Defensin-Rich Dense Granules of Human Neutrophils

By William G. Rice, Tomas Ganz, Joseph M. Kinkade, Jr, Michael E. Selsted, Robert I. Lehrer, and Richard T. Parmley

Defensins are a newly recognized class of small, cationic polypeptides that have in vitro microbicidal activity toward certain bacteria, fungi, and viruses. Human neutrophil granules were separated into 13 density fractions by using a high-resolution Percoll gradient centrifugation procedure, and the distribution of the three defensin polypeptides in these fractions was determined. Levels of defensins and several granule marker proteins were estimated in each fraction from relative staining intensities of bands following acid-urea and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total acid-extractable proteins. These results were confirmed by enzyme immunoassay measurements of defensins and quantitative determinations of the typical azurophil granule enzyme immunoassay measurements of defensins and had distinctive biochemical and ultrastructural properties.

Defensins, termed human neutrophil defensins, are abundant constituents of human neutrophil and rabbit heterophil granules.11-14 Defensins possessed in vitro microbicidal activity toward certain bacteria, fungi, and viruses,11,14-16 and ultrastructural immunochemistry localized these peptides to the azurophil granules11 of human neutrophils. The purpose of the present study was to investigate the distribution of defensins among the different isolated granule density fractions reported by Rice et al13 and to determine if a particular defensin-rich granule subpopulation could be identified among the peroxidase-reactive azurophil granules. We now report that one particular fraction, H5, which contains the largest and most dense granules, was especially rich in defensins and had distinctive biochemical and ultrastructural properties.

MATERIALS AND METHODS

All investigations using human material were performed with the approval of the Human Investigations Committee, Emory University, in accord with an assurance filed with and approved by the Department of Health and Human Services.

Isolation of Granule Subpopulations

Human neutrophil concentrates were obtained from leukapheresed healthy adults after informed consent. Neutrophils were further purified to >97% purity following two cycles of hypotonic lysis of erythrocytes and subsequent centrifugation through lymphocyte separation medium (Litton Bionetics, Kensington, MD) to remove mononuclear cells as described previously.17 Granule-rich postnuclear supernatants were prepared and high (H) and low (L) density gradients were performed as described previously.1 In brief, the H and L gradients each consisted of multiple-step preformed layers of different densities prepared in 50 mL polycarbonate centrifuge tubes. All gradient solutions contained 5 U heparin/mL and were made isotonically with 10 times concentrated Dulbecco’s phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 6N HCl. Appropriate volumes of the resulting 90% Percoll solution (refractive index = 1.3518) were diluted with different volumes of PBS to construct the different density layers. The top layer of each gradient consisted of a maximum of 2.5 mL of postnuclear supernatant from 2.5 x 10⁶ cells. Centrifugation was carried out in a high-resolution Percoll gradient centrifugation procedure, and the distribution of the three defensin polypeptides in these fractions was determined. Levels of defensins and several granule marker proteins were estimated in each fraction from relative staining intensities of bands following acid-urea and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total acid-extractable proteins. These results were confirmed by enzyme immunoassay measurements of defensins and quantitative determinations of the typical azurophil granule enzyme immunoassay measurements of defensins and had distinctive biochemical and ultrastructural properties.

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Acid-urea polyacrylamide gel electrophoresis of granule fraction extracts. Lane M contains ~2 μg each of purified granule proteins: myeloperoxidase (MPO), lactoferrin (LF), human neutrophil elastase (EL), the three defensins (DEF 1 through 3), and lysozyme (LZ). Lanes L1 through L8 and H1 through H8 each contain 10 μg protein from the acid extract of the respective granule fraction. Lane G contains 10 μg of the acid extract of unfraccionated granules.

Extraction of Granule Proteins and Biochemical Procedures

Granule pellets to be assayed for myeloperoxidase were homogenized in 0.3% cetyltrimethylammonium bromide (or in 0.1% Triton X-100) in PBS, pH 7.4, containing 10 μg/mL of both phenylmethylsulfonyl fluoride and 1-L-tosylamide-2-phenylethylchloromethyl ketone and 1 mmol/L EDTA. Granule lysates were assayed spectrophotometrically for myeloperoxidase using guaiacol as the electron donor and the mass amounts of myeloperoxidase were quantified according to the procedure of Schultz. Myeloperoxidase was also quantified by difference spectroscopy. Granules were homogenized in 0.1% Triton X-100, and absorption spectra from 400 to 600 nm were measured on reduced (dithionite) minus oxidized samples using an Amino DW-2a spectrophotometer (American Instrument, Silver Spring, MD). An absorbance coefficient of 75 mmol/L cm⁻¹ was used for quantifying the characteristic 472-nm peak. Activities of α-glucuronidase were measured as described previously, as were lysozyme and elastase. Protein was measured according to Lowry et al using bovine serum albumin (BSA) as a standard except for defensin studies, in which a microbiuret procedure was used. For the assay of defensin peptides, granule pellets were extracted in 10% acetic acid, which extracts 80% to 90% of defensins from mixed granules. Defensins were quantified using a recently described enzyme immunoassay which used the IgG fraction of rabbit antibody raised against the mixture of defensins 1 through 3. The insoluble granule material was taken up into NaOH for microbiuret assay of its protein content. Gel electrophoresis was performed using two different systems: a 12.5% acid-urea polyacrylamide gel and a 10% to 30% gradient sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) system. Proteins were visualized by staining with formalin-Coomassie brilliant blue. The significance of differences between means of various groups was evaluated using Student’s t test for unpaired data.

Electron microscopy

Granule pellets were overlaid with three drops of human plasma and three drops of 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After 10 minutes at 25°C, the granule-containing plasma plug was minced and submerged 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.

After informed consent from healthy volunteers, either normal bone marrow from the posterior iliac crest was aspirated into a heparinized syringe or peripheral blood was collected by routine venipuncture. Marrow and blood samples were fixed in 0.1 mol/L cacodylate buffer, pH 7.35, containing 3% glutaraldehyde and then rinsed in 0.1 mol/L cacodylate buffer, pH 7.35, containing 7% sucrose as described previously.

Samples for peroxidase staining were incubated 30 minutes in substrate medium consisting of 3 or 5 mg diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical) in 10 mL 0.05 mol/L Tris-HCl buffer, pH 7.6, to which 33 μL of 3% H₂O₂ were added immediately before use as described previously. Control samples were incubated in medium lacking H₂O₂. Staining for sulfate was accomplished using Spicer’s high iron diamine (HID) method as
described previously. Bone marrow and blood samples processed for staining of vicinal glycol-containing complex carbohydrates were incubated en bloc in α-amylase for removal of glycogen as described previously, whereas this step was omitted for isolated cytoplasmic granules. Incubation en bloc in α-amylase for removal of glycogen as described for staining of vicinal glycol-containing complex carbohydrates were not postfixed. All samples were routinely dehydrated and embedded in Spurr low-viscosity medium. Thin sections of morphologic and peroxidase-stained samples were collected on copper grids. Peroxidase-stained specimens were not counterstained or were only briefly stained with UALC. Some thin sections of morphologic preparations were stained with aqueous 1% uranyl acetate (pH 4.5) and/or lead citrate, without or with prior tannate treatment to enhance staining of cationic substances. This thin sections of specimens processed for vicinal glycol staining were collected on stainless steel grids and stained with a periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method as described previously. HID staining was enhanced on thin sections by sequential exposure to TCH-SP as described previously. All specimens were examined on a Phillips 301 electron microscope or a Zeiss EM 109 electron microscope at an accelerating voltage of 60 kV.

**RESULTS**

*Unusual Composition of High-Density Granule Fraction H5*

Human neutrophil granules were separated into 13 isopycnic density fractions using a recently described two-gradient density centrifugation method. The two outer lanes contain mol-wt marker proteins whose values are indicated times $10^{-5}$. Lanes L1 through L8 and H1 through H5 contain 20 µg acid-extracted protein from each neutrophil granule fraction (fraction 6 was omitted because it did not contain sufficient protein). Lane G contains 20 µg acid-extracted protein from unfractionated neutrophil granules. Lane M contains purified lactoferrin, LF, 2 µg; myeloperoxidase, MPO, 4 µg; elastase, Ei, 4 µg; lysozyme, LYS, 2 µg; and 2 µg each of the three defensins (DEF) that migrate together in this gel system. The neutrophil granules used were obtained from a different donor than that in Fig 1.

**Table 1. Quantification of Defensins and Typical Primary Granule Markers in High-Density Granule Fractions**

<table>
<thead>
<tr>
<th>Granule Fraction</th>
<th>MPO (g)</th>
<th>β-GU (g)</th>
<th>LZ (µg)</th>
<th>EL (U)</th>
<th>DEF (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>72.4 ± 3.7*</td>
<td>4.7 ± 0.8*</td>
<td>25.6 ± 2.8*</td>
<td>0.30 ± 0.06*</td>
<td>349 ± 101</td>
</tr>
<tr>
<td>H2</td>
<td>53.4 ± 6.4*</td>
<td>4.8 ± 0.8*</td>
<td>24.5 ± 4.2*</td>
<td>0.36 ± 0.06*</td>
<td>355 ± 74</td>
</tr>
<tr>
<td>H3</td>
<td>61.7 ± 7.8*</td>
<td>4.1 ± 0.7</td>
<td>21.8 ± 2.6*</td>
<td>0.26 ± 0.01*</td>
<td>422 ± 74</td>
</tr>
<tr>
<td>H4</td>
<td>46.6 ± 4.2*</td>
<td>4.1 ± 0.4*</td>
<td>23.4 ± 6.4</td>
<td>0.20 ± 0.04*</td>
<td>403 ± 111</td>
</tr>
<tr>
<td>H5</td>
<td>34.6 ± 3.3</td>
<td>2.8 ± 0.4</td>
<td>13.2 ± 3.1</td>
<td>0.10 ± 0.02</td>
<td>472 ± 123</td>
</tr>
</tbody>
</table>

Mass amounts of protein, myeloperoxidase (MPO), lysozyme (LZ), and defensins (DEF), and enzymatic activities of β-glucuronidase (β-GU) and elastase (EL) were measured in fractions H1 through H5, as described in the Materials and Methods section. Values represent mean protein content or specific activity ± SE derived from the number of different experiments indicated in parentheses. The values for MPO are expressed as microgram per milligram of protein and β-GU as units per milligram of protein, where 1 U equals the amount of enzyme required to hydrolyze 1 nmol of substrate in 1 minute; LZ as micrograms per milligram of protein; EL as nkat per milligram of protein, where 1 nkat equals the amount of enzyme required to hydrolyze 1 nmol of substrate in 1 second and defensins as micrograms per milligram of protein. The values for total protein (µg ± SE, n = 6) for fractions H1 through H5, respectively, are: 732 ± 58, 599 ± 69, 696 ± 94, 519 ± 20, and 521 ± 61.

*P < .05 as compared with value for granule fraction H5.

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Percoll centrifugation procedure. Acid-extractable proteins were obtained from each of these isolated granule fractions and were electrophoresed into a 12.5% acid-urea polyacrylamide gel (Fig 1). Relative staining intensities of protein bands indicated that fractions L1 through L8 contained most of the specific granules as shown by their high content of lactoferrin. In contrast, fractions H1 through H4 were more enriched with myeloperoxidase and elastase, indicating that they contained most of typical peroxidase-containing azurophil granules. Generally, the intensities of the bands corresponding to defensins (DEF 1 through 3) increased from the lowest density fraction (L1) to the highest density fraction (H5). Based on staining intensity, the five highest density fractions (H1 through H5) were significantly enriched in defensins.

To quantitate this observation, the content of defensins was measured in each of the 13 isolated granule fractions by enzyme immunoassay (Fig 2). Whereas defensins accounted for ~10% of the total protein in each of the low-density fractions (L1 through L8), they comprised between 35% and 50% of the total granule protein in the high-density fractions, H1 through H5.

Defensin concentrations were compared with those of the known azurophil granule components, myeloperoxidase, elastase, lysozyme, and β-glucuronidase (Table 1). Whereas the relative amounts of these azurophil granule components tended to decrease as granule density increased, the converse was true of defensins. Indeed, defensins were the predominant soluble protein in the H5 granule fraction illustrated in Fig 1. This progressive enrichment in defensins, relative to other soluble granule components as granule density increased, was especially well demonstrated using a gradient SDS-PAGE system (Fig 3). In this case, equal amounts of protein from each fraction were subjected to electrophoresis, although similar results were obtained when equal aliquots of each fraction were used.

Ultrastructural Features of Defensin-Rich Peroxidase-Reactive Granules

Morphology. Morphologic staining using methanolic UALC showed that the granules in fraction H5 were the largest human neutrophil granules. These granules had a mean diameter of 0.32 ± 0.08 μm (mean ± SD, n = 200) as compared with the other typical peroxidase-reactive granule fractions, H1 through H4, which had a combined average diameter of 0.26 ± 0.07 μm (n = 200 for each fraction) (P < .01). Staining with methanolic UALC showed that 70% of the H5 granules (n = 200) had an electron-dense peripheral area and an electron-lucent globular central region (Fig 4). This morphologic staining pattern was quite distinctive, since granules in the more typical peroxidase-reactive fractions demonstrated a more homogenous, dense staining pattern (see below). This rim-staining pattern of H5 granules was not evident in specimens stained with lead alone.

![Fig 4](image-url)
DEFENSIN-RICH DENSE GRANULES

Fig 5. Electron micrographs of granules from fractions H5 and H3 after reaction with DAB for peroxidase activity. Approximately 70% of the granules in fraction H5 (panel A) demonstrated a peripheral (rim) staining pattern, whereas most of the granules in fraction H3 (panel B) exhibited more uniform and intense reactivity. Thin sections were not counterstained with uranyl acetate or lead citrate. Bar = 1 μm (panels A and B).

or with aqueous uranyl acetate, or with a tannate-lead citrate sequence (a procedure that stains cationic proteins) that appeared to result in relatively more central staining than was observed with methanolic UALC (Fig 4, insert).

Peroxidase. Staining for peroxidase with DAB was carried out to determine if the predominant granule morphology type identified in fraction H5 had a reactivity consistent with the quantitative biochemical analyses. As shown in Fig 5A, these granules demonstrated peripheral peroxidase activity and essentially negative reactivity in the central region; no staining was observed in granules incubated in DAB without H2O2. This rim-staining pattern was analogous to that which occurred with the methanolic UALC morphologic staining but was different from the more homogenous DAB staining pattern that occurred in the more typical peroxidase-reactive granules of fraction H3 (Fig 5B). This weak ultrastructural peroxidase reactivity of fraction H5 granules was in agreement with the relatively low level of myeloperoxidase measured biochemically in this granule fraction (Table 1).

The number of granules exhibiting the peroxidatic rim-staining pattern was quantitated independently by two different observers in each of the five high-density fractions. These data, shown in Fig 6, indicated that fraction H5 was enriched about sevenfold in the rim-stained type of granule as compared with fraction H1. Furthermore, the percentage of DAB rim staining granules in fraction H5 (70%) was in good agreement with the percentage (70%) of the granules showing a rim-staining electron density with the UALC morphologic procedure. The remaining 30% of granules in the H5 fraction consisted of large granules that lacked DAB staining, but otherwise resembled H5 granules in their electron density and size (15%), and granules with the more typical homogeneous DAB staining pattern mentioned previously (15%, of Fig 5B). The finding of rim-stained granules in fractions other than H5 suggests that density heterogeneity exists even among this granule subclass.

Complex carbohydrates. The large characteristic granules of fraction H5 exhibited only weak matrix reactivity...
when stained for vicinal glycol-containing complex carbohydrates using the PA-TCH-SP procedure, similar to that shown previously for granules in intact neutrophils. In addition, these same granules stained negatively when reacted for the presence of sulfate-containing macromolecules using an HID procedure.

**Intact cells.** Methanolic UALC and/or DAB staining of mature peripheral blood neutrophils (Fig 7) showed the presence of rim-stained granules in the intact cell, similar to those observed previously in stained preparations of isolated H5 granules (Figs 4 and 5). If the peroxidase reaction was allowed to continue for 18 hours (rather than 30 minutes) this rim-staining pattern was completely obscured. Similarly, human bone marrow promyelocytes, when stained with methanolic UALC, contained numerous rim-stained granules (Fig 8). The rim staining was of variable intensity and was obscured by overstaining or omission of methanolic uranyl acetate from the staining procedure. Together, these results confirmed that characteristics of this granule type were not due to artifacts imposed by the fractionation procedure.

**DISCUSSION**

We recently described a Percoll fractionation procedure that separates human neutrophil granules into 13 fractions by density. With this technique, most granules in the eight fractions of lower density (L1 through L8) are peroxidase-negative and most granules in the five fractions of higher density (H1 through H5) are peroxidase-reactive. Examination of the acid-extractable proteins from each of the 13 granule fractions by acid-urea and gradient SDS-PAGE (Figs 1, 3) showed qualitative staining patterns that indi-
cated the higher density granule fractions were enriched in cationic defensins, in agreement with the ultrastructural localization of defensins to large azurophil granules reported previously by Ganz et al.\textsuperscript{11} Quantification of the defensin content in these granule fractions using a specific enzyme immunoassay demonstrated that the five higher density fractions contained about four times more of these microbicidal peptides than did the eight lower density fractions (Fig 2). In particular, the highest density fraction, H5, was especially rich in defensins (~50% of total protein), but was relatively deficient in the typical azurophil granule marker proteins myeloperoxidase, β-glucuronidase, lysozyme, and elastase when compared with the other four high-density fractions (Table 1).

In addition to their distinctive biochemical and physical properties, the granules of fraction H5 demonstrated distinctive ultrastructural properties. Morphometric analyses showed that they had the largest mean diameter (0.32 ± 0.08 μm) of any human neutrophil granules. Also, when a UALC morphologic stain was used, ~70% of these granules exhibited an electron lucent central area whereas their periphery was more electron dense (Fig 4). As anticipated, many of these granules stained positively for peroxidase using DAB. Under conditions of limited DAB exposure (30 minutes of incubation), 70% of the H5 granules demonstrated only peripheral peroxidase reactivity and negative staining in their central region (Fig 6). This peroxidase rim-staining pattern (Fig 5A), which has been noted previously by other researchers,\textsuperscript{13,32} differed dramatically from the more typical azurophil granules, which exhibited more homogenous and intense peroxidase reactivity (Fig 5B). Hence, the biochemical and physical properties, as well as the morphologic and peroxidase ultrastructural features, distinguish these large dense granules from more typical azurophil granules. Granules with these characteristic morphologic and staining properties were observed in intact mature peripheral blood neutrophils (Fig 7), indicating that this granule type was not an artifact of the granule separation procedure. Moreover, the presence of this class of granules in intact bone marrow promyelocytes (Fig 8) indicated that they were synthesized early in myeloid development.
Previous ultrastructural studies by Ganz et al\textsuperscript{11} demonstrated the localization of defensins to large azurophil granules. For the moment, technical limitations have precluded the ultrafine structural localization of defensins within H5 granules using immunogold procedures. Nevertheless, many considerations point to the presence of defensins in the distinctive granules of fraction H5 and more specifically to the granule’s central region. First, the rim-staining pattern of peroxidase reactivity in H5 granules suggests that the contents may be nonuniformly distributed. Second, since defensins constitute approximately one-half of the total protein in H5 granules, their storage may be assumed to require approximately one-third to one-half of the total granule volume. If we consider an H5 granule to be roughly spherical, its volume will be proportional to the cube of its radius, \( r \), volume. If we consider an H5 granule to be roughly spherical, its volume will be proportional to the cube of its radius, \( r \), volume. Third, defensins are carboxyhydrate-free cationic peptides, and the tannate-citrate stain for cationic substances\textsuperscript{29} showed more staining in the central region of these granules (Fig 4, inset). In contrast, the central core of the large H5 granules exhibited only weak matrix reactivity when stained for vicinal glycol-containing carboxyhydrates by the PA-TCH-SP procedure\textsuperscript{28} and did not stain for sulfate-containing macromolecules by the HID procedure.\textsuperscript{28} Both these findings are also consistent with localization of defensins to the central core area, where they might also mask anion complex carboxyhydrate staining.

Most granules in the defensin-rich H5 fraction showed only weak cytochemical peroxidase reactivity, and defensins were also prominent in the other fractions (H1 through H4) that were rich in the more typical azurophil granules. Consequently, the defensin-rich dense granules (DRDGs) in fraction H5 may represent “immature” or incompletely developed azurophil granules. However, immature azurophil granules are more prominent in promyelocytes and demonstrate strong vicinal-glycol and sulfate staining and are strongly peroxidase reactive.\textsuperscript{26,28} In marked contrast, DRDGs were more prominent in late neutrophils, exhibited only weak vicinal glycol staining, no sulfate staining, and showed only weak peripheral peroxidase reactivity.

Alternatively, the neutrophils of certain mammals have been reported to contain abundant granules that are \textit{neither} azurophil nor specific granules.\textsuperscript{33,24} In some cases, these granules are believed to contain antimicrobial components that may prove to be defensins or their homologs.\textsuperscript{25} Other investigators have interpreted these structures to be dense azurophil granules.\textsuperscript{36} Therefore, human DRDGs may owe primary phylogenetic allegiance to organelles such as these rather than to the more typical lysosomal azurophil granules. The observation that \textasciitilde{}15\% of the granules in the H5 fraction did not appear to exhibit peroxidase reactivity is also consistent with such an interpretation.

We recently studied a patient with known “specific granule deficiency,” a condition that is associated with frequent and severe infections.\textsuperscript{39} In addition to abnormal specific and azurophil granules,\textsuperscript{38} the neutrophils of this patient almost completely lacked defensins and the DRDGs described in the present study.\textsuperscript{38} Additional experimentation will be required to establish the overall significance of defensins and DRDGs in the repertoire of neutrophil functional responses.

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Defensin-rich dense granules of human neutrophils

WG Rice, T Ganz, JM Jr Kinkade, ME Selsted, RI Lehrer and RT Parmley