Histochemistry (1986) 84:445–453

Histochemistry

Ultrastructural localization of peroxidase activity in developing neutrophil granulocytes from human bone marrow

P. Brederoo, J. van der Meulen, and W.Th. Daems
Laboratory for Electron Microscopy, University of Leiden, Rijnsburgerweg 10, NL-2333 AA Leiden, The Netherlands

Accepted October 29, 1985

Summary. Developing neutrophil granulocytes of normal human bone marrow were investigated with the diaminobenzidine technique to determine the ultrastructural localization of peroxidase activity. Neutrophil granulocytes have three types of granule: nucleated, azurophil, and specific granules. These granules are produced consecutively during the eomyelocyte stage, the promyelocyte stage, and the myelocyte stage, respectively.

The organelles involved in the production of granules, i.e., the nuclear envelope, rough endoplasmic reticulum, and Golgi apparatus, are peroxidase positive during the eomyelocyte and promyelocyte stages and peroxidase negative thereafter. This pattern differs for the granules themselves: nucleated granules are negative in the eomyelocyte and become positive in the promyelocyte. Azurophil granules become positive in the promyelocyte. Specific granules are negative.

Our observations highly suggest that small Golgi-derived peroxidase-positive vesicles are involved in the maturation of both nucleated granules and azurophil granules.

Introduction

A considerable number of reports have been published concerning the ultrastructure of developing neutrophil (heterophil) granulocytes in the bone marrow of mammalian species (Bainton and Farquhar 1966, 1968; Wetzel et al. 1967; Ackerman 1968; Dunn et al. 1968; Breton-Gorius and Guichard 1969; Breton-Gorius 1970; Scott and Horn 1970; Bainton et al. 1971; Ackerman and Clark 1971; Ackerman 1971a, b; Bentfeld et al. 1977). These investigations dealing mainly with the heterogeneity of the granule population, concerned either the morphological or the cytochemical aspects of granulopoiesis. Most of the cytochemical studies have been focused on the localization of peroxidase activity. Both aspects have only been investigated extensively in two mammalian species, the rabbit and man.

However, despite the numerous studies there is still no agreement on the total number of distinct types of granule occurring in neutrophil granulocytes of mammalian species: reports on morphological as well as peroxidase-cytochemical studies reflect the diversity of opinions about the distinct types of granule and the cell stage in which they are formed (for references on human neutrophil granulocytes, see Brederoo et al. 1983, Table 3).

Since it is difficult to distinguish the various types of granule occurring in the neutrophil granulocytes of most mammalian species, and specifically those of man, solely on the basis of morphological criteria, the finding of two types of granule in rabbit heterophils, one of them peroxidase positive (azurophil granules) and the other peroxidase negative (specific granules), led Bainton and co-workers (Bainton et al. 1971; Farquhar and Bainton 1972; see also Bainton et al. 1976) to use peroxidase cytochemistry to distinguish between granules in neutrophils of mammalian species other than the rabbit. However, this method obscured the status of a granule characterized morphologically by the presence of a nucleoid and reacting positively under incubation for peroxidase (Bainton et al. 1971; Breton-Gorius and Reyes 1976; Brederoo and Daems 1977). It should be kept in mind here that our morphological studies on bone marrow have shown that such granules, which we call nucleated granules, differ from azurophil and specific granules, at least in the guinea pig (Brederoo and Daems 1978), the rat (Brederoo and van der Meulen 1983), and man (Brederoo et al. 1983).

In the present study an attempt was made to extend our morphological findings by applying cytochemical procedures to normal human bone marrow cells to establish the localization of peroxidase activity.

Materials and methods

Bone marrow

Pieces of sternal bone obtained from hematologically healthy adults during cardiothoracic surgery were washed in phosphate-buffered Ringer solution. Cell suspensions were then centrifuged.

Prefixation

The bone-marrow cells were prefixed by resuspending the pellets in 1.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3; 370 mOsm) for 2 or 10 min at room temperature, after which the cells were washed twice by repeated suspension in phosphate-buffered Ringer solution for 5 min.

Incubation

Prefixed cells were incubated (60 min in the dark at room temperature) in a medium containing 20 mg diaminobenzidine-4HCl and

In honour of Prof. P. van Duijn

0.003% H_2O_2 per 10 ml Ringer solution with 0.025 *M Tris* (hydroxymethyl)-amino-methane (pH 7.3) (Roels et al. 1975). For the controls, hydrogen peroxide was omitted. The medium was refreshed after the first 30 min of incubation.

Postfixation

Next, the cells were pelleted, washed three times by resuspension in phosphate-buffered Ringer solution, and postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.3; 330 mOsm) for 30 min at 4° C (Millonig 1962).

To enhance the morphology, cells prefixed for 2 min were fixed in 1.5% glutaraldehyde in cacodylate buffer and washed before being postfixed.

Embedding, microscopy

Osmium tetroxide-fixed cells were embedded in Epon after pelleting in 2% Bacto-agar and dehydration in a graded series of ethanol dilutions. Ultrathin sections stained with lead hydroxide were examined in a Philips EM 410 electron microscope at 80 kV.

Results

Terminology

On the basis of our finding that three distinct types of granule appear during the development of the neutrophil granulocyte in human bone marrow, the following terminology will be used. The earliest recognizable cell of the neutrophil series is called the *eomyelocyte*. In this cell stage, *nucleated granules*, in the mature state characterized by a nucleoid, are present. *Azurophil granules* are formed in *promyelocytes*, and the formation of *specific granules* starts in the myelocyte stage (for details on the maturation of both cells and granules, the reader is referred to Brederoo et al. 1983).

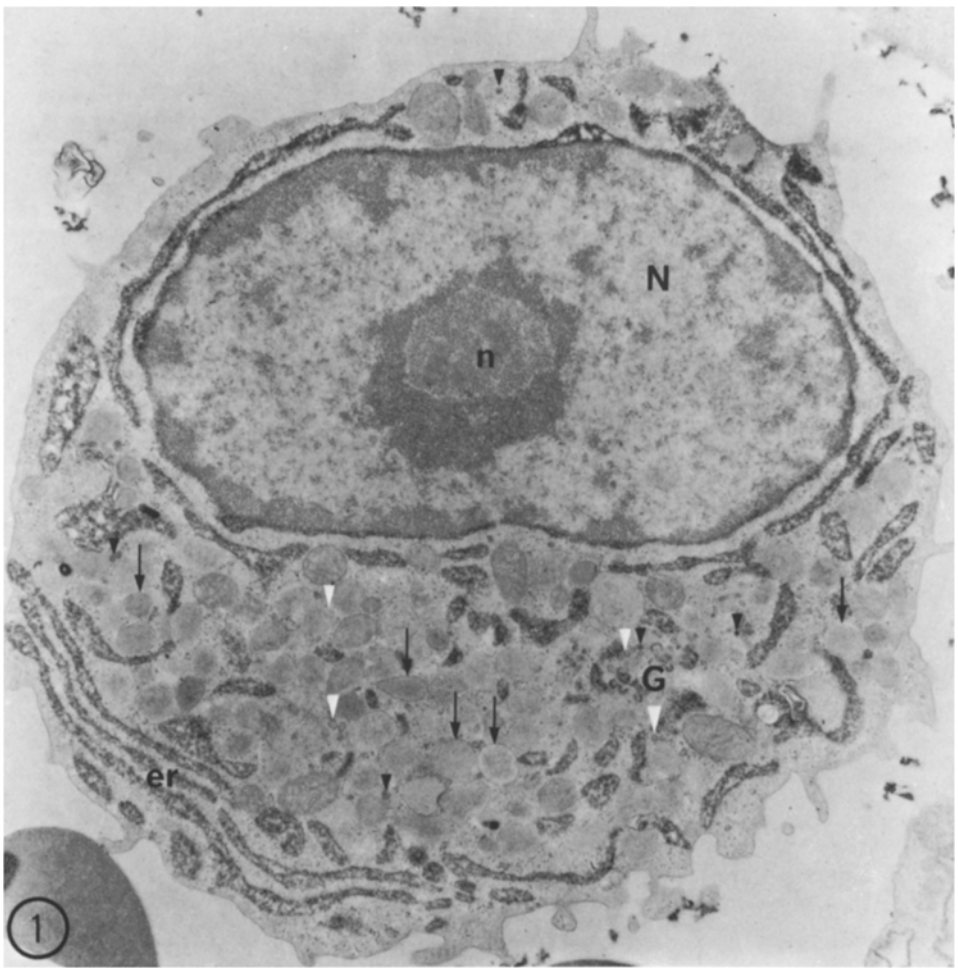
Cytochemistry (see Table 1)

The eomyelocyte. Peroxidase activity can be seen in the nuclear envelope and in the cisternae of the rough endoplasmic reticulum. The Golgi area is small and Golgi stacks consist of short, slender cisternae surrounded by small vesicles.

Table 1. Peroxidase activity of granules and organelles in the various neutrophil cell stages

Cell stage	Eomyelocyte	Promyelocyte	Myelocyte	Mature
NE/RER	+	+	−	−
Golgi	+	+	−	−
Vesicles	+/−	+	+	+
Nucleated gr	−	→+	+	+
Azurophil gr	np	→+	+	+
Specific gr	np	np	−	−

NE: nuclear envelope; RER: rough endoplasmic reticulum; gr: granule; +: positive; −: negative; →+: becoming positive; np: not present



Figs. 1–10. *N:* nucleus; *n:* nucleolus; *er:* endoplasmic reticulum; *G:* Golgi area; *c:* centriole

Fig. 1. Eomyelocyte with immature nucleated granules throughout the cytoplasm (*arrows*), a small Golgi area, and a number of cisternae of rough endoplasmic reticulum. The nuclear envelope and the cisternae of the endoplasmic reticulum are positive. A few of the vesicles in the Golgi area and the cytoplasm are positive (*arrowheads*) or negative (*white arrowheads*). Note that the granules are all negative. ×12,000

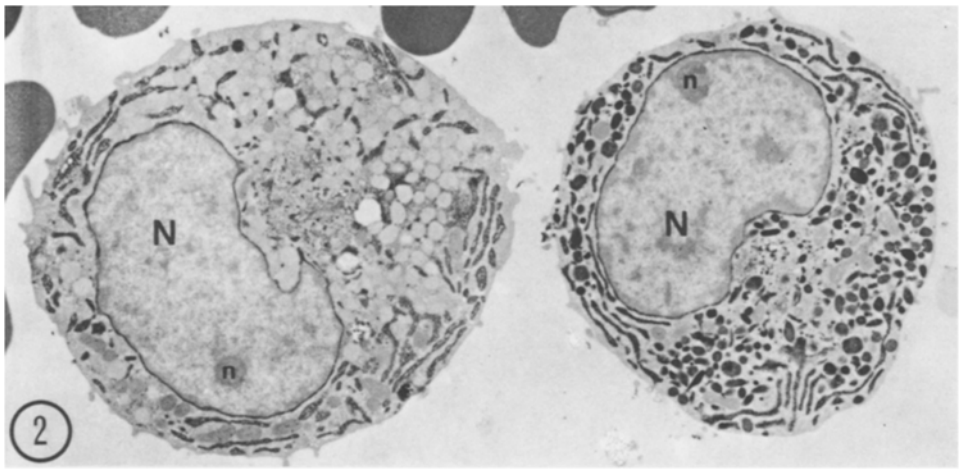
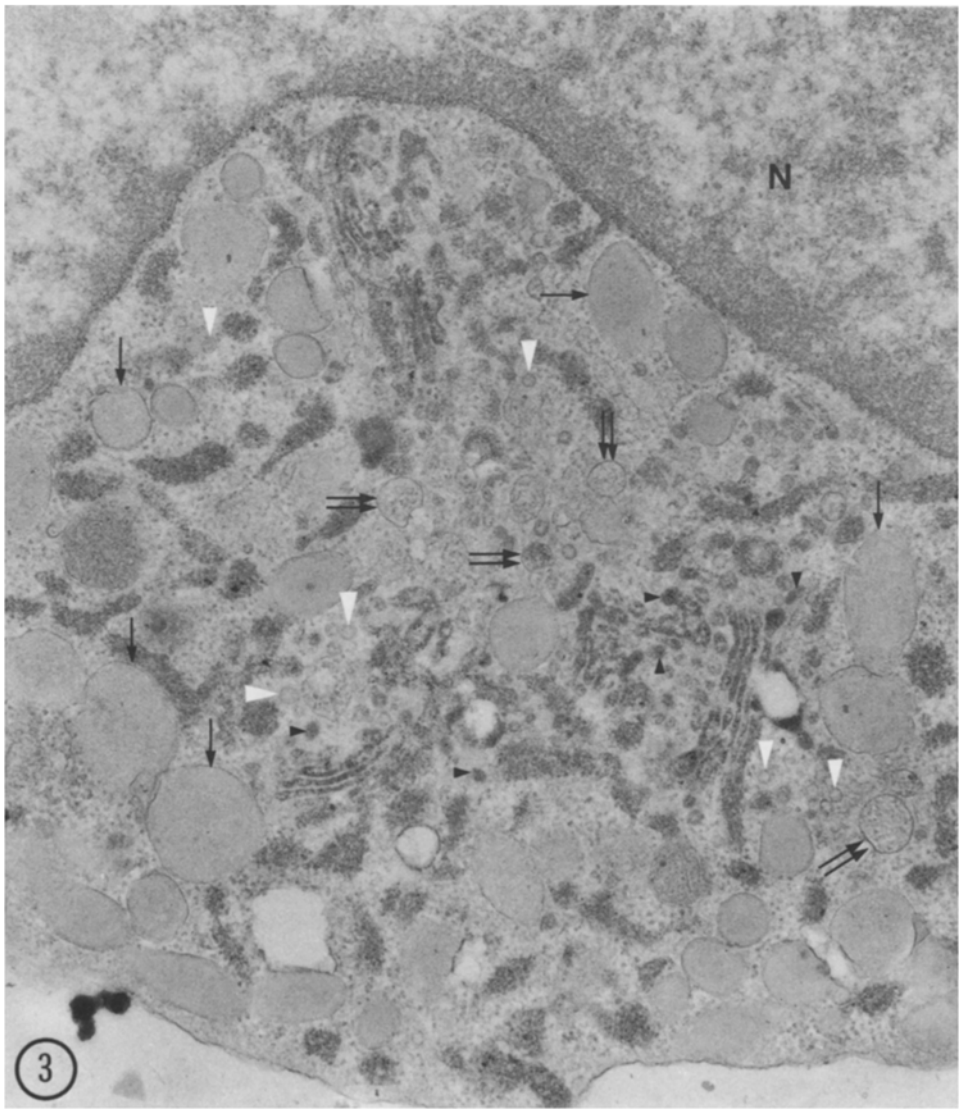


Fig. 2. Differences in staining pattern of two promyelocytes. The nuclear envelope and cisternae of the Golgi apparatus and the endoplasmic reticulum are positive in both. The cell on the left represents the beginning of the promyelocyte stage and has negative granules. The cell on the right is almost at the end of the promyelocyte stage. In this stage of maturation all granules are positive. ×4,500



Figs. 3–6. Consecutive phases of the promyelocyte stage. Note the increase in staining of the granules during this stage. The nuclear envelope and cisternae of the Golgi apparatus and endoplasmic reticulum are positive.

Fig. 3. Golgi area of a young promyelocyte with mainly negative immature nucleated granules (*arrows*) and a number of immature azurophil granules (*double arrows*). The latter show a faint peroxidase reaction, if any. Golgi vesicles are positive (*black arrowheads*) or negative (*white arrowheads*). ×26,500

Both cisternae and vesicles show peroxidase activity. A number of vesicles resembling those seen in the Golgi area occur throughout the cytoplasm, usually close to granules. Most of these vesicles are peroxidase positive, but some are negative. Nucleated granules and their precursors are negative (Fig. 1).

The promyelocyte

The localization of reaction product is the same as in the eomyelocyte: the endoplasmic reticulum, the nuclear envelope, and the Golgi cisternae are positive. The Golgi area is larger than in the eomyelocyte, and azurophil granules

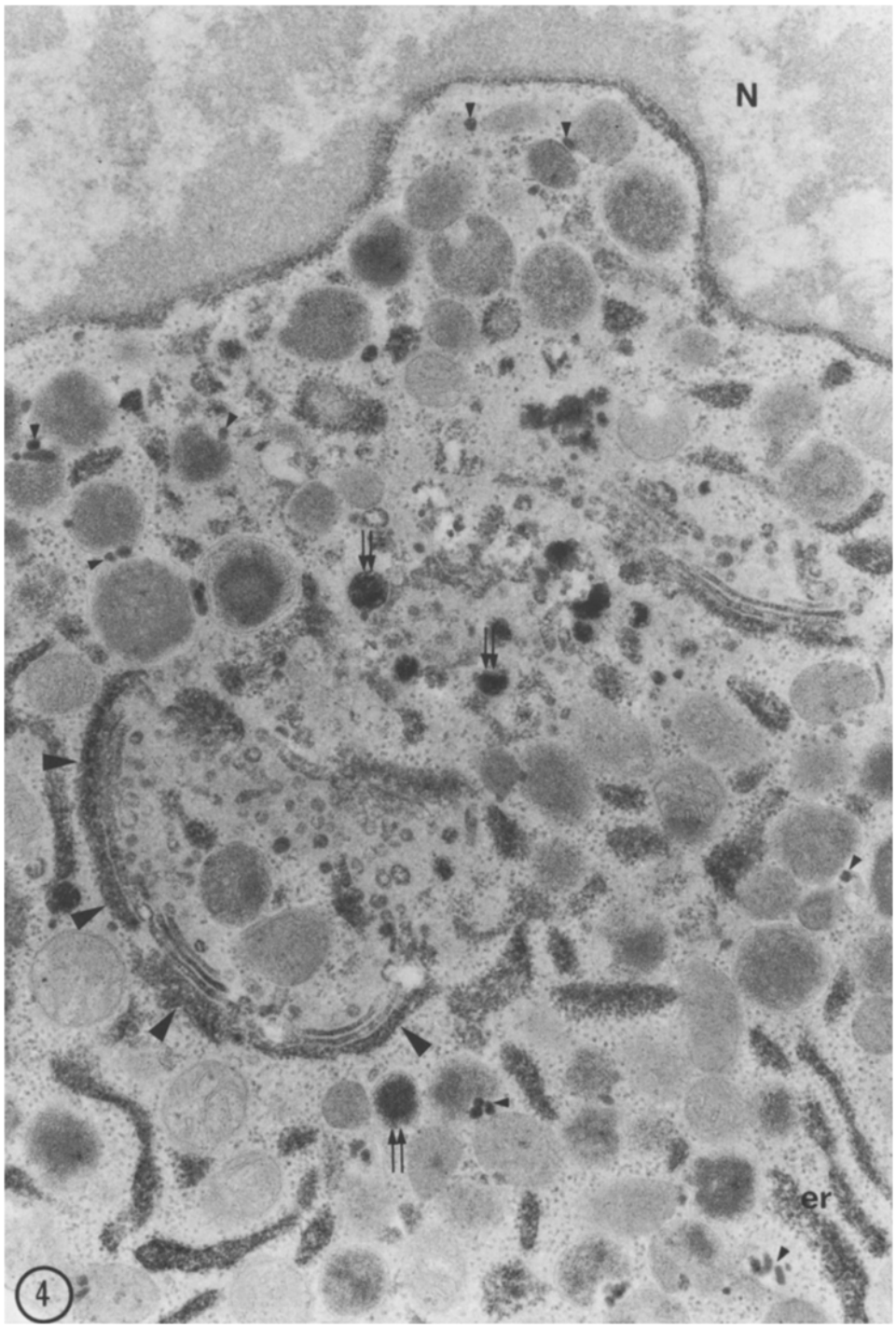


Fig. 4. Golgi area of promyelocyte in the stage of maturation following that in Fig. 3. The density of the granules is slightly increased and some are strongly stained (*double arrows*). The outer Golgi cisternae are surrounded by cisternae of the endoplasmic reticulum (*large arrowheads*). Note the distribution of positive vesicles over the cytoplasm, with a preferential proximity to granules (*small arrowheads*). ×26,000

can be seen close to the Golgi cisternae. Azurophil granules in the Golgi area are initially negative (Fig. 3), as are nucleated granules (Figs. 3 and 8). During the promyelocyte stage the intensity of the staining of the nucleated and azurophil granules increases (Figs. 2–6). The majority of the granules are moderately reactive and a few are highly reactive (Fig. 2). In this cell stage the Golgi vesicles stain intensely (Figs. 4 and 5). Their location is the same as in the eomyelocyte, i.e., in the Golgi area and close to granules (Fig. 7). At the end of the promyelocyte stage it becomes more difficult to distinguish between nucleated and azurophil granules.

Myelocyte and later stages

From the myelocyte stage on, specific granules are formed. These granules are negative after incubation for the detection of peroxidase activity, as are the nuclear envelope, the cisternae of the endoplasmic reticulum, and the Golgi apparatus (Fig. 10). Nucleated and azurophil granules are strongly positive and the distinction between them can hardly be made, although at high magnification an occasional nucleoid can be seen (Fig. 9). Vesicles are positive, and their number decreases with maturation of the cells. Multivesicular bodies are negative.

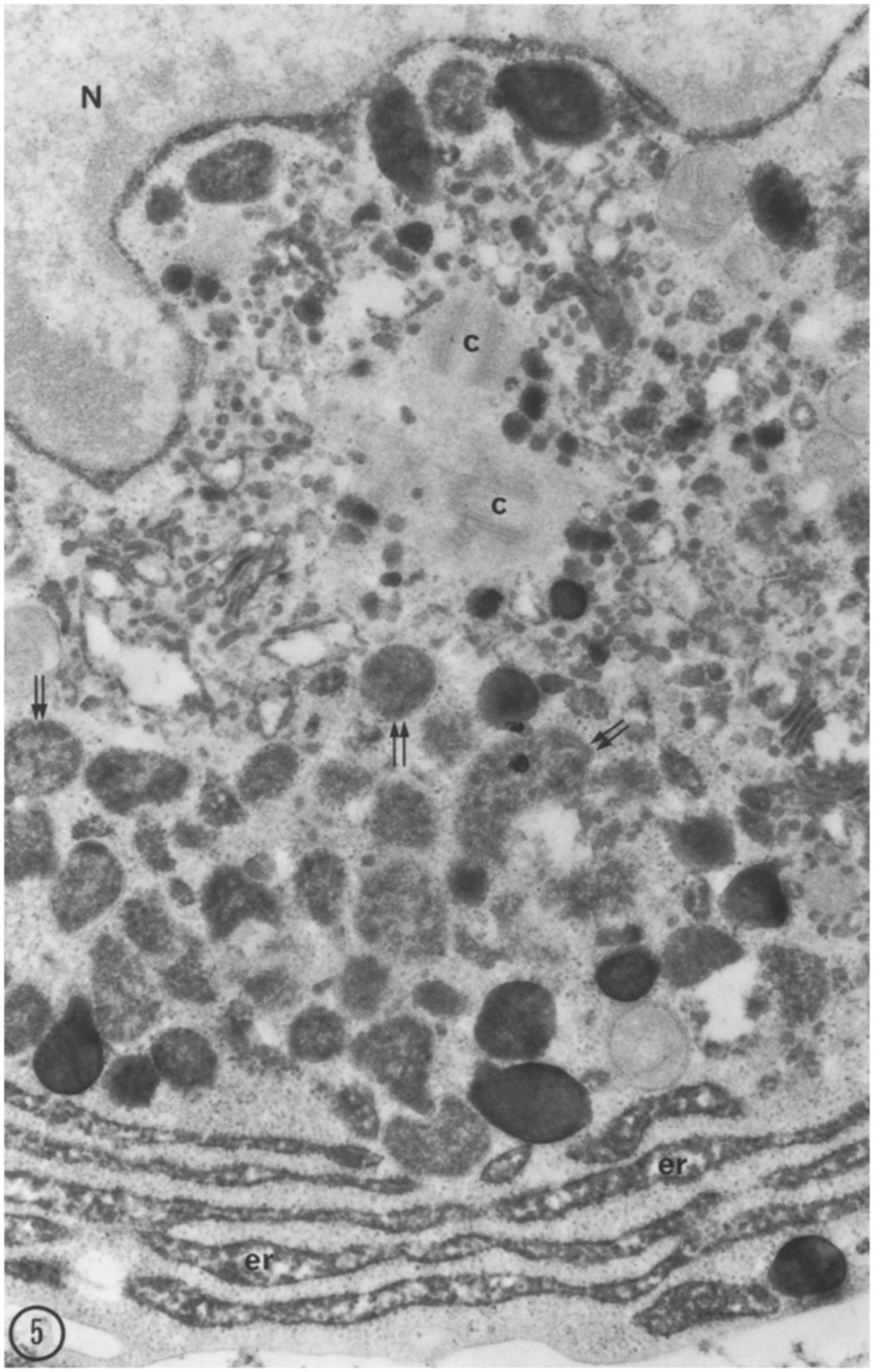


Fig. 5. Golgi area of promyelocyte in the stage of maturation following that in Fig. 4. Cisternae of the endoplasmic reticulum and Golgi vesicles are striking. Numerous granules can be seen in different stages of maturation, some of which can be recognized by the halo underneath the limiting membrane to be azurophil granules (*double arrows*). ×26,500

Control incubation

In general, the omission of hydrogen peroxide from the incubation medium gave negative results. The only exceptions were the following. In eomyelocytes weak reaction product was seen incidentally in the nuclear envelope and the cisternae of the endoplasmic reticulum and Golgi apparatus. And although the majority of the mature cells was peroxidase negative, a few had a small number (on average 5) of relatively large granules that reacted positively.

Discussion

Many ultrastructural studies have been devoted to morphological and cytochemical aspects of the maturation of the neutrophil granulocytes, and in particular the development

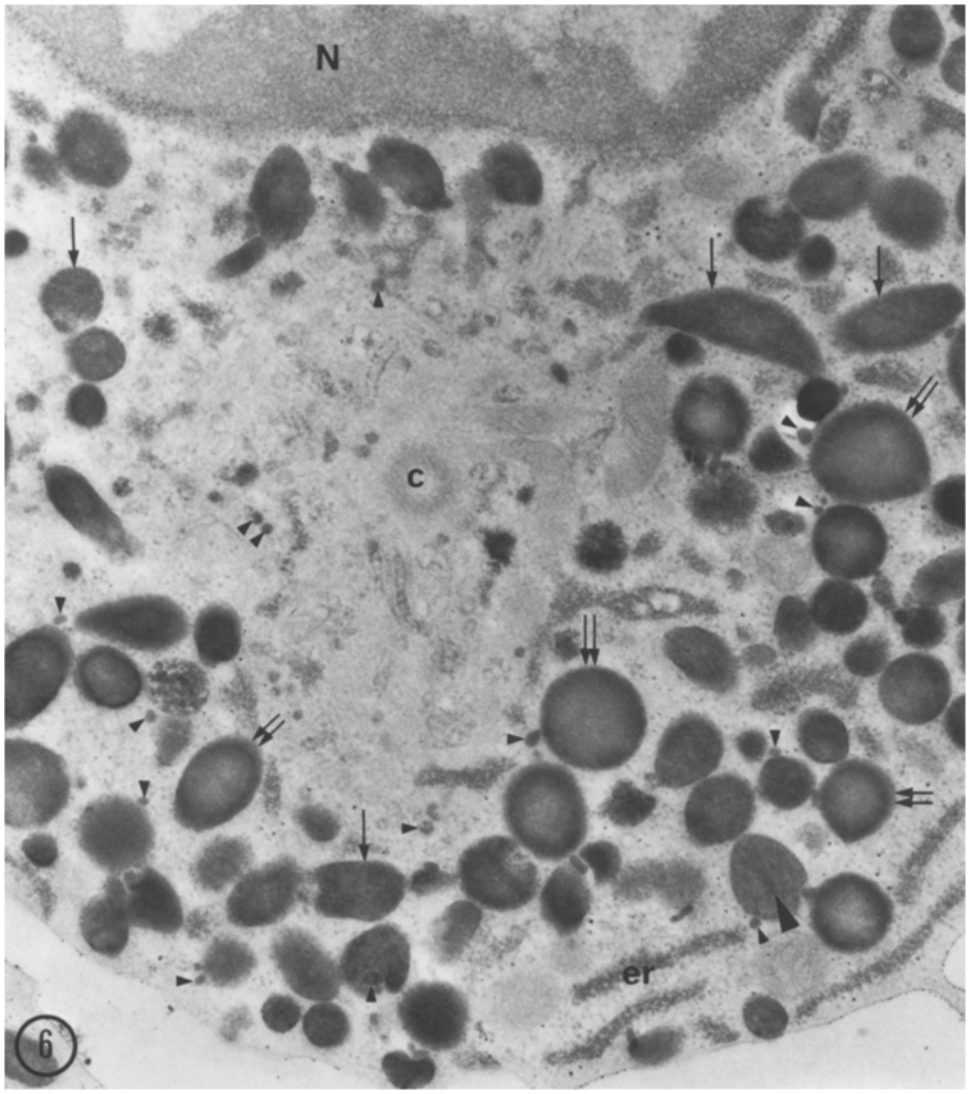


Fig. 6. Golgi area at the end of the promyelocyte stage. The endoplasmic reticulum is less striking than in Fig. 5 and the activity in the Golgi area is decreased (compare with Fig. 5). Vesicles can be seen in the cytoplasm and near granules (*arrowheads*). Granules with a flocculent matrix probably represent nucleated granules (*arrows*); those more spherical and with a more homogeneous matrix represent azurophil granules (*double arrows*) (see Brederoo et al. 1983). *Large arrowhead* points to a nucleoid in a nucleated granule. ×26,500

of the granule population (for references, see Brederoo et al. 1983). The cytochemical studies were focused mainly on the localization of peroxidase activity, and the majority of them led to the conclusion that two types of granule are produced during the development of the neutrophil granulocytes. In analogy with light-microscopical terminology these are called azurophil and specific granules. Electron microscopy provided conclusive proof that azurophil granules are formed during the promyelocyte stage and specific granules during the myelocyte stage. This has been beautifully illustrated by Bainton and Farquhar's (1966) investigation of rabbit bone marrow. Because the azurophil granules were found to be peroxidase-positive and specific granules peroxidase-negative in this species, the peroxidase method was adopted for bone-marrow studies in other mammalian species whose granules were difficult to characterize by morphology alone (Farquhar and Bainton 1972). This work led to the two-types-of-granule concept, peroxidase-positive granules being formed in promyelocytes and peroxidase-negative granules in myelocytes.

However, because such granules are stained after incubation for peroxidase, the use of peroxidase cytochemistry meant that the question of the status of a granule morphologically characterized by the presence of a crystalloid was neglected. Nevertheless, morphological studies done in our laboratory on the development of neutrophil granulocytes of the guinea pig (Brederoo and Daems 1978), rat (Brederoo and van der Meulen 1983), and man (Brederoo et al. 1983) have clearly shown that formation of such (nucleated) granules starts earlier during granulocytopoiesis, i.e., before the azurophil granules are formed. Because these nucleated granules and azurophil granules originate in different stages, we use the term eomyelocyte for the cell in which the nucleated granules and their precursors are the only granules present (Brederoo et al. 1983).

We undertook the present study to add cytochemical information to our morphological findings. Because it is generally agreed that specific granules are negative after incubation for peroxidase, as are the cell organelles involved in their production, we focused our attention on the first two cell stages, the eomyelocyte and promyelocyte.

It was found that nucleated granules in eomyelocytes and azurophil granules in promyelocytes are initially negative and both become peroxidase positive during the promyelocyte stage. The cell organelles involved in granule production, i.e., the nuclear envelope, rough endoplasmic reticulum, and Golgi apparatus, were positive after incubation for peroxidase in both eomyelocytes and promyelocytes.

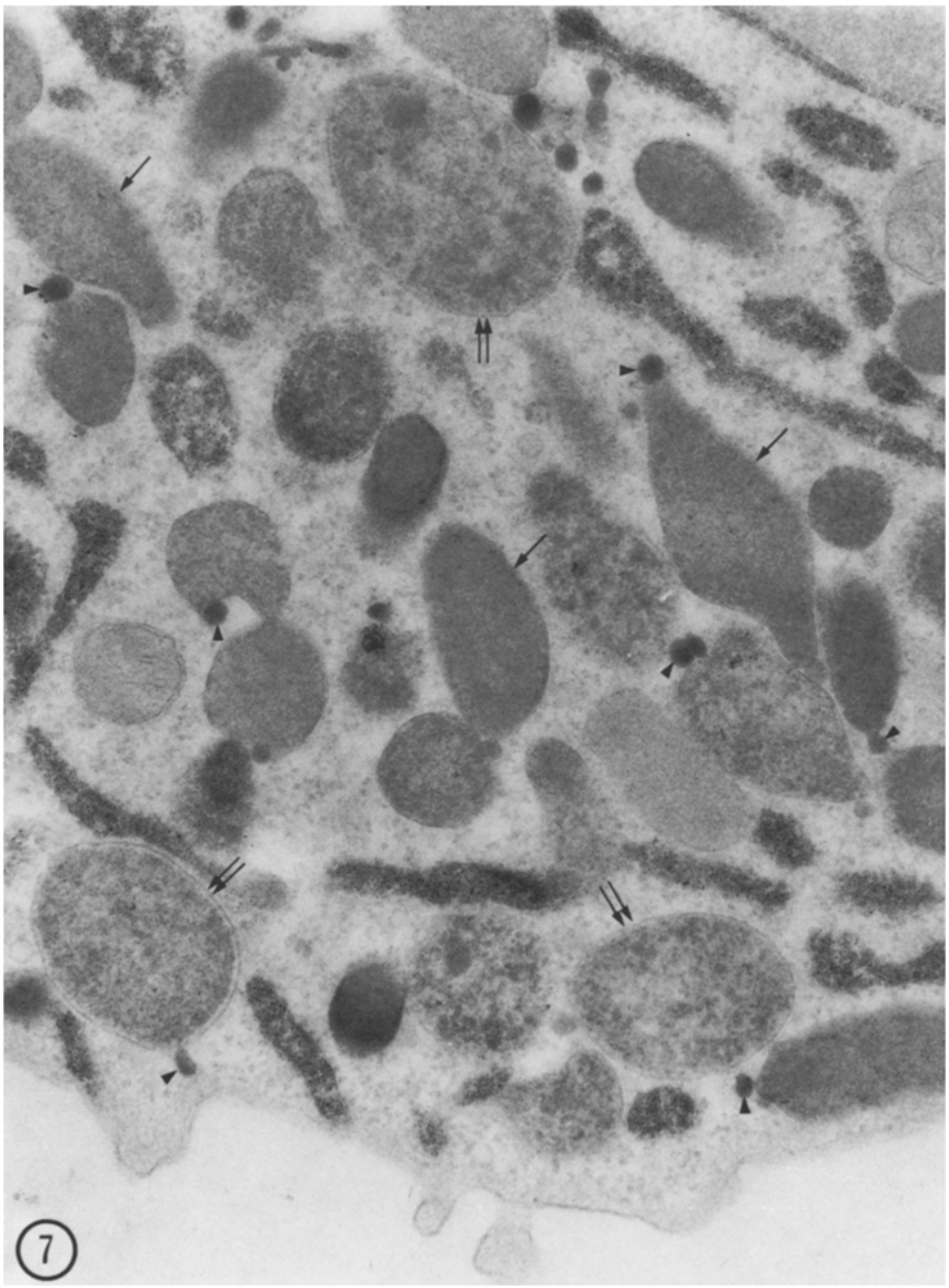
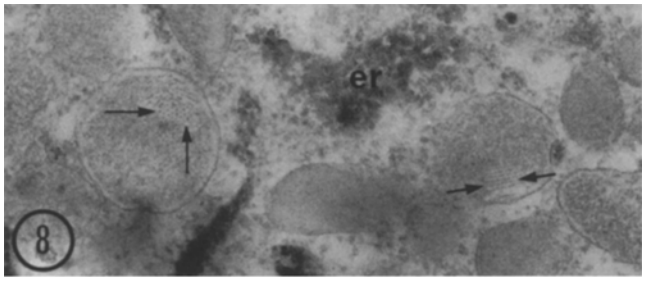
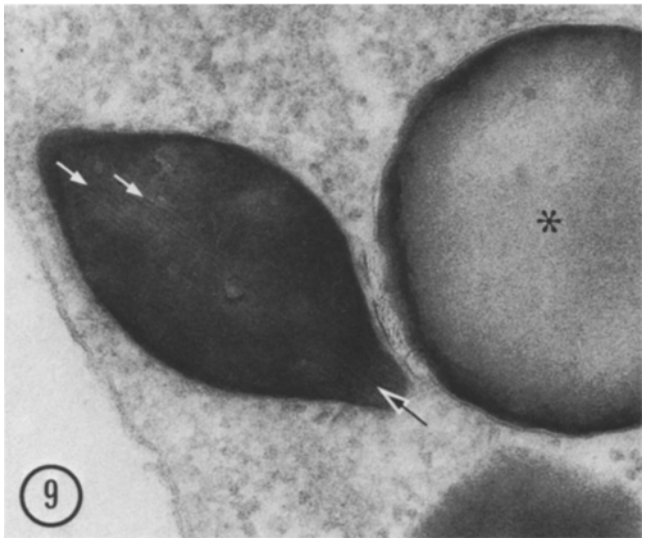


Fig. 7. Detail of a promyelocyte showing the location of vesicles (*arrowheads*) near the granules. *Arrows* point to nucleated granules, *double arrows* to azurophil granules. The latter are recognized by the halo occurring underneath the limiting membrane. ×41,000

The stacks of Golgi cisternae did not show polarity with respect to the presence of reaction product. The increase in staining intensity in the organelles during the promyelocyte stage corresponded with the staining intensity of the nucleated and azurophil granules, thus reflecting the involvement of these organelles in granule production and maturation (Figs. 2–6). Furthermore, the activity of the Golgi elements ended at the end of the promyelocyte stage when production of azurophil granules ceased (compare Figs. 5 and 6). Vesicles in the Golgi area also seemed to be involved in the maturation of both nucleated and azurophil granules (Fig. 7). Some of these vesicles are positive before the appearance of the azurophil granules (Fig. 3), and although fusion of these vesicles with granules has not been observed with certainty, their close proximity to both nucleated and azurophil granules strongly suggests such a process (Fig. 7). Furthermore, these vesicles decreased in number during the cell stages following the promyelocyte (Fig. 10), and although this is undoubtedly also a result of mitotic processes (see Brederoo et al. 1983), it is possible that these vesicles eventually fuse with granules. In this context it should be kept in mind that in rabbit heterophil promyelocytes Golgi-derived vesicles fuse to form multicored immature azurophil granules (Bainton and Farquhar 1966, 1968) and that vesicles resembling our Golgi vesicles in size and location have been seen in other studies on the development of neutrophil granulocytes (e.g. Ackerman and Clark 1971; Bainton et al. 1971; Morris et al. 1975).

Nucleated granules have been seen by others. Daems (1968) described the fine structure of these granules and considered them – despite their peroxidase positivity – to be a distinct type on the basis of his study on blood neutro-





phils, whereas Bainton et al. (1971) and Breton-Gorius and Reyes (1976) consider these granules to be only a variety within the class of azurophil granules. Watanabe and Enomoto (1974) found granules with a fine structure to be negative after incubation for peroxidase activity and considered them to represent a distinct type of granule, but reported that such granules appear after the formation of the azurophil granules.

In sum, the present results support our earlier findings (Brederoo et al. 1983) indicating that nucleated granules are a distinct type of granule and the first to be formed during the development of neutrophil granulocytes, i.e., before the formation of the azurophil granules and thus confirm the formation of three types of granule during granulocytopoiesis. In addition, the following conclusions can be drawn:

1. Nucleated granules are peroxidase negative in the eomyelocyte and become peroxidase positive in the promyelocyte stage.

Fig. 8. Detail of a young promyelocyte showing two negative nucleated granules recognizable by the presence of two nucleoids, one cut longitudinally (*left, arrows*), and the other transversely (*right, arrows*). ×40,000

Fig. 9. Detail of a young myelocyte, showing a nucleated granule with characteristic fine structure (*arrows*) and part of an azurophil granule (*asterisk*). ×77,000

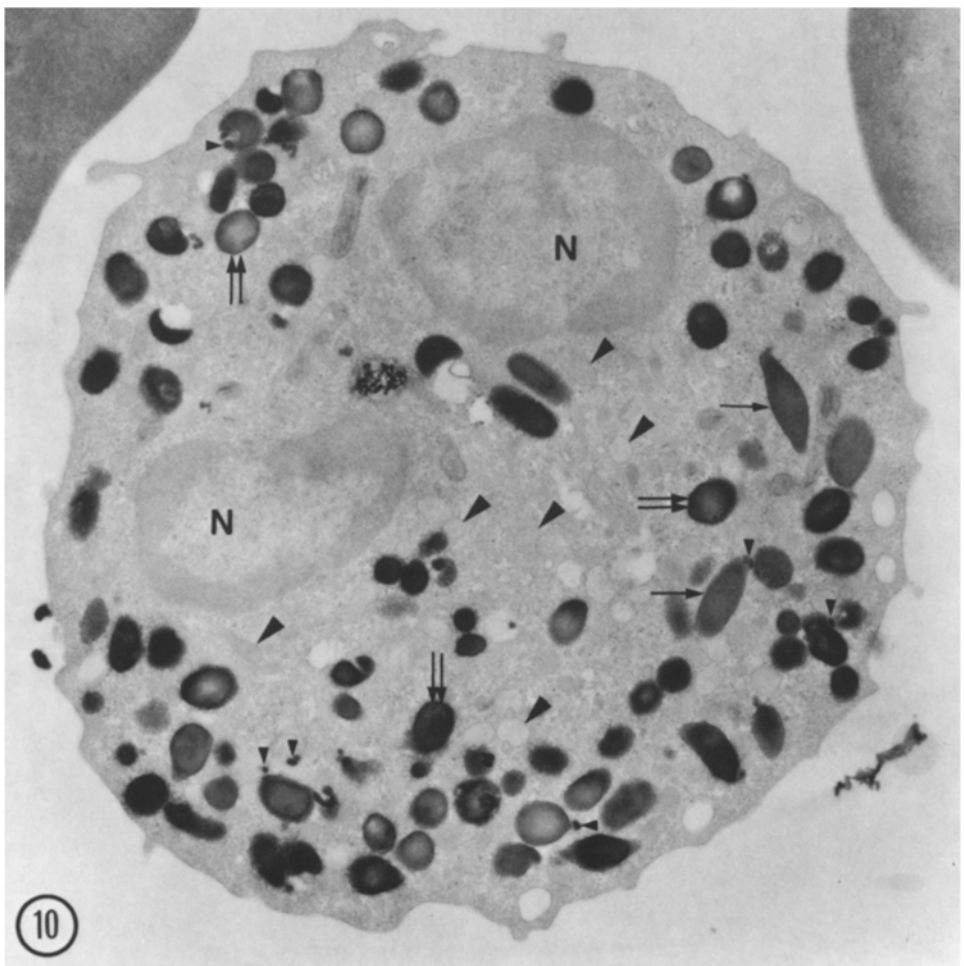


Fig. 10. Mature neutrophil granulocyte. Some nucleated granules can be recognized by their elongated shape (*arrows*). Azurophil granules are generally spherical (*double arrows*), homogeneously dense, or have a bright center. Specific granules are formed after the promyelocyte stage and are peroxidase negative (*large arrowheads*). A few vesicles are present (*small arrowheads*). ×13,000

2. Azurophil granules become peroxidase positive during the promyelocyte stage.

3. Golgi vesicles are involved in the process of maturation of nucleated and azurophil granules.

Acknowledgements. The authors are greatly indebted to their colleagues W.C. de Bruijn, L.A. Ginsel, and H.K. Koerten for discussion of the manuscript and to Prof. Dr. H.A. Huysmans (Department of Thoracic Surgery, Leiden University Hospital) for his cooperation. They also thank Mrs. I. Seeger for reading the English text, Mrs. G.C.A.M. Spigt-van den Bercken for typing the manuscript, and Mr. J.J. Beentjes and Mr. L.D.C. Verschragen for preparing the photographic material.

References

Ackerman GA (1968) Ultrastructure and cytochemistry of the developing neutrophil. Lab Invest 19:290–302

Ackerman GA (1971a) The human neutrophilic promyelocyte. A correlated phase and electron microscopic study. Z Zellforsch 118:467–481

Ackerman GA (1971b) The human neutrophilic myelocyte. A correlated phase and electron microscopic study. Z Zellforsch 121:153–170

Ackerman GA, Clark MA (1971) Ultrastructural localization of peroxidase activity in normal human bone marrow cells. Z Zellforsch 117:463–475

Bainton DF, Farquhar MG (1966) Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J Cell Biol 28:277–301

Bainton DF, Farquhar MG (1968) Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. J Cell Biol 39:299–317

Bainton DF, Ullyot JL, Farquhar MG (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J Exp Med 134:907–934

Bainton DF, Nichols BA, Farquhar MG (1976) Primary lysosomes of blood leukocytes. In: Dingle JT, Dean RT (eds) Lysosomes in biology and pathology, vol 5. North Holland, Amsterdam Oxford, pp 3–32

Bentfeld ME, Nichols BA, Bainton DF (1977) Ultrastructural localization of peroxidase in leukocytes of rat bone marrow and blood. Anat Rec 187:219–240

Brederoo P, Daems WTh (1977) A new type of granule in guinea pig heterophil granulocytes. Cell Biol Int Rep 1:363–368

Brederoo P, Daems WTh (1978) The ultrastructure of guinea pig heterophil granulocytes and the heterogeneity of the granules. I. The development in the bone marrow. Cell Tissue Res 194:183–205

Brederoo P, Meulen J van der (1983) Development of the granule population in heterophil granulocytes from rat bone marrow. Cell Tissue Res 228:433–449

Brederoo P, Meulen J van der, Mommaas-Kienhuis AM (1983) Development of the granule population in neutrophil granulocytes from human bone marrow. Cell Tissue Res 234:469–496

Breton-Gorius J (1970) Aspects morphologiques de la granulopoïèse. Pathol Biol 18:433–440

Breton-Gorius J, Guichard J (1969) Etude au microscope électronique de la localisation des peroxydases dans les cellules de la moelle osseuse humaine. Neuv Rev Fr Hématol 9:678–687

Breton-Gorius J, Reyes F (1976) Ultrastructure of human bone marrow cell maturation. Int Rev Cytol 46:251–321

Daems WTh (1968) On the fine structure of human neutrophilic leukocyte granules. J Ultrastruct Res 24:343–348

Dunn WB, Hardin JH, Spicer SS (1968) Ultrastructural localization of myeloperoxidase in human neutrophil and rabbit heterophil and eosinophil leukocytes. Blood 32:935–944

Farquhar MG, Bainton DF (1972) Cytochemical studies on leukocyte granules. In: Takeuchi T, Ogawa K, Fujita S (eds) Histochemistry and cytochemistry 1972. Proc 4th Int Congr Kyoto, pp 25–26

Millonig G (1962) Further observations on a phosphate buffer for osmium solutions in fixation. In: Breese SS (ed) Fifth Int Congr on Electron Microscopy Philadelphia 1962, vol 2. Academic Press, New York London, pp 8–9

Morris RB, Nichols BA, Bainton DF (1975) Ultrastructure and peroxidase cytochemistry of normal human leukocytes at birth. Dev Biol 44:223–238

Roels F, Wisse E, De Prest B, Meulen J van der (1975) Cytochemical discrimination between catalases and peroxidases using diaminobenzidine. Histochemistry 41:281–312

Scott RE, Horn RG (1970) Ultrastructural aspects of neutrophil granulocyte development in humans. Lab Invest 23:202–215

Watanabe Y, Enomoto Y (1974) Fine structure of human neutrophilic granulocytes and leukemic cells. In: Sanders JV, Goodchild DJ (eds) Eighth Int Congr on Electron Microscopy, vol 2. Academic Press, New York, pp 416–417

Wetzel BK, Horn RG, Spicer SS (1967) Fine structural studies on the development of heterophil, eosinophil, and basophil granulocytes in rabbits. Lab Invest 16:349–382