Peroxidase-Containing Microgranules in Human Neutrophils: Physical, Morphological, Cytochemical, and Secretory Properties

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A microgranule fraction, isolated from human neutrophils by using a novel high-resolution Percoll density gradient system contained granules with the lowest density and diameter when compared with 12 other isopycnic granule fractions. Ultrastructurally, from 34% to 50% of the microgranules showed homogeneous diaminobenzidine (DAB) staining under conditions for localizing peroxidase reactivity. The presence of myeloperoxidase (MPO) was further confirmed by biochemical and spectral analysis and immunodiffusion methods. Periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) intensely stained vicinal glycols in the matrix of granules in contrast to the weak or absent staining seen in larger primary granules. Directly sampled segmented neutrophils contained small DAB- and PA-TCH-SP-positive granules, which often appeared in clusters. These DAB-positive microgranules selectively remained within the cells after stimulation of exocytosis with the calcium ionophore A23187. The enriched DAB-positive microgranule fraction recovered from A23187-treated cells also contained lysozyme and β-glucuronidase but lacked vitamin B12 binding protein activity. A similar small, DAB- and PA-TCH-SP-positive granule type was also identified in normal promyelocytes and was the predominant or only granule type observed in leukemic or preleukemic myeloid cells from four patients. This study demonstrates a unique subpopulation of MPO-containing microgranules in normal and leukemic human myeloid cells that are distinguished from (other) primary granules by their extremely low density, small size, content of complex carbohydrates, and resistance to secretion.

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Pervious Ultrastructural1-5 and biochemical studies6,7 of human neutrophils have identified two major types of cytoplasmic granules, large myeloperoxidase (MPO)-containing primary granules and smaller secondary granules lacking peroxidase activity. However, a number of recent studies8-10 indicate considerably more heterogeneity among the granules. Rice et al.,11 using a high-resolution Percoll density gradient system, isolated and characterized 13 granule fractions. Among the five higher-density MPO-containing granule fractions, a unique, large granule enriched in microboidal defensin polypeptides was observed.11 At least three different forms of MPO have been identified biochemically in human neutrophils.11,12,13 These forms show differences in subunit structure and detergent extractability, which indicates differences in compartmentalization or association with granule membranes.11 In addition, these different forms of MPO appear to be under separate secretory control and are differentially distributed among isolated granules of differing density.12,13 Taken together, these results indicate the existence of several physically and functionally distinct MPO-containing neutrophil granules rather than a single MPO-containing granule previously termed a primary or azurophil granule.

A variety of apparently distinct small granule populations containing sulfated glycosaminoglycans, acid hydrolases,16,17 gelatinase,18 alkaline phosphatase,19 elastase,20 and catalase21 have been reported in biochemical and/or ultrastructural studies. Ackerman has previously observed small granule profiles (0.1 to 0.25 μm) in early promyelocytes1 and clusters of small diaminobenzidine tetrahydrochloride (DAB)-reactive granules in metamyelocytes and segmented neutrophils2; however, little attention has been directed to these granules since their original description. Recently, these granules have been further characterized with the observation of microtubules connecting the granules when observed by stereo high-voltage electron microscopy.22 The present study reexamines the microgranule population in neutrophils and demonstrates that their DAB staining corresponds to the presence of MPO and that the granules differ dramatically from typical primary granules in size, density, carbohydrate cytochemistry, and response to secretory stimuli. The granules were identified in isolated neutrophil granule preparations as well as in intact neutrophils, leukemic myeloid cells, and ionophore-treated neutrophils. A preliminary report of this observation has been previously published.23

MATERIALS AND METHODS

Separation of granules. Concentrated samples of human neutrophils were obtained from healthy adults undergoing continuous-flow leukapheresis. Neutrophils were further purified to >97% purity after two cycles of hypotonic lysis of residual erythrocytes24 and subsequent centrifugation through lymphocyte separation medium25 to remove monocytes and lymphocytes. Pulled cell layers were lysed, the granule-rich postnuclear supernatant (PNS) was prepared, and the high-(H) and low-(L)-density Percoll gradients were performed as described previously by Rice et al.25 The H and L gradients consisted of fused multiple-step preformed layers of different densities. These gradients were designed to maximize resolution of 12 isopycnic granule fractions that had been initially identified by using continuous, self-generated Percoll density gradients. The gradients were fractionated, and appropriate fractions were pooled and centrifuged for three hours at 100,000 g at 4°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc, Palo Alto, CA). The granule pellet was collected, washed with phosphate buffered saline (PBS) and recentrifuged at 27,000 g for 15 minutes at 4°C in a DuPont/Sorvall (Willimington, DE) SM-24 rotor to obtain a purified granule pellet.
Assay of granule components. Granule pellets that were to be assayed for MPO were suspended in 0.3% cetyltrimethylammonium bromide in PBS, pH 7.4, and exposed to five cycles of freezing and thawing. The granule lysate was spectrophotometrically assayed for MPO activity by using guaiacol as the electron donor, and an increase in 1 absorbance unit at 470 nm in one minute was defined as 1 unit of activity. Protein concentrations were measured according to Lowry et al.26

MPO protein was quantitated by spectroscopy. Difference spectra from 400 to 600 nm were obtained in dithionite-reduced minus oxidized samples by using an Aminco DW-2a spectrophotometer (American Instrument Co, Silver Spring, MD). The samples were homogenized in 0.1% Triton X-100, and an absorbance coefficient of 75/mmol/L/cm was used to quantitate the characteristic MPO peak at 472 nm.27 MPO in microgranules was also identified immunologically by using Ouchterlony double-immunodiffusion plates and a monospecific rabbit antibody prepared against purified normal human neutrophil MPO as previously described.31

Activity of β-glucuronidase was measured as described elsewhere,14 and vitamin B₉ binding proteins were measured by the rapid charcoal assay described by Kane et al.28 Amersham, Arlington Heights, IL, supplied the 32Co-vitamin B₉ (specific activity, 10 to 20 μCi/μg). Lysozyme activity was determined by using the lysozyme method of Osserman and Lawlor with egg white lysozyme as a standard.

Electron microscopy. Granule pellets were overlayered with 30 to 40 μL human plasma and 30 to 40 μL 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After ten minutes at 25°C the granule-containing plasma plug was minced and submersed in fresh glutaraldehyde-cacodylate buffer for an additional 50 minutes. The minced plug was then washed and stored in 0.1 mol/L cacodylate buffer containing 7% sucrose.

Normal bone marrow was obtained in a heparinized syringe after informed consent by needle aspiration from the posterior iliac crest of a healthy volunteer (n = 1) and from patients undergoing routine staging of solid tumors (n = 2) or evaluation of lymphoid leukemia in remission and not receiving chemotherapy (n = 2). Bone marrow samples were similarly obtained from five untreated patients with myeloid leukemia (French-American-British classification M2) and a patient with a pancytopenic, preleukemic myeloproliferative disorder. Peripheral blood was collected from healthy volunteers in a patient with a pancytopenic, preleukemic myeloproliferative disorder.

RESULTS

Isolation and properties of microgranules. Monodispersed granules of the PNS from normal human neutrophils were separated on an L-type Percoll density gradient which resulted in eight low-density isopycnic granule fractions as described previously by Rice et al.25 The lowest density fraction, L1, was isolated and recentrifuged on an identical L-gradient, which resulted in remigration to its original characteristic isopycnic density (mean density, 1.046 ± 0.003 g/mL; n = 25 experiments). The majority of granules in the L1 fraction appeared homogeneously electron dense in UA-LC-stained morphological preparations (Fig 1). Morphotometric analysis yielded a mean granule diameter of 0.15 ± 0.04, 0.14 ± 0.05, and 0.11 ± 0.05 μm for UA-LC-, DAB-, and PA-TCH-SP-stained specimens, respectively, and indicated that the L1 granule fraction consisted of a novel class of microgranules. In each granule preparation, 100 consecutively identified granules were scored in ran-
domly selected photographs. All membrane-bound structures were scored, which would include tangential sections. Such scoring was used to prevent observer bias. Although repeat experiments did not clearly demonstrate a significant difference in diameter of PA-TCH-SP-stained preparations, the tendency for smaller size in these preparations may be related to the lack of OsO₄ postfixation before dehydration.

When the microgranules were reacted for peroxidase by using a DAB ultrastructural procedure, it was found that 34% to 50% (n = 4 experiments, 200 granules scored in each experiment) of the granules demonstrated intense staining (Fig 2A), thus indicating the existence of a DAB-reactive subclass of microgranules. The presence of DAB reactivity (30-minute incubation) at pH 7.6 rather than pH 9.7 alone indicated that the reactivity was not attributable to catalase alone, which was previously described as reacting at only the higher pH. No DAB reactivity was observed in granules or neutrophils incubated 30 minutes without H₂O₂ or in the presence of 10 mmol/L KCN.

Biochemically, the microgranules of fraction L1 had a specific activity for peroxidase (units per milligram protein; electron donor, guaiacol) about five times less than that of typical, large primary granules (15.6 ± 73.0, n = 6). This accounted for approximately 3% of the total granule-associated peroxidase activity of the neutrophil. The latency of MPO in this fraction was >95% after disruption of granules with 0.1% Triton X-100, which indicated that the peroxidase was localized within an intact, membrane-bound granule.

To identify the nature of the peroxidase activity associated with the microgranules, several characteristics of this fraction were investigated. First, the reduced minus oxidized
difference spectrum for the microgranule fraction displayed a prominent absorption maximum at 472 nm, which indicated the presence of MPO (Fig 2B). Quantitation of the spectral data demonstrated that the microgranules contained 11 μg MPO/mg protein (range, 6 to 20; n = 3), which accounted for approximately 3% of the total granule-associated MPO on a per milligram protein basis (consistent with the enzymatic determination of MPO activity shown earlier). The absence of a trough at 430 nm due to absorption of oxidized MPO may be due to the presence of a b type cytochrome that has been reported to be colocalized with vitamin B12 binding protein; however, because of technical difficulties in dealing with small absorbances in turbid suspensions, a definitive explanation is not possible. Interestingly, Vitamin B12 binding protein was concentrated in these lowest fractions, and we have also confirmed the presence of the b type cytochrome in these same fractions by spectral analysis (data not shown). Second, a positive immunologic reaction for MPO was demonstrated on Ouchterlony plates when using a monospecific rabbit polyclonal anti-MPO (purified IgG fraction). Taken together, these results suggest that (a) the DAB reactivity seen in the ultrastructural preparations and that measured biochemically are due to the presence of MPO in the microgranule fraction and (b) this MPO appeared to be equivalent to that associated with typical primary granules.

Vicinal glycol-containing complex carbohydrates in the microgranule fraction stained strongly in greater than 97% of the granules when using the PA-TCH-SP procedure (Fig 2C). In marked contrast, typical, large primary granules in the denser granule fractions showed only very weak to negative PA-TCH-SP staining. Furthermore, when isolated microgranules and larger, more dense, DAB-positive granules (fractions L1 and H3, respectively) were mixed and then stained by using the PA-TCH-SP procedure, each type of granule retained its characteristic staining pattern (Fig 2D). These results demonstrated that the peroxidase-positive microgranules were not fragments or tangential sections of the larger primary granules but belong to a newly recognized distinct subclass of MPO-containing granules that stain intensely for both peroxidase and periodate-reactive complex glycoconjugates.

**Microgranules in intact normal cells.** Intact neutrophil precursor cells of normal human bone marrow and mature peripheral blood neutrophils were examined ultrastructurally for the presence of microgranules. Small, intensely DAB- (Fig 3A, B) and PA-TCH-SP-reactive granules (Fig 3C) varying from 0.08 to 0.20 μm in diameter were observed...
at all stages of development beyond the myeloblast and appeared to correspond to the microgranules isolated from mature neutrophils. In the promyelocyte, these small granules were not readily apparent in that they were randomly dispersed and could not be easily distinguished from tangentially sectioned larger primary granules. In the myelocyte, the small granules were observed occasionally in clusters. This clustering phenomenon appeared to increase progressively with maturation. The small, DAB-reactive granules were more apparent in some profiles of fully mature neutrophils where they often appeared in clusters of three to more than ten granules. In the late (segmented) neutrophil, these clusters were observed in the central portion of the cell near the DAB-unreactive Golgi region as well as at the periphery of the cell.

Resistance of DAB-positive microgranules to secretion. Because the DAB-positive microgranules were found to be physically and chemically different from the large primary granules, the possibility they might exhibit differences in exocytosis in response to a secretory stimulus was examined. The calcium ionophore A23187 was chosen because this secretagogue has been reported to cause the differential secretion of secondary and primary granules in a dose-dependent manner (1 μmol/L and 10 μmol/L respectively). Consistent with these previous reports, 1 μmol/L ionophore resulted in the loss of most DAB-negative granules and variable loss of DAB-positive granules, although DAB-positive microgranules consistently remained within the cell (Fig 4A). Again, consistent with previous reports, exposure of the cells to 10 μmol/L ionophore resulted in virtually complete secretion of large (>0.20-μm-diameter) DAB-positive granules. In marked contrast the DAB-positive microgranules remained within the cells and frequently were redistributed radially to the subplasmalemmal region. These ultrastructural results were supported by Percoll density gradient centrifugation of granules prepared from 10 μmol/L ionophore-treated neutrophils in which the only visibly apparent granule fractions were the two lowest density granule fractions, L1 and L2.

Additional support for the selective retention of these MPO-containing microgranules was obtained by quantitating the distribution of MPO in isolated granule fractions from untreated and treated cells. In untreated cells, 70% of the total MPO activity was associated with the larger, high-density granules found in fractions H1 to H5, 22% with the intermediate-density fractions (L3 to L8) and 8% with the two lowest density fractions (L1 and L2). After treatment with 10 μmol/L ionophore, only fractions L1 and L2 retained significant amounts of their original MPO activity (Fig 4B); fraction L1 retained 96% ± 36% (n = 5), whereas fraction L2 retained 36% ± 10% (n = 5). In other experiments (n = 2) using neutrophils from different donors, treatment with 10 μmol/L ionophore resulted in the retention of 104% and 63% of the β-glucuronidase activity in the

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Fig 4. (A) Approximately 20% and 80% of neutrophil profiles contain predominantly DAB-reactive microgranules (arrows) after exposure to 1 and 10 μmol/L of calcium ionophore A23187 respectively (1 mmol/L sample illustrated). DAB-negative granules and large DAB-positive granules have degranulated. The granule matrices of some larger primary granules are evident in vacuoles (V) or channels into which they have degranulated. Less dense secondary granules are not evident and have presumably degranulated. The thin section is lightly counterstained with UA and LC. Bar, 1 μm. (B) Granules from untreated neutrophils (control) or from neutrophils after treatment with 10 μmol/L calcium ionophore A23187 for 45 minutes at 37°C (10 μmol/L A23187) were separated by using L and H Percoll gradients to prepare fractions L1 to L8 and H1 to H5, respectively, as previously described. Granule fractions were isolated and assayed for their content of MPO. The amount of MPO activity in each fraction is expressed as a percentage of the sum of MPO activity in all fractions of control samples (ordinate). The abscissa identifies the individual granule fractions.
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L1 fraction compared with control samples. Similarly, 70% and 72% of lysozyme (n = 2 experiments) was retained in the L1 fraction after 10 μmol/L A23187 compared with control values. In contrast, only 6% and 0% of vitamin B12 binding protein activity (n = 2 experiments) was retained in the L1 fraction after 10 μmol/L A23187 compared with simultaneous controls. The percent retention of a particular marker was based on the following control values (mean ± SD) for the L1 granule fraction derived from 2 × 10⁷ neutrophils in both the control and ionophore-treated experiments: MPO (2.6 ± 0.6 units, n = 5), β-glucuronidase (2.0 ± 0.7 mU, n = 4), vitamin B12 binding proteins (28,260 ± 4,820 cpm bound, n = 3), and lysozyme (84 ng and 76 ng, n = 2). The results obtained with MPO indicated that although DAB-positive microgranules were localized primarily in fraction L1, a portion was also associated with fraction L2 (Fig 4B). Ultrastructurally, the DAB+ microgranules found in fraction L2 had identical morphometric, DAB, and PA-TCH-SP staining properties as those found in fraction L1. Granules from fraction L1 were isolated from 10 μmol/L ionophore-treated cells and were processed for ultrastructural staining using DAB. Of the remaining granules, 71% ± 8% (100 granules scored, n = 3) were DAB-positive. Mitochondria were more prominent in these preparations and were excluded from granule scoring. Thus, the DAB-positive microgranules were highly resistant to secretion relative to the larger, more typical primary granules (fractions H1 to H4), thereby demonstrating differences in secretory control between the two granule types.

Microgranules in intact leukemic myeloblasts and myelodysplastic neutrophils. Ultrastructural studies were performed on intact leukemic myeloblasts from the bone marrow of five myeloid leukemia patients (French-American-British classification M2). In three cases, the predominant granule type observed was a small, DAB- and PA-TCH-SP–reactive granule varying from 0.08 to 0.20 μm in diameter (Fig 5A, B). Auer rods, which were present in two of the patient samples, were uniformly and intensely stained with DAB and PA-TCH-SP (Fig 6). Examination of leukemic cells from two other patients with acute myelogenous leukemia (M2 classification) revealed a more heterogeneous population of DAB-positive granules; however, distinct microgranule profiles were evident.

Myelodysplastic neutrophils from a patient with a pancytopenic preleukemic syndrome (1 year prior to developing myeloid leukemia) appeared grossly abnormal in morphological preparations and completely lacked DAB-unreactive granules. Two distinct granule populations were evident in these cells, a homogeneous electron-dense and DAB-reactive large primary granule (0.25 to 0.40 μm in diameter) and a small (0.08 to 0.20 μm in diameter) electron-dense, DAB- and PA-TCH-SP–reactive granule (Fig 7A, B). The small granule type outnumbered the larger primary granules and often appeared in clusters as seen in normal neutrophils. The increase in microgranules was also observed in promyelocytes and myelocytes from this patient.

DISCUSSION

The present studies identify a peroxidase-containing microgranule population in human neutrophils that is distinct from previously described primary granules. Small granule profiles have been previously identified in intact cells at the early promyelocyte stage of development.1 Similarly, DAB-positive microgranule clusters have been observed in

Fig 5 A, B. This leukemic myeloblast from a marrow specimen contains a cluster of small granules (arrows, enlarged in B) that stain intensely with DAB. N, nucleus; M, mitochondria. The thin section is not counterstained. Bar, 1 μm.
segmented neutrophils; however, previous studies have not isolated the granule, defined the enzyme responsible for the DAB positivity, or examined cytochemical, physical, or secretory features of the granule. The present study establishes the existence of MPO in isolated microgranules by using cytochemical, biochemical, and immunologic methods and provides a method for concentrating these granules from cells after ionophore-induced secretion. Biochemical analysis of the concentrated microgranule fraction indicates that they resemble primary granules in their content of β-glucuronidase and lysozyme and lack of vitamin B<sub>12</sub> binding protein activity. The DAB reactivity of microgranules and larger...
primary granules demonstrate that DAB reactivity and MPO content span the entire gamut of neutrophil granule size and density.

Previously described primary granules are two to three times the size of microgranules and have considerably higher buoyant densities than do the microgranules. The intense PA-TCH-SP staining of vicinal glycols in microgranules is clearly distinct from the weak to absent staining of larger primary granules and demonstrates that microgranules also differ in content from previously described primary granules. Moreover, the DAB-positive microgranules are resistant to 10 mmol/L A23187 ionophore-induced secretion, which demonstrates that these granules are under secretory control distinct from typical primary granules that were secreted under these conditions. Hence, the differences in size, density, carbohydrate content, and resistance to secretion of the DAB-positive microgranules identify them as a unique population of granules for which the terms primary or azurophil may be inappropriate, although the latter terminology has been used for all neutrophil granules with peroxidase activity.

Neutrophil granules contain several potentially DAB-reactive substances. In addition to containing at least three forms of MPO, neutrophils also contain catalase-reactive peroxisomes, which may correspond in size to the microgranules in this study. Although some of the microgranules in the lowest density fraction may, in fact, be peroxisomes, the DAB reactivity of the granules at pH 7.6, the presence of biochemically, spectrally, and immunologically demonstrable MPO in these granules, and the great abundance of these granules in some leukemic myeloblasts would appear to exclude this possibility for the DAB-reactive microgranule described in this study. Alternatively, a b-type cytochrome has been identified as a component of secondary granules and/or other small, low-density granules, and cytochromes are known to be DAB-reactive. The b-type cytochrome has been shown to translocate to the plasma membrane after stimulation of the cell with either a phorbol diester or the calcium ionophore A23187; however, we found that stimulation of neutrophils by the ionophore did not result in secretion of the DAB-reactive microgranules. Furthermore, the abundance of similar granules in leukemic cells (presumably lacking secondary and/or cytochrome b-containing granules) also suggests that a b-type cytochrome is not responsible for the staining of microgranules in this study. Thus, the cytochemically demonstrable DAB reactivity is most consistent with the localization of MPO identified biochemically, spectrally, and immunologically in this granule fraction.

The presence of DAB-reactive microgranules in leukemic myeloblasts indicates the synthesis of at least some of these granules early in myeloid development. Conversely, the lack of DAB reactivity in Golgi lamellae and the endoplasmic reticulum beyond the myelocyte stage of development indicates that the granules are not synthesized in late neutrophils. Recent studies have suggested that microgranules are synthesized in late promyelocytes after synthesis of larger primary granules; however, this hypothesis does not readily account for the previously observed synthesis of small granules (0.10 to 0.25 μm in diameter) in the earliest promyelocytes and the observation in this study of abundant microgranules in some leukemic myeloblasts. Similarly, cultured myeloid leukemic HL-60 cells contain predominantly small MPO-positive granules of comparable density to the microgranules reported in this study. Alternatively, the microgranules could be synthesized throughout the promyelocyte stage of development or exist as subpopulations being synthesized in early and late promyelocytes, respectively.

The microgranules were redistributed into clusters, observed both in normal and myelodysplastic neutrophils in this study. Similar clusters of DAB-reactive small granules have been observed in monocytes and have been taken to indicate functional subcompartmentalization. Although clustering may represent worm-like structures in monocytes, the lack of such elongated profiles in the present study of intact neutrophils and isolated granules or in previous high-resolution electron microscopic studies of microgranules indicates differences in the small DAB-reactive granules of monocytes and neutrophils. The clustering phenomenon presumably has functional significance in neutrophils and may occur in preparation for endocytosis or as a result of this process. The extreme resistance of microgranules to secretion is similar to that observed with residual bodies or secondary lysosomes and may indicate prior involvement in an endocytic event. However, the lack of inclusions or ultrastructural heterogeneity within the microgranule matrix would argue against this possibility. Alternatively, the DAB-positive microgranules could represent fragmented primary granules; however, the intense PA-TCH-SP reactivity of the microgranules and the virtual lack of similar reactivity in primary granules does not support this hypothesis. The sequence of granule production has in the past correlated with resistance to granule secretion in that primary granules are secreted most readily, followed by secondary granules and primary granules. The observation of small granule profiles in the earliest promyelocytes and leukemic myeloblasts in this study would suggest that these are among the first granules synthesized and the last secreted at all.

NOTE ADDED IN PROOF

Recently, White and Krumwiede have ultrastructurally demonstrated failure of peroxidase positive "vesicles" and granules to fuse with giant lysosomes in neutrophils from patients with Chediak-Higashi Syndrome. Many of these granules were of similar size to microgranules reported in this study. We would suggest that their observation represents in vivo resistance to secretion comparable to that observed in vitro in our study.

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