ABSTRACT

Giant round pink inclusions (=2 μm) 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 H2O2 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 glycosaminoglycans.

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. Their lack of azurophilia is due to the absence of sulfated glycosaminoglycans.

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 azurophil (primary) granule population. In addition, these 3 cases did not have DIC.

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 μm) 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-β-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 x 10⁹/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 $\times$ 10⁹/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 electron microscopic examination in May 1976.

The patient was treated with thioguanine, vincristine, prednisone, 1β-d-arabinofuranosylcytosine, and cyclophosphamide 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 differential 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 contained 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 WBC count. He received chemotherapy for the first time on March 9, 1976. Initially, he was given cyclophosphamide, thioguanine, prednisone, and vincristine every 4 weeks, but, beginning in November 1977, he received the combination of cyclophosphamide, thioguanine, prednisone, 1β-D-arabinofuranosylcytosine, and cyclophosphamide and was in remission for 6 months. During this period, 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°C, 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 previously 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 endogenous products of the leukocytes was easily resolved by electron 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 azurophil (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 (3a, inset), nor dialyzed iron (not illustrated), 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 $\rightarrow$ vesicles $\rightarrow$ Golgi complex $\rightarrow$ 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>
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<th>Bands + polymorphonuclear leukocytes</th>
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Table 1  
Summary of initial blood data for 3 patients with AML

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Abnormal Granules in AML

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 diazoyed 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 mucosubstances(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).

Schmaizl 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...
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. 28). 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


Electron Microscopic and Peroxidase Cytochemical Analysis of Pink Pseudo-Chediak-Higashi Granules in Acute Myelogenous Leukemia

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