BRIEF REPORT

Ultrastructural Localization of Myeloperoxidase in Human Neutrophil and Rabbit Heterophil and Eosinophil Leukocytes

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Rabbit heterophil leukocytes contain three distinctive types of granules, which have recently been designated primary, secondary, and tertiary granules according to their chronological appearance during cell development. The primary and secondary granules were conventionally designated azurophil and specific granules, respectively. Primary granules are present exclusively in early heterophils (promyelocytes); primary and secondary granules are present in intermediate heterophils (myelocytes, metamyelocytes, and bands); and tertiary granules are added to the primary and secondary granule population in late heterophils (fully mature segmented forms). These granule types differ not only in the time of their appearance during cell development, but also in their composition as revealed by cytochemical methods. Thus, primary granules contain acid phosphatase, acid mucosubstance, and strongly basic protein; secondary granules contain alkaline phosphatase; and tertiary granules contain acid phosphatase and acid mucosubstance. The morphologic heterogeneity of granules in human neutrophils has been established, and correlation of granule types in human with those in the rabbit leukocytes has been suggested. However, human neutrophil granules have not been classified on the basis of their cytochemical properties and their chronologic appearance during leukopoiesis as in the rabbit heterophil, and correspondence between granule types in the two species has not been established on these criteria.

Strong myeloperoxidase activity is demonstrable at the light microscope level in neutrophil and eosinophil granulocytes and monocytes. An ultrastructural method now available for visualizing peroxidase activity was utilized in the present study to determine which type of granule contains this enzyme in rabbit heterophils and human neutrophils. The results of this investigation reveal that peroxidase activity is present in primary granules of...
Fig. 1.—Rabbit heterophil leukocyte in an intermediate (metamyelocyte) stage of development. Peroxidase preparation of bone marrow. Granular deposits indicative of peroxidase activity fill the relatively large primary granules (PG), imparting electron opacity selectively to these granules. The smaller granules containing material of low density are identified morphologically as secondary granules (SG). The nucleus (N), cytoplasm, and secondary granules lack evidence of peroxidase activity. × 23,500.

polymorphonuclear neutrophil leukocytes of both species and that the presence of this enzyme activity can therefore be used as a marker for identification of primary granules in human neutrophils.

METHODS

Bone marrow and buffy coat specimens obtained from healthy, male rabbits and human subjects (medical students) were fixed at 4 C. for 4 hours in a solution of 10 per cent formalin and 2 per cent calcium acetate or at 25 C. for 5 hours in a solution of 4 per cent paraformaldehyde and 5 per cent glutaraldehyde. Essentially similar results were obtained with either fixative.

Sections 40 μ thick, prepared with a Smith-Farquhar tissue sectioner, were rinsed briefly in cold 7 per cent sucrose and incubated 3–10 minutes at 25 C. in a saturated solution of 3,3′-diaminobenzidine (free base) (K and K Laboratories, Plainview, N. Y.) in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.01 per cent H2O2. Sections from the same block were incubated comparably in 0.05 M Tris-HCl buffer containing 0.01 per cent H2O2 as controls. Following incubation in substrate, sections were post-fixed 1 hour in 2 per cent O3O4 adjusted to pH 7.4 with s-collidine buffer, dehydrated by routine procedures, and embedded in Epon. Thin sections were examined in an AEI-6B electron microscope with and without lead citrate staining.

Note: Cells illustrated in Figs. 1, 2, and 4–8, are from specimens fixed with the paraformaldehyde-glutaraldehyde solutions. Cells shown in Figs. 3 and 9 are from specimens fixed with calcium acetate formalin. The thin sections micrographed for Figs. 1–3 and 9 were unstained. The thin sections micrographed for Figs. 4–8 were stained with lead citrate.
Fig. 2.—Intermediate (metamyelocyte) stage rabbit heterophil. Buffer control preparation of bone marrow. Some of the primary granules (PG) contain moderately dense material which is easily distinguished from the more dense and granular deposits of reaction product (Cf. Fig. 1.). The nucleus (N), cytoplasm, and secondary granules (SG) possess the same density in this control as in the peroxidase preparation. × 25,000.

RESULTS

Rabbit Heterophils

Electron-opaque material indicative of myeloperoxidase activity was observed in granules of all heterophils of rabbit bone marrow and buffy coat. These peroxidase positive granules can be identified as primary granules, because they appeared in the same proportions at the same stages of cell development and they possessed the same morphologic features as rabbit primary heterophil granules described in a previous study.1 Thus, the newly formed primary granules, which comprise the exclusive granule type in early heterophils, the somewhat older primary granules, which comprise about one-half of the granule population in intermediate heterophils (Fig. 1), and the still older primary granules, which constitute a minority of the granule population in mature heterophils (Fig. 3) exhibited myeloperoxidase activity. However, in some mature cells (Fig. 3), not all primary granules revealed reactivity; some primary granules appeared partially or completely extracted. Secondary granules and other cytoplasmic organelles lacked reaction product indicative of myeloperoxidase activity (Figs. 1,3), and tertiary granules with reaction product were not observed.

In control preparations (Fig. 2), some of the primary granules possessed moderately dense material, but it was readily distinguished from the more dense and granular deposits of reaction product. Many of the primary granules in the buffer control preparations also appeared extracted to a variable extent.
Fig. 3.—Mature (segmented) rabbit heterophil. Peroxidase preparation of buffy coat. The presence of five nuclear lobes consisting largely of heterochromatin and the paucity of mitochondria and ribosomes in this profile identify this heterophil as a mature cell. The peroxidase positive primary granules (PG) are less numerous in mature heterophils than in early and intermediate heterophils. (Cf. Fig. 1.) Peroxidase negative secondary granules (SG) are more numerous than primary granules. × 17,300.

**Human Neutrophils**

Early neutrophils of human bone marrow were identified by the large size of the nucleus and nucleolus, the dispersion of chromatin, and the abundance of cytoplasmic ergastoplasm. Profiles of such early neutrophils often exhibited dense, granular deposits indicative of peroxidase activity in all of the numerous granules (Fig. 4). The great majority of the granules were roughly spherical and were filled with evenly distributed reaction product. However, a small minority of the granules in early neutrophils appeared elongated and revealed a cleft that was devoid of reaction product. This cleft was usually located near the center of the granule and was oriented along its longitudinal axis. This area, devoid of peroxidase, apparently corresponds with a low density area observed in morphologically similar granules of control preparations (Fig. 6). Neutrophils at an intermediate stage of development in human bone marrow revealed numerous strongly reactive granules. Nearly all of the peroxidase reactive granules in intermediate neutrophils (Fig. 5) resembled the elongated granules with the unreactive cleft—that granule type which comprised only a small percentage of the granules in the early neutrophil. In addition to the peroxidase positive granule, the intermediate stage neutrophil contained approximately an equal number of larger granules that showed no evidence of peroxidase activity. Mature neutrophils (Fig. 7) revealed fewer peroxidase reactive granules and more peroxidase unreactive granules compared with earlier cells.

Control sections (Fig. 6), which were incubated with a buffer solution
Fig. 4.—Human neutrophil at an early (promyelocyte) stage of development. Peroxidase preparation of bone marrow. The high nuclear/cytoplasmic ratio, dispersed chromatin, large nucleolus, and abundant rough endoplasmic reticulum identify this as an early developmental form. All cytoplasmic granules contain dense deposits indicative of peroxidase activity and are considered the counterpart of the primary granules in rabbit heterophils. Except for two elongated granules with an unreactive space (arrows), the granules appear more or less rounded and filled throughout with reaction product. × 6,400.

Fig. 5.—Intermediate (myelocyte) stage of human neutrophil. Peroxidase preparation of bone marrow. The reactive primary granules (PG) generally appear elongated and reveal an unreactive cleft. Somewhat larger, rounded granules (SG) with moderately dense contents, lack peroxidase and are considered comparable to secondary granules of rabbit heterophils. Mitochondria (M) and Golgi elements (G) also lack evidence of peroxidase. The prominent Golgi elements presumably are involved in biosynthesis of secondary granules, which were not present at the early stage of cell development. (Cf. Fig. 4). × 18,000.
Fig. 6.—Intermediate (metamyelocyte) stage human neutrophil. Buffer control preparation of bone marrow. Two types of granules are evident. The relatively small type (PG) with a central area of low density apparently corresponds with the peroxidase positive primary granule. The other larger, more rounded type (SG) with a homogenous content apparently corresponds with the peroxidase negative secondary granule. × 18,800.

lacking the peroxidase substrate, exhibited no dense deposits comparable to those developed in the presence of substrate. Intermediate stage neutrophils (Fig. 6) in such control preparations revealed two granule types, one with an empty central cleft, apparently corresponding with the peroxidase posi-

Fig. 7.—Mature (segmented) human neutrophil. Peroxidase preparation of buffy coat. The segmented nucleus consisting largely of condensed chromatin and the small number of mitochondria and ribosomes in this profile identify this neutrophil as a mature cell. The peroxidase positive primary granules (PG) are less numerous in mature neutrophils than in early and intermediate neutrophils. (Cf. Figs. 4 and 5.) Peroxidase negative secondary granules (SG) are more numerous than the primary granules. × 18,800.
Monocyte from human bone marrow. Peroxidase preparation. The cytoplasm exhibits a single peroxidase reactive type of granule that is often elongated. Empty appearing vacuoles representing dilated elements of smooth endoplasmic reticulum and Golgi associated vesicles are characteristically prominent in these cells. $\times$ 18,800.

tive granule, and the other similar morphologically to the peroxidase un-reactive granule.

**Human Monocytes**

Monocytes in human bone marrow contained a uniform population of granules with strong peroxidase reactivity (Fig. 8). These granules appeared more elongated than neutrophil primary granules.

**Rabbit Eosinophils**

In confirmation of Bainton and Farquhar’s observations,\(^1\)\(^9\) granules and rough endoplasmic reticulum in rabbit eosinophils showed myeloperoxidase activity (Fig. 9). However, two distinct types of eosinophil granules as described by these investigators\(^1\)\(^9\) were not observed in this study, but rather developmental stages of a single type of granule, as previously reported.\(^1\)\(^2\)

Both the spherical, immature eosinophil granule and the angulated, mature granule—which apparently forms as a result of development of the crystalloid—revealed deposits indicative of peroxidase activity. The center of the spherical, immature granule generally appeared somewhat less reactive than the periphery in these briefly incubated sections.

**Discussion**

Light\(^3\) and electron microscopic\(^4\) cytochemical studies have visualized sulfated acid muscosubstances in newly formed primary granules of early rabbit heterophils. However, late rabbit heterophils reveal very few stained granules apparently because the primary granule population becomes diluted with unreactive secondary granules during cell development and the stainability of primary granules diminishes with their maturation. Light microscope cytochemical studies\(^1\)\(^1\) have indicated that a similar situation prevails in human neutrophils and therefore suggest the existence of a granule type
Fig. 9.—Rabbit eosinophil leukocyte in an early stage of development. Peroxidase preparation of bone marrow. Dense granular deposits indicative of peroxidase are present in the spherical granules which precede the angulated form in the development of the eosinophil granule. The deposits resemble those of heterophil granules in opacity to the electron beam. Reaction product is present in most of the granules in this cell profile and appears heavier peripherally. Rough endoplasmic reticulum in the cytoplasm and nuclear envelope reveal heavy deposits (arrows). $\times$ 27,000.

in these cells which corresponds with the primary granule of rabbit heterophils in regard to its sulfated mucosaccharide.

The present study provides evidence for the existence of a granule type in human neutrophils that corresponds with the primary granule of rabbit heterophils in regard to its peroxidase reactivity. A previous ultrastructural study\textsuperscript{12} suggested that peroxidase was localized in specific (secondary) granules of human neutrophils. Two lines of evidence are presented here supporting the concept that the peroxidase reactivity is located in primary granules of human neutrophils. 1) The most direct evidence is that peroxidase activity was present only in the granule type formed exclusively in early human neutrophils (promyelocytes). Since primary granules are by definition the exclusive granule type formed in promyelocytes, the peroxidase positive granules are then primary granules. 2) Rabbit heterophils contain peroxidase reactive granules that can be definitely identified as primary granules, because they are formed exclusively in promyelocytes and they possess the ultrastructural features of rabbit primary granules described in previous studies.\textsuperscript{1,13} Assuming that a specific enzyme occurs in the same granule type in leukocytes of both rabbit and man, peroxidase activity should then be present in primary granules of human neutrophils.

Peroxidase activity can therefore be used as a marker for the identification of primary granules in human intermediate and late neutrophils, in which distinction between the granule types on a morphological basis is difficult.

Despite the cytochemical and developmental evidence for equating the peroxidase reactive granule with the human primary (azurophil) granule,
doubt might exist about such a conclusion from the common light microscope experience that segmented polymorphonuclear leukocytes in peripheral blood smears exhibit strong peroxidase reactivity but show very few azure-stained granules. This apparent discrepancy can be explained by the observation that azurophil staining (due to the presence of acid mucosubstances) of human primary granules progressively diminishes during maturation, whereas peroxidase reactivity of these granules is retained in mature circulating neutrophils. Although primary granules are not detectable in mature neutrophils of blood smears stained with basic dyes, presumably because of their masked basophilia or azurophilia, electron micrographs of such cells show the primary granules to comprise about one-third of the total granule population. Therefore, the intensity of azurophil staining cannot be equated with the number of primary granules in neutrophils. Since primary granules comprise a significant proportion of the total granule population in mature neutrophils, they could account for the strong peroxidase reactivity observed in peripheral blood smear preparations.

In rabbit eosinophil leukocytes, peroxidase reactivity appeared more prominent in the rough endoplasmic reticulum and perinuclear space than in the cytoplasmic granules, possibly as a result of dilution of enzyme or inaccessibility of enzyme to the substrate in the granules. However, eosinophils observed in human bone marrow in this study showed peroxidase activity in the granules but not in the rough endoplasmic reticulum or perinuclear space. In contradistinction to the postulate of Miller, et al., the crystalloids in granules of mature rabbit eosinophils did not reveal peroxidase activity, indicating that this enzyme does not account for the crystalline material within the granules. The reactivity in eosinophil and monocyte granules encountered in these studies serves to validate the ultrastructural technic, since these are the other cells in bone marrow known to contain this enzyme.

**Summary**

Ultrastructural cytochemical observations revealed peroxidase reactivity in primary (azurophil) granules, but not in secondary (specific) granules, of rabbit and human polymorphonuclear leukocytes. Peroxidase reactivity was also observed in the rough endoplasmic reticulum and granules of rabbit eosinophils and in granules of human monocytes.

**SUMMARIO IN INTERLINGUA**

Cytochemic observationes ultrastructural revelava reactivitate peroxydasic in granulos primari (azurophilic) sed non in granulos secundari (specific) de leucocytos polymorphonucleari de conilios e humanos. Reactivitate peroxydasic esseva etiam observate in le crude reticulo endoplasmatic e le granulos de eosinophilos ab conilios e in le granulos de monocytes ab humanos.

**REFERENCES**


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