Heterogeneity of peroxidase positive granules in normal and pathologic human neutrophils

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Abstract: Studies have demonstrated significant heterogeneity in neutrophil granule morphology and physical density. Using cytochemical methods to localize peroxidase and vicinal glycol containing complex carbohydrates we examined the heterogeneity of neutrophil granules from intact human neutrophil granules in 13 isolated granule density fractions, calcium ionophore A23187 treated neutrophils and neutrophils from patients with Chediak-Higashi Syndrome and Specific Granule Deficiency. At least four distinct populations of peroxidase positive granules (PPG) were identified based on peroxidase staining, vicinal glycol staining, morphology, β-glucuronidase and defensin content, and physical density characteristics. The smallest (0.15 μm diameter) PPG was the least dense granule, had a unique peroxidase/β-glucuronidase ratio, reacted intensely for vicinal glycols, resisted ionophore degranulation and was not consumed in giant granule formation in Chediak-Higashi Syndrome. The largest (0.3 μm average diameter) and most physically dense PPG was rich in defensins, stained weakly for vicinal glycols, and was absent in specific granule deficiency. These studies demonstrate and correlate morphologic, biochemical, functional, and pathologic differences in PPG populations.

Key words: human; neutrophil; Chediak-Higashi Syndrome; Specific Granule Deficiency.

Introduction

Neutrophils function as mediators of phagocytosis and inflammation, and therefore play a major role in host defense. Central to these functional roles are the neutrophil’s cytoplasmic granules. These granules contain a broad spectrum of hydrolytic enzymes and microbicidal factors that function at both intracellular and extracellular sites. Two major types of granules, azurophil (primary) and specific (secondary), have been characterized in human neutrophils based on ultrastructural morphology and cytochemistry and separation by Percoll gradient centrifugation procedures. Azurophil granules have been characterized by their content of myeloperoxidase (MPO) and lysosomal enzymes such as β-glucuronidase. The most common markers for the specific granules have been lactoferrin and vitamin B12 binding protein. Lysozyme has been identified in both azurophil and specific granules.

The purpose of this report is to review studies from my laboratory and collaborations which have described significant heterogeneity in neutrophil granules. This review will attempt to demonstrate the entire spectrum of azurophil or peroxidase-positive granules (PPG) in normal and selected pathologic conditions.

Materials and Methods

Preparation of cells for electron microscopy

Blood samples from healthy volunteers, a child with Chediak-Higashi Syndrome and an adult patient with Specific Granule Deficiency, were centrifuged, pelletized, fixed in 3% glutaraldehyde, processed for either 1) morphology, 2) pre-embedment myeloperoxidase staining with diamino benzidine (DAB) as substrate or 3) post-embedment staining for vicinal glycols using periodate-thiocarbohydrazide silver proteinate (PA-TCH-SP) as described previously.

Preparation of granule fractions

Purified cells were lysed, the granule-rich post nuclear supernatant was prepared and high (H) and low (L) density Percoll gradients were prepared as described previously. The gradients were fractionated to maximize resolution of 13 isopyknic granule fractions that had been identified using continuous self-generated Percoll density gradients. Fractions were centrifuged and washed to form granule pellets, which were fixed and processed for electron microscopy as described above.

Ionophore treatment

Since previous studies have demonstrated secretion of peroxidase-negative granules with calcium ionophore, A23187, we examined intact neutrophils after ionophore treatment and granules from ionophore-treated neutrophils. The latter allowed the assessment of a purer preparation of PPG. Neutrophils were suspended 45 min at 37°C in PBS with 1 M Ca++ and 1 M Mg++ with or without 1 or 5 μM A23187 as described by Gilbert et al.
**Biochemical studies**

Purified granule fractions were extracted and assayed for MPO and β-glucuronidase activity as described by Rice et al. [7]. Defensins were quantified using an enzyme immunoassay which used an IgG fraction of rabbit antibody raised against the mixture of defensins 1 through 3 [8].

**Results**

**Electron microscopy of granules**

<table>
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<th>Table 1</th>
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<td><strong>Density g/cm³ (range of means)</strong></td>
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<td>Granule Type I</td>
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<td><strong>Cross-sectional area/μm² (range of means)</strong></td>
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<td>Granule Type I</td>
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<tr>
<td><strong>Prominent DAB stain pattern</strong></td>
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<td>Granule Type I</td>
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<tr>
<td><strong>PA-TCH-SP staining</strong></td>
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<td><strong>Secretion with 10 μM A23187</strong></td>
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<td><strong>β-glucuronidase/MPO ratio</strong></td>
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<td><strong>Defensin content</strong></td>
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<td><strong>Expression in specific granule density</strong></td>
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<td><strong>Expression in Chediak-Higashi syndrome</strong></td>
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PPG were identified in all 13 granule fractions. PPG in granule fractions isolated from neutrophils treated with 1 μm A23187 had similar morphology but the relative percentage of PPG was significantly increased in the low density fractions. The peroxidase staining appearance for each granule could be categorized as one of the following four patterns: (a) solid or homogeneous staining; (b) angulated unreactive core containing a central cuboidal, angulated or needle-like lucent area; (c) rim staining in which peroxidase activity appeared stronger.

![Fig. 1: Electron micrograph of granule fractions in order of increasing density from (A) L1 (the least dense), (B) L4, (C) H1, and (D) H5 (the most dense). The neutrophils were exposed to 1 μm calcium ionophore A23187 for 45 min at 37°C to remove peroxidase negative granules. The size of the granules appears to increase with physical density and various staining patterns are evident. Homogeneously stained microgranules are evident in A; granules with angulated lucent areas (arrows) are evident in B and, to a lesser extent, in C; rim-stained granules are present in D. Mitochondria (M) were observed in the two least-dense granule fractions but not in the other more dense granule fractions. Bars = 1 μm.](image-url)
in a rim distribution or (d) extracted where the intactness of the granule was in question. In general less than 10% of the granules appeared extracted. The solid pattern of peroxidase staining was most prominent (comprising over 50%) in lower density fractions L1-L7 and high density fractions H2-H4, while the angulated reactive core pattern was seen in more than 25% of granules in fractions L5-H4. Rim staining was prominent in the densest fraction, H5 (Fig. 1, Table 1).

PA-TCH-SP strongly stained most of the granules in the lowest density fractions obtained from A23187-treated neutrophils which were also presumed to be peroxidase positive (after ionophore treatment). Most of the granules in the intermediate density fractions, L7-L8 from ionophore treated cells were moderately PA-TCH-SP positive and were also DAB-positive. In contrast most of the granules in the densest fractions H3-H5 were PA-TCH-SP negative. (Fig. 2, Table 1).

Electron microscopy of intact cells from normal, Specific Granule Deficiency, and Chediak-Higashi specimens demonstrated that PPG varied widely not only in size but also in staining patterns (Fig. 3-6, Table 1).

Solid homogeneous-staining microgranules (0.1-0.15 μm diameter) often appeared in clusters of usually up to 15 granules. Larger (0.15-0.25 μm diameter) homogeneously-stained granules comprising approximately 10-30 granules/cell profile were randomly distributed in the cell. Some granules (approximately 3-10/cell profile) contained distinct central angulated lucent areas. Large (averaging 0.3 μm diameter) granules with rim peroxidase staining were usually observed at a variable frequency of 5-20 cell profile (Fig. 3).

Neutrophils in Specific Granule Deficiency generally contained smaller PPG and lacked normal appearing peroxidase negative granules. Notably these cells lacked large rim stained PPG (Fig. 5).

Giant PPG were seen in all Chediak-Higashi neutrophils. Few normal appearing PPG could be identified other than the microgranules, which did not appear to be consumed in giant granule formation (Fig. 6).

**Biochemical studies**

Calculation of the ratios of β-glucuronidase to MPO in the individual granule fractions indicated that the

![Fig. 2: Comparison of PA-TCH-SP staining of granule fractions (A) L1, (B) L3, (C) L7 and (D) H3. Before fractionation, the cells were treated with calcium ionophore A23187 (1 μM) to remove peroxidase negative granules from L-fractions. L1 shows strong PA-TCH-SP-positive staining, whereas L3 and L7 are moderately PA-TCH-SP-positive. For comparison, most H3 granules (not ionophore treated) are PA-TCH-SP-negative or only weakly positive, although two smaller positive granules are present (arrows) and presumably represent cross-density contamination. Bars = 0.5 μm.](image-url)
Fig. 3: (A) This human peripheral blood neutrophil with two nuclear lobes (N) was stained en bloc with DAB and the thin section was counterstained with lead citrate. Note the variable sizes of PPG and generally smaller peroxidase negative granules (arrowheads). (B) peroxidase negative granules are more clearly seen at higher magnification. Significant heterogeneity exists with identification of rim-stained granules (r), microgranules (m), granules with angulated unreactive or angulated lucent areas (a), and homogeneously stained granules of intermediate and large size (arrows). Bars = 1 μm.

Fig. 4: (A) This human peripheral blood neutrophil with four nuclear lobes (N) contains a heterogeneous population of granules that vary in size and electron density with uranyl acetate and lead citrate staining. Large-sized granules with a rim density (r) or homogeneous staining patterns (arrows) are observed. Other granules are of variable size and density (enlarged in B). Bars = 1 μm.

Fig. 5: Peroxidase and lead citrate staining delineates several peroxidase positive granules in the neutrophils of this patient with Specific Granule Deficiency. The lead citrate counterstaining allows identification of some peroxidase-negative small elongated vesicles/granules (small arrows) which represent abnormal 'empty' secondary or specific granules in this patient. The PPG are generally smaller than those seen in normal neutrophils and defensin rich dense granules (rim-stained large granules) are absent. Peroxidase-positive microgranules (MG) are present and are clustered similar to those described in normal neutrophils.
markers were not represented in a one-to-one manner in all fractions. The ratio was greater than two in the lightest granule fractions while it was closer to one in the densest granule fractions[6]. Defensins appeared concentrated in the densest granule fractions[8].

**Discussion**

A number of observations in these studies do not fit a simple two-granule model but are more consistent with greater granule heterogeneity. PPG were not solely restricted to the larger high density azurophil granule region, but rather spanned the entire range of granule size[7]. The larger granules generally correlated with the high density granule fractions, whereas the smaller granules were localized in the less dense granule fractions. The concentration of these large and small PPG could be significantly increased by pre-treating neutrophils with calcium ionophore A23187, which results in secretion of peroxidase-negative granules.

The heterogeneity of PPG size and density correlated with biochemical and cytochemical heterogeneity. The ratio of % β-glucuronidase to % MPO was strikingly different across the range of granule fractions. Since both markers resided in the same granule, the result indicated that their packaging varied with size and density of granules and did not change with different concentrations of secretagogue. The ultrastructural heterogeneity of peroxidase staining within the PPG supported this interpretation.

Similarly the strong glycoprotein staining using PA-TCH-SP in low density fractions and the association of small and intermediate size PPG with PA-TCH-SP staining allowed separation of PPG into at least four categories based on size and cytochemistry[6,7].

Microgranules appear to be particularly unique PPG[6,10]. In addition to being the smallest and least dense PPG they contain relatively more β-glucuronidase compared to MPO than other PPG. In contrast they contain less defensins than other PPG[8]. Functionally the microgranule is unique in that it is the only neutrophil granule type that resists secretion with high doses of ionophore. This finding appears consistent with the observations in Chediak-Higashi Syndrome, where the microgranule appears to resist depletion and/or consumption by giant granule formation/granule fusion[6].

In contrast to the microgranule the largest granule type in neutrophils stains only weakly for peroxidase and has minimal PA-TCH-SP staining of vicinal glycols[7]. These large PPG are rich in their content of defensins, a cationic peptide with microbicidal activity[8]. Defensins are deficient in neutrophils from patients with Specific Granule Deficiency. Consistent with this finding is the observation of an absence of large rim stained granules in these patients[11]. These findings have led to the term

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Fig. 6: Serial sections of Chediak-Higashi Syndrome neutrophils show small PPG or microgranules (arrows) in B, but not in sections A or C, showing that the small PPG (m) are not all projections of the giant PPG. N, nucleus; g, giant PPG. Bars = 1 μm.
defensin-rich dense granules to describe this population of PPG.

References


