited by elastase inhibitors. The enzyme is proteolytic, however, and Aoki et al. propose that it regulates the amount of active pyridoxal enzymes that play rate-limiting roles in key biosynthetic processes in the cells (2). The enzyme activity detected in our study exhibits characteristics similar to those of this elastase-like enzyme described by Aoki and co-workers. The activity on Boc-Ala-SNp is observed in the mitochondria of developing monkey bone marrow granulocytes and erythrocytes and between the inner and outer mitochondrial membranes of mature human granulocytes. This enzyme also hydrolyzes an alanine-containing ester and is inhibited by inhibitors of elastase.

Electron-dense reaction product was also observed in neutrophil granules, many of which are presumably azurophil granules. Enzymes active on Boc-Ala-SNp were also observed in phagocytic vacuoles after the cells were induced to phagocytose and the granules had fused with the phagocytic vacuole. This suggests that the granule enzyme activity localized with Boc-Ala-SNp functions in intracellular digestion, and no doubt reflects the activity of the neutrophil granule elastase that has been isolated and characterized in biochemical studies. Both elastase and cathepsin G of neutrophils have been localized in the azurophil granules by biochemical fractionation techniques (17, 21).

Reaction product was also observed in the nuclear envelope, Golgi complex, and endoplasmic reticulum in both mature and developing neutrophils. As a result of the necessary limitations of the fixation procedure used in the preparation of cells, it is difficult to determine whether the enzyme activity is associated with membranes or whether it is in the lumen of these structures. This distribution suggests that the enzyme may be involved in the process of secretion, either as a processing enzyme that acts upon newly synthesized proteins or as a protein, which itself is being synthesized and packaged for secretion or storage. Thus, it is possible that this activity may reflect a membrane-associated enzyme that plays a role similar to that of the signal peptidase involved in the processing of newly synthesized proteins for export (26). Alternatively, the enzyme activity localized with Boc-Ala-SNp may be newly synthesized enzyme, i.e., elastase, destined for packaging into granules. Evidence for this is the fact that the endoplasmic reticulum, nuclear envelope, and Golgi complex are all active in the synthesis and packaging of the neutrophilic granule enzymes (5). It is important to note that many studies of neutrophil maturation have suggested that synthesis and packaging of granule enzymes has ceased before maturation and that enzyme synthesis is negligible or absent in mature peripheral neutrophils (5, 15). There is more recent evidence, however, that mature neutrophils may continue to synthesize enzymes and other proteins (23, 24). Supporting the more recent findings, our study finds ribosomes in association with the endoplasmic reticulum and on the nuclear envelope (Fig. 10) of mature neutrophils that had not been reacted with Boc-Ala-SNp. Activity against Boc-

Ala-SNp was observed in all recognizable stages of developing monkey neutrophils in protein-synthesizing structures, further suggesting the continuous synthesis of elastase-like enzymes throughout development and into maturity.

The observation of reaction product in these membranous organelles, however, is inconsistent with biochemical fractionation studies that localize elastase activity against Boc-Ala-ONp only in association with the neutrophil granules (17, 27). This inconsistency is not surprising because the volume of neutrophil granules is considerably greater than that of mitochondria, endoplasmic reticulum, or Golgi complex, and enzyme activity not located in the granule fraction might easily be overlooked (28). Thus, because of the sensitivity of this cytochemical assay, Boc-Ala-SNp can be used to detect intracellular enzyme activity not readily detectable in biochemical fractionation studies.

In summary, our results would appear to add a new dimension to the study of enzymes with elastase-like specificity. The distribution of reaction product in the nuclear envelope, endoplasmic reticulum, and mitochondria of the various types of blood cells suggests important roles for these proteolytic enzymes in general cell function. The dynamics of proteases such as elastase can now be followed at the ultrastructural level, and the role of neutrophils in the inflammatory response and as participants in disease states may be more completely understood with the appropriate application of cytochemical substrates selective for proteolytic enzymes.

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