ABSTRACT

Giant round pink inclusions (=2 \mu m) were seen in neutrophilic myeloblasts, promyelocytes, and myelocytes from three patients with acute myelogenous leukemia. On preliminary examination of the bone marrow smears, these inclusions looked like ingested red blood cells in that they were pink and not azurophilic. The bone marrow specimens were processed for the electron microscopic demonstration of peroxidase with 3,3'-diaminobenzidine and \textit{H}2\textit{O}2 at pH 7.6. In all three cases, the inclusions were determined to be large peroxidase-positive granules since they were limited by a single unit membrane and, unlike endocytized red blood cells, were not contained within phagocytic vacuoles. The granules were homogeneously dense for peroxidase and showed no obvious crystalline structure when examined stained or unstained on grid. We believe that they correspond to the giant pink round granules Van Slyck and Rebuck observed in immature leukemic granulocytes in 1974 and termed the pseudo-Chediak-Higashi anomaly. Like the giant purple granules seen in leukemia with this anomaly, these granules also appear to be an abnormal variant of peroxidase-positive azurophil (primary) granules. Their lack of azurophilia is due to the absence of sulfated glycoaminoglycans.

INTRODUCTION

In 1974, Van Slyck and Rebuck (40) observed, on Wright's-stained smears, giant round granules in leukemic cells from 2 patients with acute myelomonocytic leukemia. Since this acquired morphological abnormality closely mimicked the giant round granules seen in the Chediak-Higashi anomaly, a rare autosomal recessive disorder, they termed it the pseudo-Chediak-Higashi anomaly. Like the giant purple granules seen in leukemia with this anomaly, these granules also appear to be an abnormal variant of peroxidase-positive azurophil (primary) granules. We have had the opportunity to study 3 patients with AML whose blasts, promyelocytes, and myelocytes contained enormous round pink inclusions, initially thought to be ingested RBC. Analysis by electron microscopy and peroxidase cytochemistry revealed that these pink structures were large peroxidase-positive granules and therefore also represented abnormal variants of the azurophilic (primary) granule population.

MATERIALS AND METHODS

Case Studies

Case 1. A 62-year-old white housewife with a history of hypertension and arthritis was sent for a hematological consultation in June 1974 because of fatigue, anemia, and recent onset of bruising. Physical examination revealed pallor and multiple ecchymoses but not organomegaly or adenopathy. Laboratory examination of the blood revealed pancytopenia (Table 1), but no abnormal circulating leukocytes were detected. A bone marrow aspiration and biopsy specimen were hypercellular, showing an increase in the granulocytic series, 5% myeloblasts, and promyelocytes containing Auer rods and salmon-pink inclusion bodies. The pink inclusions were usually large (1 to 2 \mu m) and round, but they were occasionally tear shaped or tapered at both ends. Rarely, both forms were present in a single cell. The patient's condition was diagnosed as AML.

Over the next 2.5 years, the patient was given various treatments for AML and had a smoldering course without complete remission. The Auer rods disappeared with partial remission, but the giant inclusions merely decreased in number. Initially, the patient was treated with daunomycin, vincristine, prednisone, and 1-\beta-D-arabinofuranosylcytosine. Because she came near death twice and her marrow remained abnormal, she was switched to a maintenance dose of daily cyclophosphamide and monthly vincristine. During the ensuing months, she had a number of complications, mainly infections. A bone marrow specimen was processed for fine-structural examination in November 1975.

In October 1976, the patient was found to have an endometrial adenocarcinoma, which was treated with radiation therapy. At that time, her WBC count was 0.7 \times 10^9/liter. She developed bowel obstruction and died on January 8, 1977. An autopsy revealed metastatic carcinoma to the peritoneum, liver, pleura, and bone. The marrow was hypercellular. During the course of her illness, there was no clinical or laboratory evidence of DIC.

Case 2. A white 16-year-old female was found to have AML in May 1976. She had experienced dizziness on exertion, easy bruising, and heavy menstrual flow for 6 weeks. Her WBC...
count was $5.0 \times 10^9$/liter with a marked shift to the left in the
neutrophilic series, including 20% myeloblasts. Her blood data
are summarized in Table 1. Her bone marrow was hypercellular
and showed granulocytic hyperplasia with a marked shift to the
left. The myeloblasts contained large salmon-pink cytoplasmic
inclusions and occasional typical azurophilic Auer rods. Culture
for Philadelphia chromosome was negative. Leukocyte alkaline
phosphatase was normal. Bone marrow was obtained for elec-
tronic microscopy examination in May 1976.

The patient was treated with thioguanine, vincristine, pred-
nisone, 1-β-o-arabinofuranosylcytosine, and cyclophospha-
mide and was in remission for 6 months. During this period, no
giant granules were seen in her granulocytic precursor cells. A
bone marrow specimen taken in January 1977 showed 14%
myeloblasts but no giant granules. The patient did not respond
to reinduction therapy and died in April 1977 of a pulmonary
infection. There was no evidence of DIC.

**Case 3.** In January 1976, the preoperative leukocyte differen-
tial for a white 52-year-old male scheduled to undergo
elective surgery revealed abnormal circulating leukocytes with
large round pink inclusions. About 12% of the cells were blast-
like and contained very large round pink granules. The physical
examination revealed no abnormalities. The complete blood
count is recorded in Table 1. An aspirate of sternal bone
marrow showed granulocytic hyperplasia and the same large
pink granules in blasts and promyelocytes. Some blasts con-
tained typical azurophilic Auer rods. A bone marrow specimen
was obtained for electron microscopy in March 1976. By May
1976, the patient had become symptomatic with fatigue and
bleeding and had a falling hematocrit and an increase in his
circulating blasts to 50%. He received chemotherapy for the
first time on March 9, 1976. Initially, he was given cyclophos-
phamide, thioguanine, prednisone, and vincristine every 4
weeks, but, beginning in November 1977, he received the
treatment regimen every 6 weeks. His last hematology values,
recorded on August 29, 1980, show a WBC count of $6.3 \times
10^9$/liter, including 1% bands, 51% polymorphonuclear leu-
kocytes, 32% lymphocytes, and 12% monocytes. His hema-
tocrit was 45%, and his platelet count was $140 \times 10^9$/liter. A
recent marrow specimen still showed large pink inclusion bod-
ies in many cells of the granulocytic series. There has been no
evidence of DIC during the course of his illness, and he is still
alive as of October 1980.

**Light and Electron Microscopy**

Smears of bone marrow were stained for aldehyde fuchsin
(19, 30) and dialyzed iron (17, 35). Cells from bone marrow
aspirates were fixed in 1.5% glutaraldehyde in 0.1 M sodium
cacodylate buffer (pH 7.4) with 1% sucrose for 10 min at 4°,
washed twice in the same buffer with 7% sucrose, and shipped
on ice by air freight from Washington to California. They were
incubated for peroxidase with 3,3'-diaminobenzidine and H2O2
at pH 7.6 by the method of Graham and Karnovsky (21),
postfixed in 1% OsO4, and subsequently processed as previ-
ously described (7).

**RESULTS**

The question of whether the large round pink inclusions seen
on smears in early neutrophilic precursor leukocytes (Fig. 1,
inset) were exogenous particles (i.e., ingested RBC) or endog-
ogenous products of the leukocytes was easily resolved by elec-
tron microscopy. These peroxidase-positive inclusions (Fig. 1)
were each surrounded by a single unit membrane (Fig. 2). If
they were indeed ingested RBC, there would be 2 membranes,
an inner one belonging to the RBC and an outer one belonging
to the phagocytic vacuole.

The cells with the large pink inclusions which were up to 3
µm in diameter also contained normal peroxidase-positive azur-
ophil (primary) granules (Fig. 3a), which in humans are usually
no larger than 500 nm. Some cells contained many giant
granules (Fig. 1), whereas others contained only one (Fig. 3a).
Although the content of these granules was not completely
homogeneous, since there were both light and dark areas, we
were unable to resolve crystalline structures even on unstained
material. This lack of crystalline structure differs from the case
previously reported by Tulliez et al. (39). When examined by
light microscopy, the giant granules showed no staining for
aldehyde fuchsin (Fig. 3a, inset), nor dialyzed iron (not illus-
trated), whereas the adjacent normal azurophil granules were
stained.

Having demonstrated that the giant inclusions were abnormal
peroxidase-positive granules of endogenous origin, we wanted
to further delineate their formation. In previous studies of
normal human bone marrow (5, 7), we had demonstrated that
the peroxidase-positive azurophilic, or primary, granules are
formed by the usual pathway followed by secretory products
(29) (RER → vesicles → Golgi complex → storage granules);
*i.e.*, in normal promyelocytes, the marker enzyme peroxidase
is synthesized and segregated within the RER and transported
to the Golgi region by small transitional vesicles. The enzyme
is concentrated within the Golgi cisternae (see Ref. 4, diagram);
then small Golgi-derived vesicles bud off and move to the

<table>
<thead>
<tr>
<th>Neutrophilic series</th>
<th>Bands + polymorphonuclear neutrophil leukocytes</th>
<th>Lymphocytes (%)</th>
<th>Monoocytes (%)</th>
<th>Eosinophils (%)</th>
</tr>
</thead>
</table>
| Patient | Age | Sex | Hematocrit (%) | Platelet count (× 10^12/liter) | Leukocyte count (× 10^9/liter) | Myeloblasts (%) | Promyelo-
cytes (%) | Myelo-
cytes (%) | Metamyelo-
cytes (%) | Lymphocytes (%) | Mono-
cytes (%) | Eosino-
phils (%) |
| 1 | 62 | F | 25 | 37 | 1.1 | 10 | 90 |
| 2 | 16 | F | 16 | 21 | 5.0 | 20 | 15 | 10 | 5 | 2 | 41 | 7 |
| 3 | 52 | M | 44 | 5.7 | 12 | 1 | 1 | 1 | 62 | 27 | 3 | 3 |

**Table 1**

Summary of initial blood data for 3 patients with AML

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Abnormal Granules in AML

Peripheral cytoplasm where they aggregate to form the larger mature azurophil granules. In the 3 bone marrow specimens described here, RER and Golgi cisternae contained no reaction product for peroxidase (Fig. 3a). This lack of reaction product may well reflect the procedures used, rather than a true absence of peroxidase, since 3 days elapsed between fixation of the specimens in Washington and incubation for the enzyme in California. However, it is interesting to note that other investigators have had difficulty demonstrating peroxidase in RER and Golgi cisternae in leukemic cells containing giant granules (30, 39). Clustered near the giant granules in our specimens were large numbers of small vesicles containing high concentrations of peroxidase (Fig. 3b). Parmley et al. (30) have suggested that the giant granules in leukemic cells are formed by the fusion of these small dense vesicles as well as by the fusion of intact primary granules and concomitant entrapment of cytoplasmic material. The 2 abnormal granule forms in Fig. 2 (labeled ag) may exemplify the latter mechanism.

In addition to the prominent round inclusions, the cells from 2 patients contained several other forms of abnormally large peroxidase-positive granules (Fig. 4). These granules were usually elongated, and some contained amorphous densities (Fig. 4a). Others contained a crystalloid structure (Fig. 4b), and yet others contained an obvious well-organized crystal (Fig. 4c). The structure containing the crystal could be seen by light microscopy on Wright’s-stained smears and was azurophilic with a rounded middle and pointed ends (Fig. 4c, inset). All these forms seem to be variants of Auer bodies. The form illustrated in Fig. 4c was recently termed Phi body by Hanker et al. (22, 23). Since Auer originally described and illustrated organelles of these shapes, we prefer to call them Auer bodies (see Ref. 3, Fig. 15).

The following observations in more mature neutrophils were made. Although the giant granules were observed only in myeloblasts, promyelocytes, and myelocytes, other abnormalities were seen in mature neutrophils. The most striking of the pathological abnormalities was the paucity and sometimes complete absence of peroxidase-negative specific granules (Fig. 5). This secondary granule population is usually formed during the myelocyte stage and ordinarily constitutes two-thirds of the total granule population of human neutrophils (7). In addition, there were prominent nuclear blebs, which have been reported to be more common in leukemic cells (2), usually blasts, than in normal cells.

DISCUSSION

Abnormal forms of granules have been observed in the early myeloid cells of patients with acute leukemia by many investigators (1, 3, 5, 6, 8, 9, 11–14, 24, 25, 36, 37, 41). The first form recognized and the one best characterized to date is the Auer rod (3), which is present in the myeloblasts and promyelocytes of from 10 to 15% of patients with AML. This cytoplasmic inclusion is usually needle shaped, although Auer also commented on other forms, and is easily recognized on smears prepared with Romanowsky-type stains because it is azurophilic (i.e., stains reddish purple); it also stains for peroxidase and several other enzymes (6, 8, 12, 14, 36). Only 7 cases of “giant lysosome-like structures” (26), “oval cytosomes” (36), or pseudo-Chediak-Higashi granules (18, 30, 39) in cells from patients with acute leukemia have been previously studied.

One major difference between our 3 cases and others was the color of the giant granules as observed by light microscopy. In the earlier reports, the granules were described as purple (39), reddish purple (30), or azurophilic (18), whereas the inclusions that we observed were pink. Furthermore, these inclusions did not stain for aldehyde-fuchsin, a stain for anionic glycoconjugates, or diazylized iron, a stain for carboxylated and sulfated acidic glycoconjugates. In contrast, the giant reddish-purple inclusions described by Parmley et al. (30) stained strongly for aldehyde-fuchsin. Since Dunn and Spicer (17) and Parmley et al. (31) have demonstrated that the azurophilia of normal azurophil (primary) granules is due to the presence of sulfated glycoaminoglycans, we hypothesize that the giant nonazurophilic granules we observed lack acid mucosubstance(s). It should be mentioned that these giant round granules in acute leukemia stain for other constituents of normal azurophil granules, such as peroxidases (18, 26, 30, 39), naphthol AS-D chloroacetate esterase (26, 30), acid phosphatase (18, 26), Sudan black (30), and periodic acid-Schiff (16, 18, 26).

Schmaatz et al. (36) have noted that Auer bodies also usually stain for peroxidase, naphthol AS-D chloroacetate esterase, acid phosphatase, and Sudan black and are thus closely related to normal azurophil granules. They point out, however, that not all Auer bodies in a particular specimen exhibit all of these staining properties. This variability of staining and presumably of granule content seems to apply to the pseudo-Chediak-Higashi anomaly as well.

The 2 investigations of giant granules in leukemic cells by electron microscopy and peroxidase cytochemistry (30, 39) have both shown that: (a) the granules are bound by a single unit of membrane, and the vast majority contain a matrix densely reactive for peroxidase; (b) swarms of small peroxidase-positive vesicles are prominent in the cytoplasm of cells containing such granules; and (c) peroxidase reaction product is not clearly demonstrable in RER or Golgi cisternae in these cells. There are, however, some major discrepancies among the findings. In the study by Tulliez et al. (39), no Auer bodies were observed. The giant granules contained crystalline cores, seen best on unstained grids, and exhibited a periodicity different from that of the typical Auer bodies seen in acute promyelocytic leukemia associated with DIC. The giant granules frequently contained heterogeneous membranous material and amorphous nucleoids. In our specimens, the granule matrix was relatively homogeneous and did not have a crystalline substructure. Furthermore, our patients showed no evidence of DIC. Like Parmley et al. (30), we observed Auer rods, but only rarely were both abnormal granule types seen in the same cell.

None of the investigations thus far, including our own, has provided clear evidence of the mechanism by which the giant granules are formed. Tulliez et al. (39) have suggested that they are formed by fusion of azurophil granules. Parmley et al. (30) have hypothesized that they are formed by 2 processes: (a) the fusion of small peroxidase-positive vesicles; and (b) the fusion of intact primary granules and concomitant entrapment of cytoplasmic material. It should be mentioned that these numerous small peroxidase-positive vesicles (Fig. 3b) were also a prominent feature in cells forming Auer rods (Fig. 4) as previously observed (6, 12). Also, perhaps, the previously mentioned absence of stainable glycoaminoglycans in these

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large granules may provide a clue to their formation. Even though the factors responsible for the concentration of enzymes and their packaging into normal azurophil granules are still obscure, we believe that they may be similar to the factors suggested by Reggio and Palade (34) to explain the concentration of enzymes in zymogen granules of the pancreatic exocrine cell. These investigators proposed that the concentration was not achieved by membrane ion pumps in the condensing vacuoles but possibly occurred as a result of the osmotic effects of a sulfated polyanion, a peptidoglycan. Hypothetically, this polyanion together with secretory proteins that are predominantly cationic could cause a reduction in the osmotic activity within the condensing vacuoles; the resultant efflux of water would bring about concentration. Additional data on normal human leukocytes are needed before abnormalities in the condensation of azurophil granules in leukemic cells can be further analyzed.

The formation of the megagranules in the hereditary Chediak-Higashi anomaly has been carefully studied by Davis et al. (15). Promyelocytes from persons and animals with this trait lack normal azurophil (primary) granules and, instead, contain enlarged pleomorphic granules, which may resemble azurophil granules to varying degrees. These unusual granules are not present in the blasts and become apparent in promyelocytes after the appearance of the precursor vesicles derived from Golgi cisternae. The precursor vesicles undergo a series of fusions leading to the formation of the megagranules. In humans, the characteristic crystalloid does not seem to form, and the granule matrix develops a reticulated array of membrane-like material. These granules also contain peroxidase and stain with Sudan black. On Wright’s staining of smears, they may stain reddish purple, blue, purple, or sometimes pink (10, 15, 32, 39, 40). The second stage of granulopoiesis, which gives rise to the peroxidase-negative specific (secondary) granule, is unaffected; and a full complement of these granules is present in mature neutrophils. No normal azurophil granules are present. It was recently demonstrated that some form of the megagranules also contains lactoferrin, indicating some limited fusion with specific granules (32). The defects underlying the abnormal granule fusion in Chediak-Higashi leukocytes have not yet been defined although 2 possibilities, alteration of intracellular cyclic nucleotides and dysfunction of microtubules and granule membranes, have been investigated (see Ref. 26). It should also be remembered that, in hereditary Chediak-Higashi syndrome, other hematopoietic cell types contain megagranules as do many other nonhematopoietic cells, such as melanocytes, renal tubular epithelial cells, and cells of gastric mucosa, pancreas, thyroid, and neural tissues (15).

The relative paucity of peroxidase-negative specific granules in the more mature segmented neutrophils warrants comment. It appears that cytoplasmic development has ceased after the promyelocytic stage, whereas nuclear maturation has progressed in a fairly normal fashion, a phenomenon termed “maturation anarchy” by Bessis (8). He attributes this anarchy to failure of the “program” of cell maturation, which is characteristic of acute leukemias. The absence of certain normal organelles from mature neutrophils of patients with acute leukemia has been previously documented by electron microscopy and cytochemistry (4–6) as well as by immunofluorescence methods. For example, we have demonstrated the absence of specific (secondary) granules in neutrophils from patients with AML (4, 6), and Odeburg et al. (27) and Rausch et al. (33) have observed the absence or marked decrease of lactoferrin, a substance found exclusively in the specific granule population. These 2 characteristics may permit recognition of mature neutrophils derived from leukemic clones. We have observed these abnormal neutrophils quite frequently in acute myeloid leukemia with maturation, i.e., the M2 variety, as well as in certain cases of hematopoietic dysplasia. We plan to follow patients with the latter condition to see whether the presence of such neutrophils correlates with the eventual appearance of acute leukemia.

Several of the patients in previously published case reports had acute promyelocytic leukemia with clinical and laboratory findings suggestive of DIC (30, 36, 39). In this study, we have described 3 more patients, all with well-differentiated AML, whose neutrophilic promyelocytes and myelocytes contained giant pink granules. Unlike the patients previously studied, they showed no definite evidence of DIC.

We have no explanation at present for these different clinical findings. In addition, the survival of more than 3 years in Case 3 is atypical of AML and deserves further comment. Despite persistent bone marrow morphology consistent with the diagnosis of AML and the presence of megagranules in many neutrophilic precursor cells, the peripheral blood remains relatively normal. This case may therefore be similar to the one previously described by Branda et al. (11) in which there was an indolent course as well as a marked dichotomy between the blastic morphology of the bone marrow and a relatively normal blood picture. In addition, the blasts of their patient contained prominent vacuoles and frequent Auer rods. Furthermore, when the bone marrow was cultured, the leukemic cells differentiated to form abnormal primary granules which appeared to rupture and cause cytolysis of these cells. On the basis of such observations, they suggested that the indolent clinical course might be related to blast destruction within the marrow and recommended less intensive chemotherapy. It is reasonable to postulate that our Case 3 has intramedullary destruction of leukemic cells.

REFERENCES

Abnormal Granules in AML

Fig. 1. Electron micrograph of a neutrophilic promyelocyte from bone marrow, stained for peroxidase. The cytoplasm contains at least 6 abnormally large round granules (arrows), the pseudo-Chediak-Higashi granules, in addition to normal-size azurophil granules (ag). Very small peroxidase-positive vesicles (ve) are present near some of the large spaces. nu, nucleus; m, mitochondria; er, endoplasmic reticulum. Inset, x 17,500. Inset, light micrograph of a Wright-Giemsa-stained bone marrow preparation showing 2 large pink inclusions in a promyelocyte. N, nucleus, × 3,000.

Fig. 2. Higher magnification of part of a neutrophilic promyelocyte from bone marrow, stained for peroxidase. Pseudo-Chediak-Higashi granule (p-CH) is limited by a single unit membrane (me), similar in thickness to the membranes of the adjacent azurophil granules (ag). Although the matrix of the pseudo-Chediak-Higashi granule is not entirely uniform, no crystalline structure could be detected even on unstained specimens. er, endoplasmic reticulum. × 67,000.

Fig. 3. Electron micrographs of neutrophilic promyelocytes from bone marrow, stained for peroxidase. In a, note the abnormally large (about 1 μm) round peroxidase-positive granule (p-CH) in addition to many smaller normal-size (about 500 nm) azurophil granules (ag). The perinuclear cisterna (pn), endoplasmic reticulum (er), and Golgi cisternae (Gc) are not reactive for peroxidase. × 16,000. Inset, light micrograph of an aldehyde fuchsin-stained preparation showing normal reactive granules (a–f) and 2 large unstained megagranules (arrows). × 1,300. In b, the cluster of small reactive vesicles (ve) can be seen to better advantage. These vesicles probably fuse (ve) to form the larger granules (p-CH). Other peroxidase granules (ag) are of normal size. m, mitochondria; N, nucleus. × 40,000.

Fig. 4. Portions of the cytoplasm of myeloblasts and promyelocytes, illustrating several other abnormal forms of peroxidase-positive granules. The granules in a and b may represent immature forms present before full crystallization of granule contents has occurred, as in the elongated form in c. Numerous small vesicles (ve) are present near these forming granules. ag, normal azurophil granules; m, mitochondria; er, endoplasmic reticulum; pn, perinuclear cisterna. a, × 22,000; b, × 39,000; c, × 27,000. Inset, light micrograph showing an elongated granule (arrow) like the one in c. Such granules were azurophilic. × 1,600.

Fig. 5. Electron micrograph of a more mature neutrophil from bone marrow, stained for peroxidase. Although the nuclear chromatin is condensed and segmented (a) and the cell is presumably a mature polymorphonuclear neutrophilic leucocyte, its cytoplasm contains very few peroxidase-negative specific granules (sg). In a normal mature polymorphonuclear neutrophilic leucocyte, about two-thirds of the granules would be peroxidase-negative specific granules, and about one-third would be peroxidase-positive azurophilic granules (ag). Some of these peroxidase-positive granules are also abnormal in that they tend to be larger and less homogeneous in content than normal azurophilic granules. A nuclear bleb (b) is also present. N, and N, nuclear segments; m, mitochondria. × 20,000.
Electron Microscopic and Peroxidase Cytochemical Analysis of Pink Pseudo-Chediak-Higashi Granules in Acute Myelogenous Leukemia

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