Characterization of Canine Neutrophil Granules

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The purpose of this study was to isolate distinct populations of canine neutrophil granules and to compare them with neutrophil granules from other species. Size, shape, density, and content of canine neutrophil granules were determined. Neutrophils obtained by Ficoll-Hypaque sedimentation were homogenized, and granule populations were separated by isopycnic centrifugation on a linear sucrose gradient (p, 1.14 to 1.22 g/ml). The most dense granule population (p, 1.197 g/ml) contained all of the myeloperoxidase, β-glucuronidase, and elastase, more than half of the acid β-glycerophosphatase, and most of the lysozyme. The population with intermediate density (p, 1.179 g/ml) contained lactoferrin, vitamin B₁₂-binding protein, and the remainder of the acid β-glycerophosphatase and lysozyme. The least dense granule population did not contain a major peak of any of the enzymes or binding proteins tested but was distinguished by density and morphology. The size and shape of the granules were determined from scanning electron micrographs and assessment of shape was aided by transmission electron micrographs. By these methods three populations of canine neutrophil granules were characterized and named: myeloperoxidase granules, vitamin B₁₂-binding protein granules, and low-density granules.

In 1960, Cohn and Hirsch (12) reported studies which partially characterized rabbit neutrophil granules. Since then the granules of chicken (10), horse (15, 28), guinea pig (20), and human (8, 18, 19, 23, 26, 29, 30) neutrophils have been examined and separated into distinct classes. Interesting comparisons of granule morphology and content can be made, and numerous similarities exist among species.

Dogs have become important experimental animals for the study of neutrophil kinetics and function, but their granules have not been examined. The present investigation was performed to provide this information. By using the same techniques for isolation and assays for the same enzymes and binding proteins, we compared human and dog neutrophil granules. Rabbit neutrophil granules (3, 4) are also discussed.

MATERIALS AND METHODS

Isolation and separation of granule populations. Blood was obtained from normal dogs and anticoagulated with 2 ml of 5% disodium EDTA per 50 ml of blood. Neutrophils were separated by Ficoll-Hypaque sedimentation, and contaminating erythrocytes were then lysed by suspending the cells in 10 volumes of cold ammonium chloride solution (0.155 M NH₄Cl-0.01 M NaHCO₃-0.1 mM EDTA) for 10 min. Leukocytes obtained in this manner contained greater than 95% neutrophils.

Isolation and separation of granule populations. The neutrophils were suspended in 0.32 M sucrose and centrifuged at 200 × g for 10 min. The supernatant solution was discarded and the cells were suspended in 16 ml of 0.32 M sucrose. Portions (4 ml) were homogenized at 4°C in a smooth glass vessel by a VirTis no. 23 homogenizer (The VirTis Co., Inc., Gardiner, N.Y.). Homogenization was performed for 1 min at 4,700 rpm with four up and down strokes of a Teflon pestle (diameter, 13 mm). Six milliliters of 0.32 M sucrose was added to each portion before centrifugation at 400 × g for 10 min. The supernatant solutions were removed and centrifuged at 10,000 × g for 15 min. Granule pellets were gently suspended in 0.5 ml of 1.10-g/ml sucrose. The granule suspension was layered onto 4 ml of a linear sucrose gradient (p, 1.14 to 1.22 g/ml) and centrifuged in a swinging bucket rotor (SW39) of a Beckman Model L ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) for 2 h at 75,000 × g. The bottom of the tube was punctured, and the gradient was divided into approximately 50 fractions. The gradient fractions were diluted with 0.3 to 0.7 ml of distilled water before sonication for 15 sec at 35% of full power by a Fisher Sonic Dismembrator, Model 300 (Fisher Scientific Co., Pittsburgh, Pa.). The fractions were then frozen at −80°C but were never frozen and thawed more than once.

Enzyme and binding protein assays. Myeloperoxidase (peroxidase, EC 1.11.1.7) was assayed spectrophotometrically at 560 nm by using 0.2 ml of 3.2 mM O-dianisidine as hydrogen donor, 1.2 ml of 0.1 M citric acid–sodium citrate buffer (pH 5.5) 0.2 ml of 1 mM H₂O₂ and 0.2 ml of 0.5% (vol/vol) Triton X-100 (8). Horseradish peroxidase was used as a standard.

Acid β-glycerophosphatase (acid phosphatase, EC 3.1.3.2) was reacted with 1.8 ml of 0.052 M β-
glycerophosphate in 0.05 M acetate buffer (pH 5.0) (9). Released phosphorous was measured by the method of Chen (11). Potato acid phosphatase was used as a standard.

β-glucuronidase (β-D-glucuronidase, EC 3.2.1.31) activity was measured by incubation of the sample with 0.1 ml of 0.01 M phenolphthalein-glucuronic acid (pH 7.0) and 0.6 ml of 0.1 M acetate buffer (pH 4.6). Incubation at 37°C was stopped by the addition of 2 ml of 0.2 M glycine buffer (pH 10.4) in 0.2 M NaCl (9). Optical density was measured at 550 nm. β-glucuronidase from (bovine liver) was used as a standard.

Lysozyme (EC 3.2.1.17) was measured by the amount of lysis of Micrococcus lysodeikticus in 0.1 M phosphate buffer (pH 6.5) (13). Three milliliters of the suspension (initial optical density 0.7) was incubated for 15 minutes at 37°C, and the decrease in optical density was measured at 450 nm (24). Egg white lysozyme was used as a standard.

Vitamin B₁₂-binding protein was assessed by measuring the amount of 57cobalt-labeled cyanocobalamin bound to proteins in each of the gradient fractions (14). Hanks balanced salt solution without phenol red (0.9 ml), 0.1 ml of Triton X-100, and 2 μl of 57Co vitamin B₁₂ (10 μCi/ml) were added to the sample and incubated for 30 min at room temperature. A 100-ml volume of 5% Norit A decolorizing carbon was mixed with 100 ml of 1% albumin in distilled water, and 2 ml of the mixture was added to the sample. After 10 min, the samples were centrifuged at 2,200 x g for 20 min. Two milliliters of the supernatant solution were counted in a gamma counter and compared to known amounts of 57Co-labeled vitamin B₁₂ (17).

Elastase (EC 3.4.21.11) was measured by assessing the release of p-nitrophenol from tert-butyloxy carbonyl-l-alanine-p-nitrophenyl ester (29). The substrate solution in methanol was added to 0.05 M sodium phosphate buffer (pH 6.5) for a final concentration of 0.2 mM. The sample was incubated with 2.9 ml of the substrate solution at room temperature for 10 min, and the optical density was read at 347.5 nm (16). The standard used was porcine pancreas.

We were unable to utilize an immunological assay of lactoferrin since we were unsuccessful in isolating lactoferrin from canine milk. Instead, we developed a new method of quantifying lactoferrin, which took advantage of its iron-binding capacity. In this method, 1 μl of 59FeCl₃ (0.1 μCi ⁵⁹Fe, 10 ng ⁵⁹Fe), 2 μl of 1 mM nitritriacetic acid (5, 6), and 8 μl of 2 M phosphate buffer (pH 7.6) were added to 0.2 ml of each sample, which consisted of various concentrations of human lactoferrin. The tubes were incubated at 37°C in a
Vortex mixer and applied to Sephadex G-25 superfine columns (10 column, one 59Fe-lactoferrin, the sucrose was containing shaking waterbath for 3 h, and then 0.15 ml of 50% sucrose was added. The samples were blended in a Vortex mixer and applied to Sephadex G-25 superfine columns (10 ml volume, 22 cm in height) which had been equilibrated with 2 M phosphate buffer (pH 7.6) containing 1 mM nitrotriacetic acid. To determine the characteristics of the separation of unbound 59Fe from 59Fe-lactoferrin, the following controls were done. To one column, we added 59Fe and excess (250 µg) human lactoferrin. Almost all of the counts eluted before 6.4 ml and almost none after 6.4 ml. To another column, we added an equal amount of 59Fe without lactoferrin. With this column, most all of the counts eluted after 6.4 ml was collected. When an intermediate amount of lactoferrin was added to a column, two distinct peaks occurred; the first one at 3.5 ml was 59Fe-lactoferrin and a second at 7 ml consisted of unbound 59Fe. This procedure permitted the separation of those fractions containing 59Fe-lactoferrin from those containing unbound 59Fe. By using this method, a standard curve was constructed with unsaturated human lactoferrin. Since larger samples were required by this procedure, only 18 fractions instead of the usual 50 were obtained from gradients. After sonication, the fractions were treated as described above, and the predetermined volume of eluate containing the lactoferrin-bound 59Fe2+ was collected, counted, and compared to the standard curve.

Transmission electron microscopy. Visible bands of granules were removed from the sucrose gradients and slowly diluted with 4 ml of 0.32 M sucrose. The granule suspensions were ultracentrifuged at 20,000 × g for 20 min. The supernatant solutions were removed, and the pellets were fixed with 0.5 ml of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Transmission electron microscopy was performed with an RCA EMU 4 electron microscope.

Scanning electron microscopy. Granule bands were removed from the sucrose gradients and slowly diluted with 3 ml of 0.32 M sucrose. Epon plugs were placed in the bottom of ultracentrifuge tubes to provide a flat surface on which 5-µm pore size polycarbonate membranes (Nuclepore Corp., Pleasonton, Calif.) rested. The granule suspensions were then centrifuged at 20,000 × g for 20 min, which sedimented the granules onto the membrane. The filter containing the granules was fixed with 0.5 ml of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The granules were coated first with carbon and then with a mixture of gold and palladium (21). Scanning electron microscopy was performed in a Joel JSM-35-C electron microscope.

Statistics. The distributions of enzymes and binding proteins are compared in Fig. 1 through 6. Graphs were obtained by averaging the percentage of total enzyme or binding protein activity found in the same fraction of different gradients. Averaging reduces the variability sometimes found in an individual gradient. All enzymes or binding proteins compared in a figure were determined from the same gradients, but distribution of the same enzyme or binding protein shown in different figures were not always obtained from the same gradients.

The average densities of fractions were determined by:

\[
\text{average density} = 1.22 - \left(\frac{0.08}{50}\right) \times \text{fraction number} + 1/2 \left(\frac{0.08}{50}\right)
\]

where 1.22 is the most dense sucrose concentration, 0.08 the difference in density from top to bottom, and 50 is the number of fractions. Fraction 1 is from the most dense and fraction 50 from the least dense region of the gradient.

The size of granule populations was determined by measuring granules as seen on scanning electron micrographs. By measuring the granules from scanning electron micrographs it was unnecessary to correct for the sectioning error inherent in transmission electron micrograph measurements. The means of the length and width and the 95% confidence ellipses for the bivariate means were determined (25).

Reagents. Reagents were obtained from the following commercial sources: Ficoll (type 3), elastase (type 1), acid phosphatase (type 2), β-glycerophosphate (grade 1), ammonium molybdate solution (2.5%), horseradish peroxidase (type 6), O-dianisidine dihydrochloride, β-glucuronidase (Glucurase, bovine liver), and phenolphthalein glucuronic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.); 50% Hypaque was obtained from Winthrop Laboratories (New York, N.Y.); M. lysodeikticus and egg white lysozyme were obtained from Worthington Diagnostics (Freehold, N.J.); human lactoferrin was obtained from Calbiochem-Behring Corp. (La Jolla, Calif.); tert-butylxycarbonyl-L-alanine-p-nitrophenyl ester was obtained from Vega Biochemicals (Tucson,

![FIG. 5. Comparison of the density distributions of vitamin B12-binding protein, myeloperoxidase, and lysozyme from eight gradients. Mean ± SEM.](image)

![FIG. 6. Comparison of the density distributions of vitamin B12-binding protein, myeloperoxidase, and acid β-glycerophosphatase from six gradients. Mean ± SEM.](image)
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**TABLE**

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Type</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CANINE</td>
<td>Neutrophils</td>
<td>Human low density acid $\beta$-glycerophosphatase granules</td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>Content: 1) acid $\beta$-glycerophosphatase</td>
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<tr>
<td></td>
<td></td>
<td>2) vitamin $B_12$-binding protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human low density myeloperoxidase granules</td>
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<tr>
<td></td>
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<td>Size: $342 \times 289$ nm</td>
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<td></td>
<td>Density: 1.16 g/cm$^3$</td>
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<tr>
<td></td>
<td></td>
<td>2) lactoferrin</td>
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<tr>
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<td>3) free protein</td>
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<td>3) acid $\beta$-glycerophosphatase</td>
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<td>4) $\beta$-glucuronidase</td>
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**FIG. 7.** Comparison of the size, shape, density, and content of canine, human (submitted for publication), and rabbit neutrophil granules. Sizes of granules are in nm ± SEM.

**Ariz.**; Norit A decolorizing carbon (alkaline) was obtained from Fisher Scientific; $^{57}$Co cyanocobalamin (intermediate specific activity) and $^{59}$FeCl$_3$ were obtained from Amersham Corp. (Arlington Heights, Ill.).

**RESULTS**

**Description of the gradient.** After centrifugation, a narrow white band was seen near the top of the sucrose gradient. Electron micrographs revealed that this band did not consist of intact granules. Beneath this was a second but broader white band which was shown to contain granules by electron microscopic examination. At a density of 1.179 g/ml, a beige band was noted which contained vitamin $B_{12}$-binding protein granules (see below). The most dense band of canine neutrophil granules ($\rho$, 1.197 g/ml) was green and contained myeloperoxidase (see below).

**Enzyme and binding protein distributions.** Myeloperoxidase activity (Fig. 1) had an unimodal distribution and therefore was presumed to be located in a single population of granules. The distribution of $\beta$-glucuronidase was very similar to that of myeloperoxidase and their peak activities were present in the same fraction which suggested that both enzymes were contained in the same granules. Elastase (Fig. 2) was concentrated in the same part of the gradient as myeloperoxidase, and their peaks coincided. Elastase, myeloperoxidase, and $\beta$-glucuronidase, therefore, appeared to be contained in the same granule population. Vitamin $B_{12}$-binding protein activity (Fig. 3) was unimodal and distributed in a less dense region of the gradient clearly separated from the myeloperoxidase activity. This indicated that the vitamin $B_{12}$-binding protein was contained in a population of granules distinct from that which contained myeloperoxidase. The lack of separation of lactoferrin and vitamin $B_{12}$-binding protein (Fig. 4) suggested that the two binding proteins were contained in...
FIG. 9. Electron micrographs of low-density granules. (A) Scanning micrograph. Bar equals 0.5 μm (×55,440). (B) Transmission micrograph. Bar equals 0.5 μm (×36,720).

the same granules. The majority of the lysozyme (Fig. 5) occupied the same area of the gradient as myeloperoxidase, which suggested that lysozyme was contained in the myeloperoxidase granules. A shoulder of lysozyme activity extended to the right of the peak, which raised the possibility that lysozyme might also have been present in the vitamin B₁₂-binding protein granules. Acid β-glycerophosphatase (Fig. 6) followed a trimodal distribution with one peak of activity at fraction 15 in the area of the gradient which contained the myeloperoxidase granules, a second peak at fraction 26 in the area which contained vitamin B₁₂-binding protein granules, and a third small peak at fraction 46 in the area which contained nongranule cell components (as seen by electron microscopy).

Size of canine granules. The size of canine granule populations (nanometers ± standard error of the mean [SEM]) were: 258 ± 9.1 by 205 ± 3.4 for the low-density granules, 261 ± 5.5 by 223 ± 4.0 for the vitamin B₁₂-binding protein granules, and 432 ± 15.7 by 272 ± 8.7 for the myeloperoxidase granules (Fig. 7). The vitamin B₁₂-binding protein granules and low-density granules were small relative to the myeloperoxidase granules. In Fig. 8, the mean sizes of the three granule populations were plotted. The ellipses represented the 95% confidence regions of the bivariate means. The slope of the principal axis for each ellipse denoted the trend that would be shown by points plotted as a scattergram. The slopes of the principle axis were 12.000, 3.947, and 6.496 for the low-den-
ty, vitamin $B_{12}$-binding protein, and myeloperoxidase granules, respectively. The slopes indicated that as the length of the vitamin $B_{12}$-binding protein granules increased, the width also increased, but the width of the other two granule populations remained fairly constant as their lengths increased.

**Shape of canine granules.** The low-density granules (Fig. 9a and b) were predominantly oval in shape, and many had areas of decreased electron density. Rod- or dumbbell-shaped granules also could be seen.

Micrographs of vitamin $B_{12}$-binding protein granules (Fig. 10a and b) showed a population which seemed to be enriched in dumbbell-shaped granules (as seen in Fig. 10a), but some oval-shaped granules were present.

The myeloperoxidase granules (Fig. 11a and b) were clearly larger than the other granule types and were round or oval in shape.

**DISCUSSION**

Three procedures used in this study facilitated the isolation of granules and their separation into distinct populations. First, a shallow (1.14 to 1.22 g/ml) density gradient was used. Other studies have generally used steeper gradients (such as 1.15 to 1.30 g/ml). Second, a larger number of fractions was obtained from each gradient than in most previous reports. This modification was helpful in separating granule populations with very small density differences. Third, the use of a higher speed of homogenization (4,750 rpm) than most previous studies appears to have been important in accomplishing cell lysis with minimal granule damage. At
this higher speed, only a single 1-min homogenization was necessary to obtain a high yield of granules in good condition. Optimal homogenization conditions exist over a very narrow range of speeds—too slow and the neutrophils do not lyse, too fast and the granules are lysed or damaged. Granule damage can be detected as soon as the granule pellet is obtained. If the pellet cannot be easily suspended in the 1.10 g/ml sucrose solution, the granules have been damaged or irreversibly aggregated, making separation on the density gradient impossible.

The data presented show that at least three populations of canine neutrophil granules exist. We have named the least-dense population the low-density granules, because a characteristic enzyme or binding protein has not yet been identified. These granules are small and rod or oval shaped (Fig. 7). The vitamin B12-binding protein granules are intermediate in density, small in size, rod or oval in shape, and contain vitamin B12-binding protein, lactoferrin, and some of the acid β-glycerophosphatase and lysozyme. The myeloperoxidase granules are the largest and most dense granules, oval in shape, and contain myeloperoxidase, β-glucuronidase, elastase, as well as most of the lysozyme and acid β-glycerophosphatase.

We have named the three granule populations by a characteristic marker enzyme or binding protein or by their density. This system of nomenclature is the same as that proposed for human neutrophil granules (submitted for publication). The markers proposed for two of the three populations of dog neutrophil granules (vitamin B12-binding protein and myeloperoxidase...
dase) are also markers for two of the four populations of human granules. Comparison of dog neutrophil granules with human and rabbit (2, 3, 4, 31, 32) granules reveals many similarities (Fig. 7). To facilitate the discussion, we have created three categories of granules based on their size, density, and content (Fig. 7). More than one population of granules may be found within a category, but this scheme helps compare a distinct granule population of one species to more than one distinct population of another species, e.g., canine myeloperoxidase granules are similar to both human low-density myeloperoxidase granules and high-density myeloperoxidase granules (all of which are in category I, Fig. 7).

Since our method of resolving one canine granule population from another depends on differences in density, different granule populations of the same or similar density cannot be separated. For example, if the canine granules which contain myeloperoxidase were actually made up of two populations (as in human neutrophils) but were of similar density, they would appear to be one granule population upon analysis of their biological activities.

The category I granule populations of dog, rabbit, and human neutrophils (Fig. 7) all contain myeloperoxidase (3, 4). The category I granules of each species also contain lysozyme, acid β-glycerophosphatase, elastase, and β-glucuronidase; however, in human neutrophils the lysozyme and acid β-glycerophosphatase are found in the high-density myeloperoxidase granules and not in the low-density myeloperoxidase granules. The category I granules are also the most dense and the largest granules of each species (4). Another similarity is that the category I granules of each species are circular or oval in shape (3, 4).

The four enzymes or binding proteins found in category II granules are vitamin B₁₂-binding protein, lactoferrin, lysozyme, and acid β-glycerophosphatase. All four of these proteins are found in canine vitamin B₁₂-binding protein granule (Fig. 7). In human (manuscript submitted) and rabbit (2–4) neutrophils, however, lysozyme and lactoferrin appear to be compartmentalized into a slightly more dense population of granules, and acid β-glycerophosphatase is contained in a slightly less dense population, namely the low-density acid β-glycerophosphatase granules in the human and tertiary granules of rabbits (4, 31, 32). The precise location of vitamin B₁₂-binding protein is difficult to determine, but it appears most likely that it is contained in the low-density acid β-glycerophosphatase granules. It has not been determined whether dog vitamin B₁₂-binding protein granules contain more than one distinct granule type of the same density. Electron micrographs of the canine vitamin B₁₂-binding protein granules (Fig. 10) show two types of granule morphologies, but this may be due to contamination with low-density granules. Other similarities among category II granules are that they are all less dense than the category I granules (4), generally rod or oval in shape (3, 4), and small in size relative to the myeloperoxidase-containing granules (4).

The category III granules found only in dog neutrophils are difficult to characterize since we have not identified its enzyme or binding protein content; however, the size, shape, and density make them similar to category II granules. Analysis of individual gradients revealed that minor peaks or shoulders of lactoferrin and vitamin B₁₂-binding protein were present in the area of the gradient containing low-density granules. It is not known if this was due to contaminating vitamin B₁₂-binding protein granules or the presence of the binding proteins in low-density granules.

The similarities in the character of human, dog, and rabbit neutrophil granules suggest that the nature of granules was determined early in mammalian evolution and that this pattern has been highly conserved. Since the contents of the dog, human, and rabbit neutrophil granules are similar, it appears likely that the compartmentalization of enzymes and binding proteins into discrete packages (granules) is important for cell function. This compartmentalization allows different enzymes and binding proteins to be released separately or at different rates either into the phagosome or extracellularly (7, 27). Selective release of granule contents is important because they have different functions, e.g., lactoferrin participates in the regulation of granulopoiesis (1), whereas a major function of myeloperoxidase is to facilitate the aerobic killing of microorganisms (22).

We have named the three canine neutrophil granule populations vitamin B₁₂-binding protein granules, low-density granules, and myeloperoxidase granules. This is consistent with our system of nomenclature for humans. Further work is needed to determine if more than three granule populations exist. In addition, we have compared dog, human, and rabbit neutrophils and have found extensive similarities in both morphology and content.

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LITERATURE CITED


