Abnormal Peroxidase-Positive Granules in “Specific Granule” Deficiency

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“Specific granule” deficiency (SGD) has been previously associated with lactoferrin deficiency. The antimicrobial peptides termed defensins, comprising 30% of normal primary granule proteins, have also been shown to be markedly deficient in SGD. The present study was undertaken to correlate these findings with ultrastructural morphometric analysis and peroxidase cytochemistry. Peroxidase-positive, rim-stained, large, defensin-rich dense granules, previously described as a subpopulation of azurophil or primary granules in normal neutrophils, were markedly decreased in a patient with SGD. Morphometric studies of peroxidase-positive granules indicated an average peroxidase-positive granule area (all profiles) in the patient of 0.019 ± 0.017 μm² (mean ± SD, n = 941) compared to control values from normal neutrophils of two volunteers of 0.049 ± 0.033 μm² (n = 896) and 0.050 ± 0.039 μm² (n = 873) (P < 0.001 between patient and control samples). Granule histograms showed a single peak of small peroxidase-positive granules, whereas control samples contained more prominent subpopulations of larger peroxidase-positive granules. The total number of peroxidase-positive granules per 100 μm² of cytoplasm in the patient was 255 ± 124 (mean ± SD, n = 15 cell profiles), which was similar to control values of 266 ± 63 and 212 ± 109. Thus, the defensin deficiency in SGD is associated with a decrease in size rather than number of peroxidase-positive granules; suggesting that defensins contribute to normal peroxidase-positive granule size and that SGD is a more global granule deficiency than originally thought. These observations of primary granules, together with the previously observed abundant incomplete secondary granules in this patient further suggest that although granule content influences granule size it does not similarly regulate the number of granules made.

HUMAN NEUTROPHIL “specific granule” deficiency (SGD) of apparent congenital origin has been associated with susceptibility to bacterial infection, neutrophil dysfunction, and abnormal nuclear lobulation. Ultrastructural cytochemical studies have demonstrated a marked decrease in normal appearing peroxidase-negative granules in these patients. However, the presence of numerous peroxidase-negative small elongated granules or vesicles that stained positively for glycoconjugates and were secreted with phorbol myristate acetate suggested the presence of abnormal small secondary granules. Furthermore, in this latter study an abnormal pattern of glycoconjugate staining in peroxidase-positive primary granules was identified, suggesting abnormal content and/or maturation of these granules. This was consistent with previous and subsequent studies demonstrating a lighter density of primary granules in SGD.

The granule deficiency in SGD has been associated with a marked decrease in lactoferrin and lactoferrin RNA, although a small amount of lactoferrin can be detected biochemically in neutrophils from these patients. On the other hand, myeloperoxidase, a primary granule marker, is not deficient in these patients. Recently Ganz et al reported that the antimicrobial peptides, termed defensins, which comprise up to 30% of normal primary granule proteins, were markedly deficient in two patients with SGD. Since certain defensin-rich primary granules demonstrate a characteristic morphology we used ultrastructural and morphometric methods to reexamine the neutrophils of one of these patients for primary granule abnormalities. A preliminary report of this observation has been presented previously.

MATERIALS AND METHODS

The case history of this patient was reported previously. Earlier studies of this patient have also described abnormalities of ultrastructural morphology and complex carbohydrate cytochemistry, as well as abnormal neutrophil function, deficient lactoferrin, and defensin content.

A bone marrow sample (n - 1) and blood samples (n - 3) from this patient were collected in heparinized or EDTA anticoagulated containers over a 6-year period after obtaining informed consent and according to institutional guidelines. At the time of sampling the patient lacked clinical evidence of infection; the white blood cell count ranged from 7,000 to 9,100 cells/μL with 60% to 65% neutrophils and contained <8% band forms. The hemoglobin ranged from 15 to 16 gm/dL; and the platelet count varied in the normal range up to 474,000 cells/μL. Bone marrow (n - 1) and blood (n - 2) from normal volunteers were comparatively processed as controls. The samples were centrifuged at 1,700 × G for three minutes and the buffy coat removed and fixed intact or resuspended in 3% glutaraldehyde 0.1 mol/L cacodylate buffer, pH 7.35, for one hour at 4°C to 10°C. The cells were then rinsed three times in a 0.1 mol/L cacodylate, 7 gm/dL succrose buffer. Samples processed for peroxidase cytochemistry were resuspended 30 minutes in a solution prepared by adding 5 mg of 3,3‘diaminobenzidine tetrahydrochloride (Sigma Chemical Co, St Louis) to 10 mL 0.05M Tris HCl buffer, pH 7.6, containing 0.01% H₂O₂ (added immediately before use) as described previously. After rinsing in 0.1 mol/L cacodylate succrose buffer all samples were post fixed in 1% OsO₄ in 0.1 mol/L cacodylate, dehydrated in graded alcohols and propylene oxide, and embedded in Spurr low viscosity resin. Thin sections of morphologic preparations were stained with methanolic uranyl acetate and aqueous lead citrate as described previously whereas peroxidase preparations were not counterstained or stained only with lead citrate. Thin sections were then examined in a Philips 301 electron microscope.
microscope (Philips Electronic Instruments, Inc, Mahwah, NJ) or a Zeiss EM109 electron microscope (Carl Zeiss, Inc, Thornwood, NY) at accelerating voltages of 60kV and 50kV respectively.

For morphometric studies DAB-lead citrate stained, EDTA anti-coagulated resuspended cells were examined from the patient and one normal control blood sample whereas the second control was processed as a minced specimen. The first 15 neutrophils observed with two or more nuclear lobes were consecutively scored using a Philips 301 electron microscope. Photographs were taken of all scored cells at an original magnification of 11,000 and printed 2.5 times the original negative size on photographic paper using standard darkroom techniques. A calibration sequence for the Philips 301 was used to assure the actual final magnification. The plasmal-emma, granule membrane, and nuclear envelope were then outlined using a Numonics 2210 electromagentic tablet and Sigma Scan v2.3 Software (Jandel Scientific, Corte Madera, CA) on an IBM-PC or IBM-AT computer. The data were tabulated using Sigma Scan v2.3, and Lotus 1-2-3 v2.01 (Lotus Development Corp., Cambridge, MA). Lotus 1-2-3 standard deviation was corrected to n-1. Final statistical manipulations and checks were run using SPSSX v2.2 on a DEC VAX 8600 computer. All granules with identifiable membrane (including presumed tangential sections) were evaluated. The total cell area, cytoplasmic area, nuclear area, and peroxidase-positive granule areas and the number of organelles were recorded for each cell. Similarly, the number of small peroxidase-negative granules and/or vesicles and mitochondria were scored in these preparations, although these negatively stained structures were not as readily seen as in uranyl acetate and lead citrate-stained preparations. Unfortunately, counterstaining with both metals resulted in such an increase in granule density that definitive peroxidase staining could not be distinguished from granule density imparted by metal counterstaining. Consequently, morphometric analysis was performed on peroxidase-stained cells counterstained with lead citrate alone.

RESULTS

Ultrastructural morphology and cytochemistry. The present study compared primary granules in SGD with recently described normal peroxidase-positive granule sub-populations including large defensin-rich dense granules, \textsuperscript{3} intermediate-size homogeneous and crystalloid containing granules. \textsuperscript{14} Neutrophils contained few (0 to 1 per cell profile) large (0.25 to 0.40 μm diameter) rim

Figs 1 and 2. The blood neutrophil depicted in Fig 1 is from the SGD patient and contains several electron dense granules (large arrows, Fig 1) presumed to be primary granules. A few of these granules contain a less dense central area (arrowheads, Fig 1), however, these granules are infrequent and smaller than certain previously described defensin-rich dense granules. \textsuperscript{10} The patient neutrophil lacks larger primary granules with rim staining seen in a similarly stained normal neutrophil depicted at the same magnification in Fig 2 (large arrows). The patient neutrophil also contains abundant small elongated granules/vesicles (small arrows, Fig 1) presumed to be incomplete secondary granules, \textsuperscript{5} which would correspond to some of the smaller granules in the normal neutrophil (small arrows, Fig 2). Thin sections stained with uranyl acetate and lead citrate. Bars = 1 μm. N, nuclear lobes.
stained granules in morphologic preparations (Fig 1) in contrast to normal neutrophils, which contained three to 15 such granules comprising approximately 20% of the primary granule population (Fig 2). These rim-stained granules were similar to those previously described in normal human myeloid cells and recently isolated and associated with a high content of defensins. The patient neutrophils contained occasional small (0.15 to 0.25 μm) rim-stained granules (Fig 1). The large granules with dense rims were similarly decreased in most marrow promyelocytes (Fig 3) and myelocytes of the patient in contrast to normal myeloid precursors (Fig 4), however, a few cells did contain several of these granules.

Peroxidase staining was present in almost all cytoplasmic granules greater than 0.1 μm in diameter (Fig 5), although abundant small elongated and/or flattened granules (0.03 to 0.10 μm) lacked positive staining (Fig 6) as described previously. Larger rim-stained peroxidase-containing granules present in normal myeloid cells (Fig 7) were absent or only rarely seen in the patient myeloid cells (Figs 5, 6). Small peroxidase-positive granule profiles (0.08 to 0.19 μm diameter) were seen in both patient (Fig 6) and normal neutrophils, however, more small granule profiles were observed in the patient sample. Some of these granules often appeared in clusters (Fig 6) as described previously for peroxidase-positive microgranules in normal neutrophils. Granules of a more intermediate size stained homogeneously or contained a central lucent crystalloid in both patient and normal neutrophils.

**Morphometry.** Morphometric studies indicated a significant (P < 0.001) smaller average peroxidase-positive individual granule area in the patient of 0.019 ± 0.017 μm² per granule (n = 941, mean ± SD) compared with normal neutrophils from two volunteers of 0.049 ± 0.33 μm² (n = 896) and 0.050 ± 0.039 (n = 873) (Table 1). The average individual granule area per cell was 0.020 ± 0.006 μm² (n = 15 cell profiles) for the patient and was significantly smaller compared with control values of 0.049 ± 0.007 and 0.050 ± 0.013 (n = 15 cell profiles for each control). Thus, the average (mean) diameter of peroxidase-positive granules in the patient sample was 0.16 μm compared with 0.24 μm in control neutrophils. The total number of peroxidase-positive granules per 100 μm² of neutrophil cytoplasm in the patient and controls was not significantly different (Table 1). Granule histograms showed a single peak of small peroxidase-positive granules, whereas normal neutrophils contained a more heterogeneous population of primary granules. Thus, the majority of the total peroxidase-positive granule area was occupied by peroxidase-positive granules less than 0.20 μm in diameter in the patient samples, whereas granules greater than 0.25 μm in diameter comprised the majority of the peroxidase-positive granule area in normal neutrophils (Fig 8). Mitochondria were more frequently observed in the patient neutrophils (Table 1). The number of

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**Figs 3 and 4.** This marrow late promyelocyte/early myelocyte from the patient lacks larger rim-stained granules seen in normal myeloid cells at a comparable developmental stage and at the same magnification (Fig 4, arrows). Several immature condensing granules and smaller dense granules are present. The cytoplasm contains a moderate amount of endoplasmic reticulum and the nucleus contains dispersed chromatin and a nucleolus (Nu). Thin section stained with uranyl acetate and lead citrate. Bar = 1 μm.
peroxidase-negative granules resembling normal secondary granules in the patient was markedly decreased compared to controls. The frequency of very small elongated granules or vesicle-like structures (excluding Golgi) in DAB-lead citrate stained patient samples was significantly increased compared to controls. However, the true frequency of these peroxidase-negative granules and elongated vesicles may have been higher since these organelles were not positively stained and uranyl acetate counterstaining was not performed thereby preventing clear demarcation of this small organelle at the relatively low magnification used in the analysis. This appears to be particularly true in the patient sample in which the small abnormal granules outnumbered primary granules by a ratio of 2 to 3:1 in uranyl acetate-lead citrate stained specimens (Fig 1). Furthermore, previous studies in this laboratory have demonstrated an abundance of these organelles when positively stained for vicinal glycol containing glycoconjugates. Unfortunately, adequate methodology for combining DAB and glycoconjugate staining does not currently exist, since the DAB reaction product is also Schiff reactive resulting in false positive staining of DAB for vicinal glycols.

**DISCUSSION**

The present study demonstrates an abnormal morphology and a decrease in size of peroxidase-positive granules in a patient with SGD. The number of peroxidase-positive granules per 100 μm² of neutrophil cytoplasm was not significantly different from normal neutrophils indicating that a decrease in granule number was not associated with the observed decrease in granule size. Previous studies have demonstrated considerable heterogeneity in peroxidase-positive granule morphology and biochemistry, which extends across the entire gamut of neutrophil granule size and density. The decrease in granule size observed in the patient’s neutrophils of this study could then result from either a lack of synthesis of a large peroxidase-positive granule subpopulation, or the failure to package certain granule constituents into a normal number of granules because of lack of synthesis or ineffective synthesis of those constituents.

The marked decrease in large rim-stained granules in the patient is consistent with the high defensin content in this morphologic counterpart and the reported deficiency of defensins in this patient and another patient with SGD. The presence of a similar decrease of these granules in marrow precursor cells suggests a decrease in synthesis and argues against a selective loss of these granules during cell differentiation. Since defensins comprise approximately 30% of granule proteins the failure to package defensins into these granules either as a result of decreased or ineffective synthesis would then account for a major portion of the decrease in total peroxidase-positive granule area observed in this study. The previous observation of lighter density-primary granules in these patients is also consistent with modification of the defensin-rich granules, which normally comprise the densest of granules on Percoll gradients.

Previous studies suggested that the abnormal content of primary granules resulted in an increase in staining of vicinal glycol containing glycoconjugates in these granules. Normal defensin-rich dense granules are relatively deficient in glycoproteins and stain weakly for vicinal glycols using the periodate-thiocarbohydrazide-silver proteinate method. The increased staining of glycoconjugates observed in primary granules in this patient may then account for a major portion of the decrease in total peroxidase-positive granule area observed in this study. The marked decrease in large rim-stained granules in the patient is consistent with the high defensin content in this morphologic counterpart and the reported deficiency of defensins in this patient and another patient with SGD. The presence of a similar decrease of these granules in marrow precursor cells suggests a decrease in synthesis and argues against a selective loss of these granules during cell differentiation. Since defensins comprise approximately 30% of granule proteins the failure to package defensins into these granules either as a result of decreased or ineffective synthesis would then account for a major portion of the decrease in total peroxidase-positive granule area observed in this study. The previous observation of lighter density-primary granules in these patients is also consistent with modification of the defensin-rich granules, which normally comprise the densest of granules on Percoll gradients.

The marked decrease in normal peroxidase-negative secondary granules in our patient has been reported previously. This has been associated with a deficiency of lactoferrin, presumed to be a component restricted to secondary granules in most but not all studies. Similarly, the patient neutrophils are comparably deficient in vitamin B₁₂ binding protein. Also, previous studies have indicated that this patient’s neutrophils are deficient in gelatinase a component presumed to be in tertiary granules. In addition to deficiencies in primary, secondary, and tertiary granule components, the patient is also known to have deficient neutrophil alkaline phosphatase activity, which is localized in a compartment distinct from the other granules and the
Figs 6 and 7. At higher magnification peroxidase and lead citrate staining delineate several peroxidase positive granules in the patient neutrophils (Fig 6), however, these neutrophils are deficient in rim-stained granules present in normal neutrophils and depicted in Fig 7 (large arrows) at the same magnification. Peroxidase-positive microgranules in the patient neutrophil (MG, Fig 6) are clustered similar to those described in normal neutrophils.\(^{13}\) The lead citrate counterstaining allows identification of some peroxidase-negative small elongated vesicles/granules (small arrows, Fig 6) and secondary granules (small arrows, Fig 7) in patient and control neutrophils, respectively. Bars = 1 \(\mu\)m. N, nuclear lobes.

### Table 1. Morphometric Analysis of Neutrophil Organelles in SGD

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Significance Between Patient &amp; Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP granule area in (\mu)m(^2) per granule, mean ± SD (n = granule number)</td>
<td>0.019 ± 0.017 (941)</td>
<td>0.049 ± 0.033 (896)</td>
<td>0.050 ± 0.039 (873)</td>
<td>(P &lt; .001)†</td>
</tr>
<tr>
<td>Average PP granule area (\mu)m(^2) per cell, mean ± SD n = 15 cell profiles</td>
<td>0.020 ± 0.006</td>
<td>0.049 ± 0.007</td>
<td>0.050 ± 0.013</td>
<td>(P &lt; .001)†</td>
</tr>
<tr>
<td>Average PP granule diameter</td>
<td>0.16</td>
<td>0.24</td>
<td>0.24</td>
<td>—</td>
</tr>
<tr>
<td>PP granule number per 100 (\mu)m(^2) cytoplasm mean ± SD, n = 15 cell profiles (range)</td>
<td>256 ± 124 (93-605)</td>
<td>266 ± 63 (171-377)</td>
<td>212 ± 109 (52-371)</td>
<td>(P = .44)‡</td>
</tr>
<tr>
<td>Mitochondria number per 100 (\mu)m(^2) cytoplasm mean ± SD, n = 15 cell profiles</td>
<td>33 ± 17</td>
<td>10 ± 10</td>
<td>9 ± 8</td>
<td>(P &lt; .001)†</td>
</tr>
<tr>
<td>PN granules per 100 (\mu)m(^2) cytoplasm mean ± SD, n = 15 cell profiles</td>
<td>27 ± 19</td>
<td>456 ± 147</td>
<td>354 ± 75</td>
<td>(P &lt; .001)†</td>
</tr>
<tr>
<td>PN vesicle-like structures§ per 100 (\mu)m(^2) cytoplasm mean ± SD, n = 15 cell profiles</td>
<td>127 ± 64</td>
<td>10 ± 14</td>
<td>4 ± 6</td>
<td>(P &lt; .001)†</td>
</tr>
</tbody>
</table>

Abbreviations used are SGD, specific granule deficiency; PP, peroxidase positive; PN, peroxidase negative.

*Parametric tests performed on the data indicated violations of the parametric model. Since the distributions were non-normal, the statistical analysis of the data is reported from nonparametric tests.

†Mann-Whitney U test of significance applied.\(^{16}\) No significant difference was observed between controls.

‡Kruskal-Wallis one way ANOVA test of significance applied.\(^{16}\) No significant difference was observed between controls.

§Golgi vesicles were not included.
SPECIFIC GRANULE DEFICIENCY

Fig 8. This bar graph relates the frequency of different size granules (n = 941 granules for the patient and 873 granules for the control) to the total area occupied by peroxidase-positive granules. The results indicate that morphologically most of the peroxidase-positive cell area is accounted for by granules greater than 0.25 μm in diameter in control neutrophils, whereas granules less than 0.20 μm in diameter account for most of this area in the patient’s neutrophils.

The presence of incomplete and small primary and secondary granules in this patient suggests a common mechanism for a pan-granule defect that presumably is similar in other patients with SGD. Although previous studies have not morphometrically examined other patients with SGD, vesicle-like structures or “constricted sacs” presumed to be abnormal secondary granules in this patient, have been seen in two other patients with this disorder. The decrease or absence of defensins in our patient and one other patient with SGD further suggests a common primary granule defect, although a relative decrease in elastase content in neutrophils from our patient compared to another SGD patient raises the possibility of disease heterogeneity. Nevertheless, the present study indicates that granule content alone does not regulate the number of granules made. Thus, this patient and presumably other patients with SGD provide a biologic model demonstrating that the formation of neutrophil granules, albeit incomplete and small granules, can occur independently of the availability of material to be packaged in the granules.

REFERENCES

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