High Resolution of Heterogeneity Among Human Neutrophil Granules: Physical, Biochemical, and Ultrastructural Properties of Isolated Fractions

By William G. Rice, Joseph M. Kinkade, Jr., and Richard T. Parmley

Previous studies on the fractionation of human neutrophil granules have identified two major populations: myeloperoxidase (MPO)-containing azurophil, or primary, granules and MPO-deficient specific, or secondary, granules. Peripheral blood neutrophils from individual donors were lysed in sucrose-free media by either hypotonic shock or nitrogen cavitation. Using a novel two-gradient Percoll density centrifugation system, the granule-rich postnuclear supernatant was rapidly (ten minutes) and reproducibly resolved into 13 granule fractions (L1 through L8 and H1 through H6). Granule flotation and recentrifugation experiments on both continuous, self-generated and multiple-step gradients using individual and mixed isolated fractions demonstrated that the banding patterns were isopycnic and nonartifactual. Isolated granules were intact based on the findings that biochemical latency of several granule enzymes was >95%, and thin-sectioned electron micrographs demonstrated intact granule profiles. Biochemical analyses of the granule marker proteins MPO, β-glucuronidase, lysozyme, and lactoferrin indicated that a number of the fractions were related to the major azurophil and specific granule populations. Lactoferrin was found in ten of 13 fractions (L1 through L8, H1 to H2), whereas MPO was found in every fraction. Consistent with these biochemical data, all fractions exhibited varying degrees of heterogeneity based on ultrastructural morphology and cytochemistry, including diaminobenzidine (DAB) reactivity for peroxidase and periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining for complex glycoconjugates. A variable but significant percentage (23% to 70%) of the granules in fractions L1 through L8 and H1 and H2 showed DAB reactivity, while about 90% of the granules in fractions H3 through H6 were peroxidase positive. These results demonstrated that DAB-reactive granules spanned the entire range of granule size and density. Ultrastructural PA-TCH-SP staining of isolated granule fractions revealed patterns similar to those of granules in intact neutrophils at different stages of development. Granules from human acute promyelocytic leukemia cells (HL-60) exhibited a surprisingly low density compared with typical azurophil granules from normal, mature neutrophils. The data suggest that both functional and maturational differences contribute to granule heterogeneity, and provide a new practical and conceptual framework for further defining the phenomenon of neutrophil granule heterogeneity.

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heterogeneity of neutrophil granules. Portions of this work have been published previously in preliminary communications.21-23

MATERIALS AND METHODS

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

Preparation of Granules

Human neutrophils were obtained by continuous-flow leukapheresis from normal, healthy male and female adults giving informed consent. Most experiments used cells from a single donor to eliminate possible heterogeneity resulting from genetic diversity. Residual erythrocytes were removed by two cycles of hypotonic lysis, with a resulting purity of >95% neutrophils.24 Eosinophils were absent in leukapheresed blood samples due to pretreatment with steroids (dexamethasone). In some cases, peripheral blood was obtained by routine phlebotomy, and neutrophils were recovered in >97% purity after HES/PAN sedimentation, centrifugation through LSM, and hypotonic lysis as previously described.24 Human leukemic HL-60 cells were cultured as described previously,25 except that they were supplemented with 10% defined bovine calf serum (HyClone). Cell staining, identification, and counting were carried out as previously described by Borregaard et al,9 and the resulting PNS was used as a source of cytoplasmic granules. Altenna-cytosis from normal, healthy male and female adults giving informed consent. Most experiments used cells from a single donor to eliminate possible heterogeneity resulting from genetic diversity. Residual erythrocytes were removed by two cycles of hypotonic lysis, with a resulting purity of >95% neutrophils.24 Eosinophils were absent in leukapheresed blood samples due to pretreatment with steroids (dexamethasone). In some cases, peripheral blood was obtained by routine phlebotomy, and neutrophils were recovered in >97% purity after HES/PAN sedimentation, centrifugation through LSM, and hypotonic lysis as previously described.24 Human leukemic HL-60 cells were cultured as described previously,25 except that they were supplemented with 10% defined bovine calf serum (HyClone). Cell staining, identification, and counting were carried out as previously reported.24 All subsequent preparation and fractionation procedures were at 4°C unless stated otherwise. Purified neutrophils were resuspended at a concentration of 1 x 10^6/mL in ice-cold hypotonic buffer (10 mmol/L Tris-HCl, pH 7.5, 0.84 mmol/L NaCl, 9.2 mmol/L KCl, 1.5 mmol/L MgCl₂, 1.44 mmol/L β-mercaptoethanol, 5 units heparin per milliliter) and allowed to swell in an ice bath for about ten minutes. The cells were gently lysed using a hand-held Teflon-glass homogenizer. The procedure was continually monitored by phase contrast microscopy to ensure maximal lysis (75% to 85%). Isotonicity was restored by adding one-tenth volume of hypertonic buffer (230 mmol/L Tris-HCl, pH 7.5, 102 mmol/L NaCl, 1.12 mol/L KCl, 40 mmol/L MgCl₂, 7.18 mmol/L β-mercaptoethanol), and the lysate was centrifuged for four minutes at 80 g. The resulting postnuclear supernatant (PNS) was used as a source of cytoplasmic granules. Alternatively, neutrophils were disrupted by nitrogen cavitation exactly as described by Borregaard et al,9 and the resulting PNS was used as the source of granules.

Fractionation of Granules

All gradient solutions contained 5 units heparin per milliliter and were made isotonic by a 1:10 dilution with ten times concentrated Dulbecco's phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 6N HCl, producing a 90% Percoll solution (refractive index = 1.3518). Several of the Percoll solutions had densities greater than the commercially available stock (Pharmacia Fine Chemicals, Piscataway, NJ, and Sigma Chemical Co, St Louis). The stock was concentrated by immersing a dialysis tube filled with the solution in dry Sephadex G-150. Solutions of desired density were prepared by further dilution with PBS. Densities were determined by refractive index using a calibration curve prepared by weighing Percoll solutions of known volume.

A variety of continuous, self-generated Percoll density gradients were used for studies of granule fractionation. In these studies, the PNS was mixed with different initial concentrations of Percoll and centrifuged at different relative centrifugal forces for varying periods. In the example shown in Fig 1, 0.3 mL of PNS was mixed with 8.0 mL of 79% Percoll (7 mL 90% Percoll plus 1 mL PBS) and centrifuged in a 15-mL Corex tube for 60 minutes at 27,200 g in a JA-20 rotor (34° fixed angle, Beckman, Palo Alto, Calif).

Granule fractionations on multiple-step gradients were carried out in 50-mL polycarbonate centrifuge tubes. The two gradients reported in this study were the low-density gradient (L-gradient) and the high-density gradient (H-gradient). During centrifugation, the density profiles of the multiple-step gradients rapidly fused such that shallow, essentially linear gradients were formed over a major portion of the tubes. Details for the preparation of H- and L-gradients are given in Table 1. The lowest-density layer (top) in each gradient system was the sample layer. This corresponded to a maximum of 2.5 mL of PNS from 2.5 x 10⁶ cells made to the indicated volume with isotonic Percoll. Dilution of the PNS in the sample layer with isotonic Percoll helped to prevent aggregation by decreasing granule concentration. Also, by more nearly equalizing the densities of the sample and its adjacent layer, a transient increase in granule concentration at the interface was avoided. Centrifugations were carried out using a microprocessor-controlled Beckman J-21 or centrifuge and a JA-20 rotor (34° angle). The instrument was programmed to run at 39,200 g for ten minutes, thus providing constant conditions from experiment to experiment. Gradients were fractionated by slowly pumping (1 mL/min) the gradient from the bottom of the centrifuge tube using a blunt-ended, 20-gauge, stainless steel spinal needle. Usually about 75 samples (0.5 mL) were collected from each gradient.

**Table 1. Preparation of H- and L-Gradients**

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<th>Diluent (mL)</th>
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Each layer contained 5 units heparin per milliliter. Except for the top layers, the diluent was PBS. RI, refractive index.

*These layers are derived from concentrated Percoll.
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One unit of MPO activity (electron donor = due to Percoll, since the Percoll was precipitated from samples on the described. One unit of βGU activity was equivalent to 1 pmol 27,200 g for I agitation at 100,000 g in a and electron microscopy. This was accomplished by centrifuging and phosphate was measured as described by Duck-Chong.34 Protein Total phospholipids were extracted as described by Bligh and Dyer,33 cholesterol was determined according to Allain et al.32 Activity was determined using the lysoplate method of Osserman and Lawlor3 with egg white lysozyme as a standard. Protein was determined according to Lowry et al,' using bovine serum albumin as a standard. Cholesterol was determined according to Allain et al.2 Total phospholipids were extracted as described by Bligh and Dyer,35 and phosphate was measured as described by Duck-Chong.34 Protein samples from isolated, washed, and detergent-extracted granule fractions (as described earlier) were electrophoresed into 7.5% polyacrylamide gels in the presence of SDS,13 and silver stained.

Latency of Granule Enzymes

Granule integrity was assessed by determining the amount of several granule enzymes in the postgranule supernatant during granule isolation and by measuring the latency of these same granule enzymes. Freshly prepared PNS was centrifuged at 27,200 g for 15 minutes at 4°C and the postgranule supernatant and granule pellet were recovered. Myeloperoxidase, LZ, and βGU were quantitated in the postgranule supernatant, as described earlier. Granule latency of these same three enzymes, as well as that of total protein, was determined according to the following relationship:

\[
\% \text{ latency} = \frac{b - a}{b} \times 100,
\]

where \(b\) and \(a\) were the amount of protein or enzyme released from granules in the presence (b) and absence (a) of 0.1% Triton X-100 (Mallingkrodt, Paris), respectively.

Electron Microscopy

Granules were separated from Percoll by high-speed centrifugation and washed with PBS as described earlier. The granule pellet was overlaid with three drops of autologous plasma and several drops of 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After standing at 25°C for ten minutes, the granule-containing plasma plug was removed and fixed for an additional 50 minutes in fresh glutaraldehyde–cacodylate buffer. The plug was washed in 0.1 mol/L cacodylate buffer, pH 7.35 containing 7% sucrose, subsequently minced with a razor blade, and rinsed in cacodylate–sucrose buffer. Samples for peroxidase staining were incubated for 30 minutes in substrate medium consisting of 3 mg of diaminobenzidine (DAB) in 10 mL of 0.05 mol/L Tris-HCl, pH 7.6, to which three drops of 3% H2O2 were added immediately before use.35 Control samples were incubated in medium lacking H2O2. Specimens processed for routine morphology and peroxidase staining were postfixed with 1% OsO4 in 0.1 mol/L cacodylate buffer, whereas those processed for glycoconjugate staining were not postfixed. All specimens were routinely dehydrated in graded ethanol and propylene oxide and embedded in Spurr low-viscosity medium.

Thin sections of morphological and peroxidase preparations were collected on copper grids. Morphological preparations were counterstained with methanolic uranyl acetate and lead citrate (UALC), whereas peroxidase preparations were examined without counterstaining. Sections of unomitted specimens (5 to 7 nm) were collected on stainless steel grids for periodate-thiocarbohydrazide–silver proteinate (PA-TCH-SP) staining as described by Thiery.36 The sections were oxidized for 45 minutes in 1% periodate, rinsed in distilled water, and then treated with 2% thiocarbohydrazide in 20% acetic acid. Specimens were subsequently washed in acetic acid, followed by three washes in distilled water. After the last wash, the samples were exposed to 1% silver proteinate for 30 minutes in the dark and rinsed thoroughly in distilled water. Control sections were run in parallel with the periodate oxidation step omitted. All specimens were examined on a Phillips 301 electron microscope or a Zeiss EM109 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Preparation and Integrity of Isolated Granules

Neutrophils were lysed by a procedure involving hypotonic cell swelling in sucrose-free medium, followed by gentle hand homogenization. This method has the advantage that cell disruption may be carefully and continually monitored by phase-contrast microscopy. In this way, we routinely achieved disruption of about 85% of the cells without any significant lysis of nuclei or cytoplasmic granules. This was verified biochemically by analyzing the post-granule supernatant for several granule proteins. No more than 5% of total granule MPO, βGU, and LZ were found, indicating that relatively little disruption of the granules had occurred.

Two additional criteria were used to further assess the integrity of granules isolated by the hypotonic swelling method. First, granules were pelleted from the PNS and the latency of granule-associated MPO, βGU, and LZ activities was determined in the presence of 0.1% Triton X-100. Latency for each of these three enzyme activities, as well as that of total granule protein, was found to be >95%. Second, no significant (<5%) difference spectrum for MPO could be observed in granules treated with dithionite unless they had also been treated with 0.1% Triton X-100.35 Essentially the same latency and spectral results were obtained using either unfractionated granules pelleted from the PNS or individual granule fractions subsequently isolated by Percoll density gradient centrifugation.

Identification and Isolation of Granule Density Fractions

Continuous, self-generated gradients. Our initial approach to fractionating cytoplasmic granules in the PNS was to use a wide variety of different isotonic Percoll density gradients that were self-generated during centrifugation. After several experiments, it was evident that the spectrum of densities exhibited by the total granule population was
quite broad. Values ranged from approximately 1.040 to 1.150 g/cm³ and were considerably lower than the densities reported for neutrophil granules isolated in sucrose-containing media. However, these lower densities were consistent with the observation that subcellular organelles isolated in isotonic Percoll media exhibit significantly lower densities than the same organelles isolated in sucrose-containing media and were similar to the values reported for human neutrophil granules isolated in sucrose-free media.

Figure 1 shows a representative profile obtained when the granule-rich PNS was fractionated on a gradient generated during 60 minutes of centrifugation (initial Percoll concentration was 79% of stock). The membrane fraction (M), together with two major diffuse bands (S and A), were obtained. A significant amount of light-scattering material was present in the interband regions as well. These gradients were separated into approximately 75 samples, and each was analyzed for its content of four granule protein markers: LF, LZ, βGU, and MPO. Representative biochemical profiles are shown in Fig 2. These markers identified two major regions of granules that corresponded to the two visible areas previously shown in Fig 1: (1) a lower-density region (1.000 to 1.095 g/cm³), which contained 88%, 64%, 29%, and 27% of the total recovered LF, LZ, βGU, and MPO, respectively, and (2) a higher-density region (1.095 to 1.160 g/cm³), which accounted for 12%, 36%, 71%, and 74%, respectively, of the total recovered LF, LZ, βGU, and MPO. Based on these marker protein analyses, it was evident that the lower- and higher-density regions corresponded, respectively, to the two major populations of granules fractionated by others using both sucrose and Percoll procedures: smaller, low-density-specific granules and larger, denser azurophil granules. It was evident from these same profiles that the specific and azurophil granule regions exhibited a marked degree of heterogeneity. The presence of multiple peaks was reproducible over many experiments, suggesting that they might represent distinct density fractions.

Fig 1. Fractionation of granules using a continuous, self-generated Percoll gradient. The granule-rich PNS from 3 × 10⁶ neutrophils was resolved on a continuous, self-generating Percoll gradient as described in Materials and Methods. The density profile was determined by measurement of the refractive index, and the position of each sample was expressed as the percentage of the total number of samples (75) collected. The gradient resolved three major fractions: membrane (M), specific granules (S), and azurophil granules (A). Note the presence of material in the interband regions.

Multiple-step gradients. Attempts to obtain improved resolution of these multiple peaks using a single gradient system were frustrated by constraints of commonly available centrifuge tube lengths and the dynamics of self-generated Percoll gradients. In these self-generated gradients, optimization of shallowness and linearity over the low-density region was gained only at the expense of a steeper, nonlinear region in the higher density region, and vice versa. Thus, no single gradient could adequately separate all of the fractions.

In order to overcome this problem, we used a two-gradient system in which the individual gradients were designed to provide a highly resolving linear expansion of the low- and high-density regions of the granule spectrum (L- and H-gradients, respectively). Further optimization was achieved by using a series of preformed density layers, which provided several advantages over self-generated gradients. First, it allowed the construction of a shallower gradient over a greater length of the centrifuge tube. Second, because the density differences between the layers were relatively small, fusion of the layers to form an essentially linear gradient was complete in ten minutes, rather than one to two hours. Third, separation of the highest-density granule fractions in a
continuous, self-generated gradient required long centrifugation periods (one to two hours) of high Percoll concentrations (>85% of stock). This resulted in formation of a large Percoll pellet at the bottom of the tube, making gradient fractionation difficult and leading to a loss of resolution between fractions. This problem was not encountered with the multiple-step H- and L-gradients. Fourth, the controlled introduction of slight discontinuities in the linear gradient resulted in a sharpening of the banding pattern, which, in turn, allowed for a more reproducible and facile fractionation of the granule bands. It is important to realize that the pattern of these minor discontinuities was specifically designed to provide increased resolution of peaks whose mean densities had already been determined on continuous, self-generated gradients such as demonstrated previously in Figs 1 and 2. A similar strategy is often used in ion-exchange chromatography, where stepwise gradients are used to minimize excessive peak tailing with eluants of fixed composition. As with such chromatographic procedures, the nonartifactuality of these gradient separations was documented by appropriate recentrifugation and mixing experiments described below.

Figure 3 shows representative density profiles and granule banding patterns obtained when a granule-rich PNS was centrifuged on high-density (H, range 1.095 to 1.150 g/cm³) and low-density (L, range 1.040 to 1.095 g/cm³) gradients. The uppermost band (M) near the meniscus corresponded to a membrane fraction similar to that observed by others during gradient fractionation of neutrophil PNS.4,5 The H-gradient resolved six additional bands. Bands H1 through H5 represented five higher-density fractions, which corresponded in mean density to five peaks identified earlier by biochemical marker analyses of fractions obtained from continuous, self-generated Percoll gradients (see Fig 2, 60% to 100%). The sixth band consisted of a mixture of lower-density granule fractions (L1 through L8), which could be resolved on an L-gradient, also shown in Fig 3. On the L-gradient, the high-density bands H1 through H5 migrated to a small region toward the bottom of the tube. Analogous to the five high-density fractions, the eight low-density fractions were also initially identified as biochemically detectable peaks on continuous, self-generated gradients. However, the more complex nature of these lower-density fractions precluded identification of all eight peaks using only a single continuous gradient such as the one shown in Figs 1 and 2.

For comparative purposes, a granule-rich PNS prepared from human promyelocytic HL-60 leukemia cells was centrifuged on an H-gradient. Surprisingly, these granules migrated as a single band near the top of the gradient. When these same granules were centrifuged on the lower-density L-gradient, they exhibited a density range of approximately 1.040 to 1.060 g/cm³, which was much lower than typical high-density azurophil granules of normal human neutrophils (1.100 to 1.142 g/cm³; see Fig 3).

**Authenticity of Granule Density Heterogeneity**

*Cell lysis.* A series of experiments was carried out to examine the possibility that the multiplicity of granule density fractions might be artifactual. To test whether the hypotonic lysis procedure could have contributed to the observed banding heterogeneity, we also isolated a granule-rich PNS using the nitrogen cavitation procedure for human neutrophil granules described by Borregaard et al.9 When such cavitates were centrifuged on either the H- or L-gradient, banding patterns and mean densities were the same as those previously shown in Fig 3. Conversely, we took the granule-rich PNS isolated by our hypotonic cell lysis procedure and centrifuged it on the two-step discontinuous Percoll density gradient described by Borregaard et al.9 As

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**Fig 3.** High resolution of neutrophil granules using high-density (H) and low-density (L) multiple-step Percoll gradients. Gradients were prepared as described in Table 1. Gradients from 2 × 10⁸ neutrophils were fractionated on each of the two multiple-step gradients. The H-gradient resolved five fractions (H1 through H5), and the L-gradient resolved eight fractions (L1 through L8); M designates the membrane fraction. The mean density of each fraction was determined from 25 separate experiments; ranges were never more than ±0.003 g/cm³ from the means. Density profiles and the percentage distance were determined as described in Fig 1.
reported by these workers, we obtained three major bands, corresponding in order of increasing density to a membrane fraction, a specific granule fraction, and an azurophil granule fraction, similar to those we had observed using a continuous, self-generated Percoll density gradient (see Figs 1 and 2). These granule fractions were isolated, and the combined membrane and specific granule fractions were recentrifuged on an L-gradient; also, the azurophil granule fraction was rebanded on an H-gradient. The results of these experiments were the same as shown in Fig 3, ie, the combined membrane and specific granule fractions contained bands M and L through L8, while the azurophil granule fraction contained bands H1 through H5. These data demonstrated that our hypotonic lysis procedure did not produce artifactual granule fractions.

Flotation, recentrifugation, and mixing. The following experiment was designed to test whether the various granule bands had reached true isopycnic equilibrium positions or whether they were being separated on the basis of some rate zonal process. Instead of placing the granule-rich PNS on top of the preformed density layers, the unfractionated granules were placed in the lowermost, high-density Percoll layer before beginning the centrifugation. Granules were then allowed to float upward to their buoyant density positions during centrifugation. After centrifugation of both H- and L-gradients, the number of bands and their corresponding mean densities were identical to those observed using the standard procedure (Fig 3). These results verified the isopycnic nature of the fractionation procedure.

Mixing experiments were carried out to assess whether interactions between different granule populations might influence the banding profiles. A wide variety of L-fractions (single or combined fractions) were mixed with individual or variously combined H-fractions and subjected to rebanding on both H- and L-gradients. The data shown in Fig 4 are representative of this group of experiments and show the results of recentrifuging three isolated H-gradient fractions (H2, H3, H4) on an identical H-gradient (right) and a mixture of two L-gradient fractions (L5 and L6) and two H-gradient fractions (H2 and H3) on an H-gradient (left). Bands H2, H3, and H4 recentrifuged to their original mean densities (1.108, 1.118, and 1.128 g/cm3, respectively), whereas bands L5 and L6 moved as a single band to a density position at the top of the gradient, where unfractionated L-bands were observed to run on the H-gradient (Fig 3). In these and other experiments (see below), biochemical analyses for protein, MPO, βGU, LZ, and LF confirmed the density positions of each fraction and indicated that no additional bands were present.

It was important to be certain that density fractions isolated on the preformed, multiple-step gradients were not artifacts generated by the procedure itself. To investigate this possibility, various individual fractions were isolated from both H- and L-gradients and were then recentrifuged on a continuous, self-generated gradient under conditions identical to those previously described in Fig 1. Photographs and a representative biochemical marker protein profile for a gradient from this group of experiments are shown in Fig 5. Band H2, initially isolated from an H-gradient (H, left photograph) appeared as a single band at the identical mean density (1.108 g/cm3) when recentrifuged on a continuous, self-generated gradient (S, right photograph). Biochemical analyses for βGU and MPO indicated essentially quantitative recovery of both marker proteins in a single symmetrical peak, further confirming the characteristic mean density of fraction H2 and the fact that no other granule bands were present. It should be noted that the shape of Percoll gradients (self-generated and preformed) is a function of, among other factors, the initial Percoll concentration, the time of centrifugation, and the relative centrifugal force. For this reason, as shown in Fig 5, band H2 appeared at different relative positions in these two gradients, even though these positions corresponded to the identical measured mean density.

**Distribution of Biochemical Markers in Granule Fractions**

**H-gradient.** Granules were separated using both H- and L-gradients, and the amounts of granule-associated LF, LZ, βGU, and MPO were quantified across each gradient (approximately 75 samples per gradient). Representative biochemical profiles are shown in Figs 6 and 7, and the amount of each protein or activity in a particular sample was expressed as a percentage of the total amount of that protein–activity recovered in all samples of the gradient. Total recoveries of marker proteins or activities in the H- and L-gradients, expressed as the percentage recovered (mean ± S.D.), were MPO (103 ± 5, n = 15), βGU (92 ± 7, n = 15), LZ (99 ± 6, n = 15), LF (93 ± 8, n = 9), and protein (96 ± 3, n = 4). In Fig 6, the three highest-density fractions obtained on the H-gradient (H3 through H5) contained MPO, βGU, and LZ, but no LF. This composition was characteristic of the typical high-density MPO-containing azurophil granules described by other investigators and indicated that the H-gradient provided an increased resolution of these granules. Furthermore, fractions H3 through H5 banded in a density range (mean 1.118 to 1.142 g/cm3) that corresponded to the major, high-density azurophil granule region (A) observed using a continuous, self-generated gradient (see Figs 1 and 2; 73% to 100%). In contrast, the
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Fig 5. Recentrifugation of a granule fraction from a multiple-step gradient on a continuous, self-generated gradient. Granule fraction H2, isolated from an H-gradient (H, left), migrated as a single band when recentrifuged on a continuous, self-generated gradient (S, right) identical to the one shown in Fig 1. The upper panel shows the density profile of the gradient, and the mean density of fraction H2 (1.108 g/cm³) is indicated by the cross-hatch. MPO and β-GU activities and the percentage distance of samples were expressed as described in Fig 2. Marker protein activities were observed only in the H2 region (percentage distance, 70%), and recovery of both activities was essentially quantitative (see Fig 6).

lower-density granules (L1 through L8), which cofractionated near the top of the H-gradient, contained 85% of the total granule LF and about 30% of the total granule MPO. The relative content of these two marker proteins was essentially the same as that reported by others for specific granules. Thus, this density region contained the majority of typical LF-containing granules. Based on its biochemical profile, a third region was apparent on the H-gradient that corresponded to granule fractions H1 and H2. These two fractions contained substantial amounts of both LF and MPO, indicating a region of major overlap in which LF-containing specific and MPO-containing azurophil granules of equivalent densities cofractionated. Consistent with this view was the observation that the density range of H1 and H2 corresponded to the interband region between the specific (S) and azurophil (A) granule bands observed on continuous, self-generated gradients (see Figs 1 and 2; 60% to 73%). Together, the five high-density fractions (H1 through H5) accounted for 70% of the total granule MPO, and thus contained the majority of typical large, high-density MPO-containing granules.

L-Gradient. A representative biochemical marker profile of granules separated on an L-gradient is shown in Fig 7. The five highest-density fractions (H1 through H5) described earlier cofractionated near the bottom of the L-gradient. Fractions L3 through L8 contained 75% of the total granule-associated LF and represented subfractions of the major specific granule band (S) previously identified using a continuous, self-generated gradient (see Fig 1). These fractions also accounted for 25% of the total granule MPO. The low-density granule fractions L1 and L2 contained 5% and 10% of the total granule MPO and LF, respectively, and corresponded to granules found in the density region between the membrane (M) and specific granule band (S) of Fig 1. In general, LF was found in

Fig 6. Biochemical marker protein profiles of high-density granule fractions separated on a typical H-gradient. Granules from 2 x 10⁷ neutrophils were separated on an H-gradient to yield five high-density fractions (H1 through H5), a membrane fraction (M), and a single low-density region in which the eight low-density fractions (L1 through L8) cofractionated (cf. Fig 3). The gradient was fractionated into 75 samples and each was assayed for the marker proteins MPO, β-GU, LZ, and LF. The amount of marker in each sample was expressed as a percentage of the total marker recovered in all samples, and these values were plotted as a function of the measured density of each sample. The total recovery of each marker was determined by expressing the sum of a particular marker measured in all samples of the gradient as a percentage of the known amount of that marker in the PNS that was loaded onto the gradient. Marker protein recoveries, expressed as the percentage recovered (mean ± SD), were MPO (103 ± 5, n = 15), β-GU (92 ± 7, n = 15), LZ (99 ± 6, n = 15), LF (93 ± 8, n = 9), and protein (96 ± 3, n = 4).
fractions L1 through L8 and H1 and H2, indicating that LF-containing granules spanned a major portion of the granule density spectrum (mean range, 1.046 to 1.108 g/cm³). On the other hand, MPO (measured enzymatically, spectrally, and immunologically), βGU, and LZ were found in all granule fractions, indicating that MPO-containing granules were localized across the entire granule density range (mean, 1.046 to 1.142 g/cm³). All four marker proteins were always observed at the top of each gradient (with the exception of recentrifuged fractions) in an area corresponding to the initial sample volume. The amounts of these proteins never accounted for more than 5% of the total recovered marker protein and probably reflected a small degree of granule damage, which occurred during the preparative procedure.

Equivalent amounts of protein from each isolated granule fraction were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). Quantitation of such staining patterns is often unreliable, particularly when dealing with a complex mixture of proteins with widely different properties. Nevertheless, the staining patterns were consistent with the quantitative biochemical data and showed MPO in all granule fractions but LF only in fractions L1 through L8 and H1 and H2. Interestingly, when the highest-density granule fraction, H5, was compared with the other four high-density fractions, it consistently appeared to be relatively deficient in MPO and elastase isoenzymes as judged by staining intensities. In addition, determination of cholesterol to total phospholipid ratios for all the granule fractions revealed a surprisingly wide range (0.62 to 1.40) and a bimodal distribution peaking in the regions of fractions L6 and H2 through H4.

It must be emphasized that varying degrees of granule heterogeneity were observed within all of the density fractions based on morphological and staining differences at the ultrastructural level (see below). For this reason, it was not possible to further extrapolate differences in the biochemical profiles between fractions to differences in the granule subpopulations themselves. However, this additional level of granule complexity was consistent with two of the biochemical observations: (1) the cofractionation of MPO-deficient–LF-containing granules with MPO-containing–LF-deficient granules in fractions L1 through L8 and H1 and H2 and (2) the finding by this laboratory and others that as much as 30% and 50% of the total granule MPO and βGU, respectively, were associated with the lower-density fractions. This observation is not merely a reflection of nonspecific adsorption of MPO to LF-containing granules, but is due to the presence of smaller, low-density peroxidase-containing granules.

**Electron Microscopy of Granule Fractions**

Characterization of the fractions was carried out at the ultrastructural level using morphological (uranyl acetate–lead citrate [UALC]), peroxidase (DAB), and complex glycoconjugate (PA-TCH-SP) staining as distinguishing criteria. In all preparations, each of the fractions contained abundant cytoplasmic granules, most of which demonstrated intact granule membranes. There were no observed eosinophil or basophil granules and only rarely were platelet alpha granules observed. Furthermore, there was minimal contamination (<1% of organelles) with mitochondria and plasma membranes.

**Morphology.** There was a wide range in the mean diameter of granules from the different isolated fractions. An example of this is illustrated in Fig 8. Approximately a two-fold difference in mean diameter was observed between the granules of fraction L1 and those of fraction H2. Figure 9 shows the results of a morphometric analysis of granules in each of the 13 isolated fractions. It can be seen that there was a generally progressive increase in mean diameter from the lowest-density fraction, L1, to the highest-density fraction, H5. Determination of mean granule diameters was obtained by measurement of the largest diameter of all membrane-bound structures (n = 200), which were consecutively scored in randomly selected photographs. To prevent observer bias, no attempt was made to exclude tangentially sectioned granule profiles; consequently, the diameters may be somewhat smaller than the actual mean diameters. Nevertheless, granules in fractions L1 and L2 (mean diameter, 0.14 μm) were clearly smaller than typical specific granules (fractions L3 through L8; mean diameter, 0.21 μm) or typical azurophil granules (fractions H3 through H5; mean diameter, 0.29 μm).

**Complex glycoconjugates.** The PA-TCH-SP procedure has been used previously with intact neutrophils to differentiate between specific and azurophil granules, which show intense and weak staining of the matrix, respectively. An example of this difference in staining intensity is shown in Fig 8. The PA-TCH-SP stain has also been used to distin-
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Fig 8. Ultrastructural demonstration of size and staining differences among isolated granule fractions. Granules were stained using a PA-TCH-SP procedure for vicinal glycol-containing carbohydrates. Granules from fraction L1 (panel A) had a mean diameter of 0.14 ± 0.05 μm, and granules from fraction H2 (panel B) had a mean diameter of 0.26 ± 0.07 μm. Note the intense staining of L1 granules compared with the weak staining of H2 granules. The arrow in (A) points to a larger granule that was trapped with fraction L1. Original magnification, 24,500×; bar equals 1 μm.

DISCUSSION

The present study describes a novel procedure for the rapid and reproducible isolation of 13 granule density fractions from normal human neutrophils of individual donors. Our preliminary studies showed that the broad spectrum of densities characteristic of these granules precluded optimal resolution when only one gradient was used. For this reason, we developed a high-resolution two-gradient system that significantly expanded the high- and low-density regions. The new system used a series of preformed density layers of isotonic Percoll in sucrose-free media. Under the conditions of centrifugation, these multiple-step layers rapidly (10 minutes) fused to produce an essentially linear gradient. This approach resulted in the isolation of eight granule fractions (L1 through L8) using the low-density L-gradient and five granule fractions (H1 through H5) using the high-density H-gradient (Fig 3).

Both the H- and L-gradients purposely used small discontinuities in the density profiles to achieve sharpening of individual granule fractions. Nevertheless, it is important to realize that this strategy did not generate artifactual banding

Fig 8. Ultrastructural demonstration of size and staining differences among isolated granule fractions. Granules were stained using a PA-TCH-SP procedure for vicinal glycol-containing carbohydrates. Granules from fraction L1 (panel A) had a mean diameter of 0.14 ± 0.05 μm, and granules from fraction H2 (panel B) had a mean diameter of 0.26 ± 0.07 μm. Note the intense staining of L1 granules compared with the weak staining of H2 granules. The arrow in (A) points to a larger granule that was trapped with fraction L1. Original magnification, 24,500×; bar equals 1 μm.

Fig 9. Relationship between mean density and mean diameter of isolated granule fractions. Mean diameters (open circles ± SD) were derived from morphometric analyses of 200 granules in each of the 13 granule fractions isolated using the H- and L-gradients. Mean densities were determined for granule fractions in 25 experiments (see Fig 3).

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profiles. Indeed, granule fractions were initially identified using a variety of continuous, self-generated gradients (see Figs 1 and 2), and a large number of mixing and recentrifugation experiments (Fig 4) demonstrated that the fractions were not artifacts of the centrifugation procedure or due to nonspecific aggregation. These experiments included recentrifugation of granule fractions isolated using multiple-step gradients on continuous, self-generated gradients and vice versa (Fig 5). Granule fractions were also shown to result from isopycnic equilibration rather than from a rate zonal process by demonstrating that flotation of granules upward during centrifugation produced the identical density patterns as when granules were loaded on top of the gradient.

Recently, Borregaard et al used Percoll as the density medium for fractionation of neutrophil granules and observed that the densities of the granules were significantly decreased compared with those isolated using hypertonic sucrose solutions. It was also shown that isolation of granules using nitrogen cavitation and isotonic Percoll in sucrose-free media yielded granules with intact membranes. This was based on the inability of exogenously added dithionite to reduce granule-associated MPO in the absence of detergent.9
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Fig 11. Quantification of ultrastructural cytochemical staining patterns typically associated with specific and azurophil granules. Isolated granule fractions from a typical experiment were PA-TCH-SP stained, and 100 granules from each fraction were scored for the percentages of the four PA-TCH-SP staining types as illustrated in Fig 10. The percentages of Types I + II (strong matrix staining, A) and Types III + IV (weak matrix staining, B) in each fraction were determined to give the total percentages of granules with staining patterns typical of specific or azurophil granules, respectively. As a positive stain for azurophil granules, isolated fractions from a typical experiment were stained with DAB for peroxidase reactivity, and 200 granules from each fraction were scored for the percentage of peroxidase positivity (C).

We have confirmed and extended these observations. We found that granules prepared from cells lysed in sucrose-free media using either nitrogen cavitation or the hypotonic lysis method gave the same two major granule fractions when separated according to Borregaard et al.9 and the same 13 granule fractions using our fractionation procedure (Fig 3). Thus, our fractions were not artifactually generated by the hypotonic cell lysis procedure. In addition, we demonstrated the intactness of isolated granule membranes using three criteria: (1) the MPO difference spectrum procedure of Borregaard et al.,9 (2) the latency (>95%) of three granule enzyme activities in the presence and absence of detergent, and (3) the observation of intact granule profiles in thin-sectioned electron micrographs. Taken together, our data strongly support the in vivo existence of substantial heterogeneity among neutrophil granules.

There is, in fact, a growing body of biochemical, cytochemical, and ultrastructural evidence that is consistent with a significant degree of granule complexity but which heretofore has not received widespread attention.10 Ultrastructural studies have demonstrated three morphologically distinct granules that form in successive stages.17,38-40 Peroxidase-positive azurophil granules were observed to form first (primary) during the promyelocyte stage of cytodifferentiation, while the specific granules formed later (secondary) during the myelocyte stage. It has also been reported that small lysosomal or “tertiary” granules, distinct from specific granules, are formed during the later stages of neutrophil development.10,16,17 More recently, it has become common to designate granules with properties different from either primary/azurophil or secondary/specific granules as tertiary granules, although the temporal sequence of their formation is uncertain.

Biochemical analyses of fractions isolated primarily by sucrose density gradient centrifugation have resolved two major granule types corresponding to the peroxidase-containing azurophil granules and peroxidase-deficient specific granules.3 On the other hand, using what are generally accepted as characteristic marker proteins, at least four classes of granules have been reported4-6,41,42: (1) azurophil granules (MPO, βGU), (2) specific granules (LF, vitamin B12-binding protein), (3) tertiary, or small lysosomal, C-particles (acid β-glycerophosphatase, N-acetyl-β-glucosaminidase), and (4) phosphomonoesters (alkaline phosphatase). Among these granule populations, only the azurophil granule fraction has been clearly shown to contain isolated subfractions, and several studies have demonstrated the existence of two MPO-containing subpopulations using sucrose density gradient centrifugation.4,5,14

Other fractionation studies and kinetics of the release of granule components have suggested further granule heterogeneity and illustrate that a major problem in defining granule subfractions has been the apparent lack of specificity of some of the marker proteins. For example, MPO and βGU are both commonly used as markers of high-density azurophil granules, but these markers have also been found in all previously isolated lower-density specific granule fractions.4,5 In addition, some fractionation studies indicate that MPO and a number of acid hydrolases exist in the same granule,6 whereas studies of the kinetics of release of these enzymes and several other fractionation studies indicate that MPO and βGU are in different granules.5,11,12 Also, gelatinase has been shown to be associated with a novel, as yet morphologically unidentified secretory unit.43 and a unique b-cytochrome.44 proposed as part of the oxidase system involved in the respiratory burst,45 was partially resolved from specific granules by rate zonal sedimentation.46

The resolution of a large number of granule fractions in the present study raises two fundamental questions. First, is this degree of heterogeneity completely at odds with the widely accepted two-granule model for neutrophils? And second, what is the biological significance of this heterogeneity? We believe that our results are consistent with many of the findings that support the two-granule model. Nevertheless, the isolation of a number of subfractions necessitates a reevaluation of the suitability of the two-granule paradigm for thinking about the origin and function of these cytoplasmic structures.

Using a single continuous, self-generated density gradient procedure, our granule-rich PNS was found to contain the two major granule populations (Fig 1, S and A) as reported by others.4-7,9 Typically, the azurophil granule region contained MPO but no LF, and the specific granule region contained LF and some MPO (Fig 2). The use of a two-gradient strategy (Fig 3) allowed further resolution of both...
the specific and the azurophil granule regions as well as resolution of additional fractions with densities between the membrane and specific granule regions and between the specific and azurophil granule regions (Figs 1 through 3). From the MPO-containing/LF-deficient azurophil granule region, three fractions were obtained (H3 through H5; Fig 6) that corresponded to relative enrichments of several large peroxidase-positive granule subtypes observed by others in their ultrastructurally heterogeneous azurophil granule fractions. From the specific granule region that was enriched in LF but still contained MPO, we resolved six fractions (L3 through L8; Fig 7). Fractions H1 and H2 were isolated from the density region between the specific and azurophil granule regions. The two lowest density fractions, L1 and L2, were derived from the density region between the membrane and the specific granule region. Hence, our data can be easily related to the fractionation of two major granule populations.

A number of observations in the present work do not fit a simple two-granule model, but are more consistent with the literature cited earlier that suggests greater heterogeneity of granules. The most obvious is the finding that peroxidase-positive granules were not solely restricted to the larger, high-density azurophil granule region, but rather spanned the entire range of size (mean diameter range, 0.14 to 0.32 μm) and density (mean density range, 1.046 to 1.142 g/cm³) of neutrophil granules (Fig 11). Specific granules have never been isolated completely free of MPO, and this has generally been attributed to contaminating azurophil granules or the adsorbed contents of damaged azurophil granules. In agreement with these previous results, our biochemical analyses of fractions L3 through L8 (which corresponded to the specific granule region) indicated that approximately 25% of the total granule MPO was associated with these fractions (Fig 6). Ultrastructural studies of these same fractions showed that, on the average, about 45% (range, 23% to 60%) of these granules demonstrated peroxidase reactivity (Fig 11). Thus, it is clear that neutrophils contain relatively large numbers of peroxidase-positive granules with sizes and densities similar to peroxidase-negative specific granules.

The distribution of peroxidase-reactive granules in the lighter-density fractions indicated that the typical correlation of this property with only high-density azurophil granules was not strictly applicable to the more complex array of isolated granule fractions. Likewise, the PA-TCH-SP staining procedure for complex glycoconjugates did not always distinguish between azurophil and specific granules. Typically, specific granules have been shown to exhibit intense matrix staining while that of azurophil granules is weak (Figs 8, 10, 11). Granules of fraction H5 were consistent with this staining pattern and exhibited >90% peroxidase positivity and >90% weak matrix PA-TCH-SP staining. In contrast, virtually all granules in fraction L1 exhibited the intense matrix PA-TCH-SP staining typical of specific granules (Fig 8), but 41% of the granules also exhibited peroxidase reactivity typical of azurophil granules (Fig 11). These data define a third major type of granule (strongly reactive for both peroxidase and complex glycoconjugates) and suggest that the terms azurophil and specific are not adequate to simply describe granules that do or do not contain peroxidase.

Similar to MPO, βGU has been considered as an azurophil granule marker, but significant amounts of this enzyme also have consistently been associated with the specific granule fraction (Fig 7). The present data do not allow us to distinguish whether βGU is colocalized with the smaller, low-density peroxidase-positive granules or whether it resides in peroxidase-negative granules, or perhaps both. However, the existence of this greater granule heterogeneity offers a possible explanation for the observations that MPO and βGU are colocalized in large, high-density azurophil granules but do not exhibit the same kinetics of secretion.

Of particular significance in the present work was the isolation and identification of two novel peroxidase-positive granule types that appear to provide further functional correlates to granule heterogeneity. Fractions L1 and L2 were found to contain a variety of small granules, which we have termed microgranules, with mean diameters (0.14 μm) and densities (1.046 to 1.053 g/cm³) considerably lower than neutrophil granules previously isolated. About 41% of these microgranules were peroxidase-positive (Fig 11), and based on their physical, biochemical, ultrastructural, and secretory properties, they represent a new class of DAB-reactive microgranules distinct from typical azurophil granules. In addition, further examination of fraction H5 revealed that it consisted of a subpopulation (>70%) of distinctively large granules that were enriched (about 50% of total protein) in a new class of microbial defensin polypeptides, but that were relatively deficient in the characteristic azurophil granule markers, MPO and elastase. Because defensins are biologically active under anaerobic conditions, these newly isolated granules may play a prominent role in nonoxidative host defense mechanisms of neutrophils. Studies are in progress to determine whether these two newly identified MPO-containing granule subtypes are related to the earlier observations of Kinkade et al regarding the differential localization and secretion of different forms of MPO.

A large proportion of peroxidase-negative granules were also observed in the region of lower size and density represented by fractions L1 through L8. Although about 85% of the total specific granule marker protein LF was found in these granule fractions (Fig 7), ultrastructural studies indicated considerable heterogeneity (Figs 8 through 11). We propose that this broad range of granules includes peroxidase-negative specific granules that typically contain LF and vitamin B₁₂-binding protein as well as peroxidase-negative granules that do not necessarily contain these specific granule marker proteins. These other granules may contain one or more of the recently described markers, such as gelatinase, histaminase, M01 glycophorin, or laminin receptors, which do not appear to be associated with typical specific granules. Furthermore, the elusive "tertiary" granule, or C-particle, which has been described as small relative to specific and azurophil granules and which contains acid hydrolases and glycosaminoglycans but not MPO, may also be localized to the peroxidase-negative granules among these lower-density fractions.

The demonstration of considerable complexity among
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neutrophil granules raises the question as to what factors contribute to the generation of this phenomenon. A number of lines of evidence indicate that heterogeneity exists among populations of normal human peripheral blood neutrophils. Presently, however, the functional implications of this observation for neutrophil biology are unclear. On the other hand, there is clear evidence that functional heterogeneity operates at the level of cytoplasmic granules. What remains to be determined is whether granule heterogeneity reflects different granule types within subsets of neutrophils, or whether there are functionally distinct subsets of granules within all neutrophils, or perhaps a combination of the two possibilities.

A second possible contributing factor to granule heterogeneity is the existence of granules at different stages of maturation. In ultrastructural studies using a cytochemical stain for complex glycoconjugates (PA-TCH-SP), Fittschen et al. identified at least four maturational forms of granules in directly sampled, intact neutrophils. Using this procedure, we observed three of PA-TCH-SP staining patterns in our isolated granule fractions (Fig 10). This observation suggests that developmental events may still be occurring in at least some of these cells and is consistent with the observation that morphologically mature bone marrow neutrophils are functionally immature compared with their peripheral blood counterparts. In this regard, it is interesting to note that the azurophil granules of undifferentiated HL-60 cells, which are predominantly immature promyelocytes, were found to exhibit a significantly lower density than typical azurophil granules from normal cells (Fig 3). Whether the decreased density of the azurophil granules from HL-60 cells reflects an abnormality associated with the leukemic state or if a lower density is characteristic of different granule types within subsets of neutrophils, or whether there is a clear need for more carefully defining which proteins should serve as selective markers for distinguishing particular granule subpopulations. We believe that our findings provide a new approach to the task of more definitively characterizing the nature of this heterogeneity and its functional correlates in normal neutrophils and in various pathological states, such as granulocytic leukemia. Also, the methodology developed for the resolution of granule heterogeneity in neutrophils may prove useful in identifying unrecognized complexity in other subcellular systems.

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High resolution of heterogeneity among human neutrophil granules: physical, biochemical, and ultrastructural properties of isolated fractions

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