Ultrastructure and Cytokinetics of Leukemic Myeloblasts Containing Giant Granules

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ABSTRACT

Leukemic myeloblasts containing abnormal granules were studied with ultrastructural, cytochemical, and thymidine labeling techniques to evaluate defects in granulogenesis and proliferation. Giant granules (1 to 3 μm in diameter) and Auer rods were observed in leukemic cells from two patients, and only rarely were both abnormal granule types observed in the same cell. The lysosomal origin of these abnormal granules was demonstrated by their content of peroxidase, esterase, and anionic glycoconjugates. Fusion of small dense granules (less than 0.2 μm in diameter) appeared to be increased in cells containing Auer rods and/or giant granules, but fusion of intact primary granules (0.2 to 0.4 μm in diameter) and sequestration of cytoplasmic contents were observed only in giant granules and not in Auer rods. Although the small granules that fused to form giant granules and Auer rods appeared similar, there was no evidence for transformation of giant granules into Auer rods. In one patient, cells with abnormal granules could easily be distinguished from the larger population of cells that lacked abnormal granules. The perturbation of these two distinct populations by chemotherapy was evaluated with thymidine labeling experiments. A high percentage (2- or 3-fold greater) of the abnormally granulated myeloblasts incorporated tritiated thymidine when compared to myeloblasts without abnormal granules in the same specimen. This difference could have resulted from an underlying metabolic defect which affected both granulogenesis and cell division. These results demonstrate that the formation of giant granules in leukemic cells is morphologically similar to that observed in the Chediak-Higashi syndrome and that leukemic cells with abnormal granules may differ cytokinetically from uninvolved leukemic cells.

INTRODUCTION

Abnormal granulogenesis is frequently observed in myeloid cells from patients with leukemia and myeloproliferative disorders (4, 32, 41). Although Auer rods were initially described as abnormal granules in lymphoid leukemia (3), they have subsequently been recognized as abnormal primary granules which form exclusively in acute nonlymphocytic leukemia (1). Ultrastructural studies of Auer rods have disclosed a unique periodicity of dense material, the presence of acid phosphatase (19, 20, 38), and an abnormal packing and concentration of peroxidase (4, 6, 11). Giant granules resembling those described in the Chediak-Higashi syndrome (12, 22) have also been observed in leukemia and myeloproliferative disorders (18, 26, 34), but they have not been observed in association with Auer rods. Since "pseudo-Chediak-Higashi" granules in leukemic leukocytes have not been studied ultrastructurally, a detailed comparison with abnormal granulogenesis in the Chediak-Higashi syndrome has not been possible.

Although the Chediak-Higashi-like granules are an acquired manifestation of the leukemia, patients with the Chediak-Higashi syndrome may develop lymphoma or myeloproliferative and accelerated lymphoma-like disorders (9, 33), which suggest the presence of an underlying defect that affects both granulogenesis and cell division. The increased destruction of leukocytes with abnormal granules in the Chediak-Higashi syndrome may result in increased proliferation (8); however, [3H]dThd³ labeling studies have not been evaluated in this disorder, nor have previous studies demonstrated proliferative differences in leukemic cells with abnormal granules.

The present study of the morphological characteristics and the proliferative activity in relationship to these characteristics was undertaken to identify defects in granulogenesis and proliferation and to determine if these abnormalities were related to those described for the Chediak-Higashi syndrome.

MATERIALS AND METHODS

Patients. Patient 1 was a 9-year, 10-month-old white girl who was well until 2 weeks prior to the diagnosis of acute myelocytic leukemia, when she developed symptoms of bruising and leg pain. Patient 2 was a 13-month-old white girl diagnosed to have acute promyelocytic leukemia after a 4-week history of increasing petechiae and anemia. After diagnosis, both patients underwent 4 to 8 weeks of induction therapy, which included vincristine, actinomycin D, 1~-D-ara-bino furanosylcytosine, 6-azauridine, L-asparaginase, and methotrexate. Despite this intensive therapy, bone marrow aspirates from the patients continued to demonstrate replacement of the marrow space with leukemic cells. Patient 1 developed severe coagulation problems and died of an intracranial hemorrhage 5 weeks after diagnosis. Patient 2 expired 21 weeks after diagnosis with progressive disease complicated by severe liver dysfunction and hemorrhage.

Light Microscopy. In addition to routine Wright’s staining of blood and marrow smears, the following cytochemical procedures were performed: (a) ethanol-formalin-fixed smears were stained for the demonstration of peroxidase according to the method of Kaplow (25); (b) naphthol AS-D chloroacetate esterase was demonstrated in buffered formalin-fixed smears, using a naphthol AS-D chloroacetate solution (Sigma Chemical Co., St. Louis, Mo.) as described by Yam et al. (40); (c) α-naphthyl acetate esterase was demonstrated in buffered formalin-fixed smears, using an α-naphthyl acetate solution (Sigma) as de-

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3 The abbreviation used is: [3H]dThd, tritiated thymidine.
Giant Granules in Leukemic Cells

Thymidine Labeling

After written consent, bone marrow samples from both patients were studied on 6 occasions during the first week of therapy as part of an ongoing protocol to evaluate the effects of chemotherapy on leukemic cell kinetics. Bone marrow from the posterior iliac crest was aspirated into heparinized syringes, and 1 ml of the aspirate was incubated at 37°C with 1 μCi of [3H]dThd (1.9 Ci/mmole; Schwarz/Mann, Orangeburg, N. Y.) for 1 hr with intermittent agitation. The cells were rinsed, routinely smeared on glass coverslips, and air dried. The specimens were dipped in NTB2 emulsion and held for 14 days in the dark. The slides were then developed with Kodak D-19 and Kodak rapid-fix solutions. Cells containing 5 or more labels were considered to be labeled. The percentage of labeled blasts was then determined by counting 1000 cells in a Wright's stain preparation, and the percentage of labeled cells containing giant granules was determined in an aldehyde fuchsin stain preparation (17).

Electron Microscopy

A portion of the heparinized marrow aspirate was transferred to 1-ml glass tubes and centrifuged at 1000 x g for 3 min to separate the buffy coat. The buffy coats were fixed and minced in 3% glutaraldehyde, pH 7.35-0.1 M cacodylate buffer for 1 hr at 4°C. This was followed by 3 rinses in 0.1 M cacodylate buffer (7 g/dl sucrose), pH 7.35.

In addition to morphological preparations, a portion of the specimen was processed for demonstration of intrinsic peroxidase according to the method of Graham and Karnovsky (21). The substrate medium consisted of 10 ml of 0.05 M Tris-HCI buffer, pH 7.6, saturated with 3 mg of 3,3'-diaminobenzidine with 0.01% H2O2 (3 drops of 3% H2O2 added immediately before use). A portion of each specimen was incubated for 30 min at room temperature in the complete substrate medium. To achieve better stain penetration, some specimens were first incubated overnight at 4°C in medium lacking H2O2, followed by a 30-min incubation in the complete medium. Control specimens were processed similarly except that H2O2 or the diaminobenzidine was omitted.

The morphological and cytochemical preparations were rinsed in buffer and postfixed for 1 hr in 1% OsO4. The specimens were then routinely dehydrated with alcohol and propylene oxide and embedded in Spurr low-viscosity medium. Thin sections of morphological preparations were counterstained with uranyl acetate and lead citrate, whereas thin sections of cytochemical preparations were not counterstained. The sections were viewed with a Philips 300 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Light Microscopy

In Patient 1, giant granules (1 to 4 per cell; up to 3 μm in diameter) were observed in 2.5% of bone marrow blasts, whereas Auer rods (1 or 2 per cell) were present in 0.2% of blasts. The majority of blasts from Patient 1 did not contain abundant cytoplasmic granules. In contrast, most of the blasts from Patient 2 contained numerous cytoplasmic granules, and 30% of the blasts contained one to several (as many as 15) Auer rods. Giant granules (1 to 4 per cell) were present in 4% of blasts, and both giant granules and Auer rods were observed in 0.3% of cells. Abnormal granules were easily identified in Wright's stained preparations as reddish purple, needle-like or round giant granules but were more frequently observed in aldehyde fuchsin-stained specimens (Fig. 1). For optimal staining of acid mucosubstance, the aldehyde fuchsin solution was allowed to ripen overnight at room temperature, was filtered, and was used within 48 hr. Giant granules also demonstrated strong reactivity for peroxidase, naphthol AS-D chloroacetate esterase, and Sudan black. In Patient 1, 80% of blasts demonstrated naphthol AS-D chloroacetate esterase staining, and 12% of blasts contained a-naphthyl esterase staining. In Patient 2, 90% of blasts stained for naphthol AS-D chloroacetate esterase, and 10% stained for a-naphthyl esterase. Leukemic blasts lacked alkaline phosphatase or periodic acid-Schiff reactivity.

Thymidine Labeling

The labeling index (determined by counting 1000 Wright's stained blasts) in Patient 1 varied from 7 to 11% during the first week of chemotherapy, whereas in Patient 2 the values varied from 3 to 7%. Background labeling was minimal, and no labeling was observed in specimens not exposed to [3H]dThd. As reported previously by this laboratory (31), a single labeling index has an inherent experimental error, which was estimated by a separate analysis of variance of 50 triplicate labeling index determinations for marrow leukemia blasts. Using this method, a difference in the labeling index is significant at the 0.05 level if 2 successive labeling indices exceed 1.86 or are less than 0.54 of the other determination.

In Patient 1, aldehyde fuchsin staining allowed differentiation of cells containing normal and abnormal granules, and labeling indices for each of these populations were determined (Fig. 2; Chart 1). Only 1 to 3% of the blasts in each sample contained abnormal granules. The labeling index of cells with abnormal granules was determined by counting an average of 170 cells per timed sample (1020 cells for the entire 168-hr period). In

![Giant Granules Graph](image-url)

Chart 1. Labeling indices for all leukemic cells, leukemic cells with giant granules, and leukemic cells with normal granules. The labeling index of blasts with abnormal granules was significantly higher in all except the 168-hr specimen. VCR DAUNO, vincristine-daunomycin; ARA-C, 1-β-D-arabinofuranosylcytosine; 6 AZUR, 6-azauridine.

OCTOBER 1979 3835

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Patient 2, the heavy granulation and intense staining of the blasts prevented accurate differentiation of these subpopulations. The labeling index of cells with abnormal granules was consistently greater than the labeling index of cells with normal granules. It was significantly elevated in all except the 168-hr sample.

**Electron Microscopy**

**Morphology.** Leukemic blasts in both patients contained nuclei with dispersed chromatin and large nucleoli. Although the majority of leukemic cells resembled myeloblasts or promyelocytes with round nuclei, abundant endoplasmic reticulum, and distinct primary granules (Fig. 3), several cells evidenced monocytic characteristics such as irregularly folded nuclei and small pleomorphic granules. Cytoplasmic maturation often exceeded nuclear maturation and resulted in nuclear-cytoplasmic asynchrony as described previously (5–7).

Primary granules 0.2 to 0.4 μm in diameter were observed in various maturational stages in the majority of cells (Fig. 3). Immature granules contained a moderately dense core with an electron-lucent rim, whereas mature granules were uniformly dense. In addition, small dense granules, less than 0.2 μm in diameter, were frequently observed in these cells and appeared increased when compared to descriptions of normal myeloid cells (5, 36). No secondary granules could be identified in leukemic blasts.

Several cell profiles contained one or 2 megagranules which were membrane limited and were up to 3 μm in diameter. Some of these granules contained a uniformly dense nucleoid (Fig. 3), and some contained less dense material, whereas others contained membrane-like material or entrapped cytoplasmic organelles (Fig. 4).

Abnormal maturation of primary granules was observed in some leukemic cells. These cells had segmented mature nuclei with primary granules that often contained myelin figures or other heterogeneous material (Fig. 5).

The morphological appearance of Auer rods was more uniform than that observed for giant granules. These granules were membrane limited and were up to 4 μm long and 1 μm wide (Fig. 6). The granules usually contained electron-dense material in the periphery and lucent or extracted material in the center. Many Auer rods contained a crystalline structure with periodic dense material similar to that described previously (11).

Cells observed at the ultrastructural level rarely contained both Auer rods and giant granules (Fig. 7). Cells with a similar finding were also infrequently seen with the light microscope. Abnormal granules were not observed in eosinophils, basophils, platelets, or megakaryocytes.

Golgi vesicles and small dense granules often contacted each other, as well as giant granules (Fig. 7). Auer rods, and normal-appearing primary granules. Entrainment of cytoplasmic material and contact and/or fusion with intact primary granules (0.2 to 0.4 μm in diameter) were observed only in association with megagranules (Fig. 7) and not with Auer rods. Fusion of small dense granules resulted occasionally in the formation of a membrane-limited circular (possibly spherical) profile which enclosed other cytoplasmic organelles (Figs. 6 and 8).

Segments of endoplasmic reticulum often contacted adjacent endoplasmic reticulum and other cytoplasmic organelles (Fig. 10), similar to those previously described in some cases of myelocytic leukemia (28). Some segments of endoplasmic reticulum appeared to be excessively dilated and contained membrane material.

**Cytochemistry.** The diaminobenzidine reaction product was often observed in primary granules, small dense granules, megagranules (Figs. 11 and 12), Auer rods (Figs. 13 and 14), and membrane-limited circular profiles (Fig. 15). Although stain penetration into tissue blocks was improved in specimens exposed to the diaminobenzidine overnight (Figs. 13 to 15), similar staining of individual cell organelles was observed with the 30-min exposure to the staining solution (Figs. 11 and 12). Infrequent staining was observed in Golgi lamellae and endoplasmic reticulum. Small dense peroxidase-positive granules (less than 0.2 μm in diameter) were frequently observed contacting and/or fusing with Auer rods (Figs. 13 and 14) and megagranules (Fig. 11). Larger peroxidase-positive primary granules were occasionally observed contacting megagranules but not Auer rods. Some Auer rods and megagranules lacked the diaminobenzidine reaction product despite the presence of reaction product in nearby normal and abnormal granules of the same cell.

No reaction product was evident in normal and abnormal granules or circular profiles of cells exposed for 30 min to control solutions lacking the H2O2 or diaminobenzidine (Fig. 16). Specimens exposed to the diaminobenzidine overnight without subsequent addition of H2O2 contained cells with a moderate amount of dense reaction product in the cytoplasmic granules. This density was not evident in overnight controls in which the diaminobenzidine was omitted from the solution.

**DISCUSSION**

These studies have compared the formation of Auer rods and giant granules in leukemic myeloblasts and identified associated abnormalities in granulogenesis. Both abnormal granules appear to evolve from exaggeration of the normal process (5, 36) of coalescence and fusion of small primary granule precursors and Golgi-derived vesicles. Although abnormal fusion of granules has been observed in leukemic cells containing Auer rods (6, 11, 20, 32) and in leukocytes with giant granules from patients with the Chediak-Higashi syndrome (15, 37, 39), the observation in this study of both types of abnormal granules in the same specimen has provided a unique opportunity for investigation of abnormal granulogenesis. We have observed that granule fusion resulting in Auer rod formation appears limited to the small dense granules less than 0.2 μm in diameter, whereas giant granule formation encompasses this process as well as fusion of intact primary granules and frequent entrapment of cytoplasmic material. Thus, the latter process may result from more extensive cellular dysfunction.

Unlike Auer rods, the formation of giant granules or pseudo-Chediak-Higashi granules occurs only rarely in leukemic myeloblasts. To our knowledge, only 2 cases have been reported in the literature (34), and, although the ultrastructural features of these granules were not investigated, the ultrastructure of giant platelet granules observed in some cases of myeloid leukemia has been related to the Chediak-Higashi anomaly (26). The granules observed here in leukemic blasts were...
Giant Granules in Leukemic Cells

histochemically and ultrastructurally similar to the abnormal primary granules of patients with the Chediak-Higashi syndrome. The presence of a limiting membrane and their content of peroxidase and esterase clearly distinguish the giant granules in this study from Dohle bodies (41) or other giant inclusions previously reported in a myeloproliferative disorder (13).

The morphology of the leukemic cell giant granules is similar to giant granules previously demonstrated in the Chediak-Higashi syndrome (15, 37, 39), in which large granules are synthesized as a single intact primary granule or form as a result of continued fusion of primary granules and/or small dense granules (15) and entrapment of cytoplasmic contents (37, 39). Although proposed explanations for the abnormal granule fusion in the Chediak-Higashi syndrome include altered intracellular cyclic nucleotides and dysfunction of microtubules (8, 27) and/or granule membranes (39), additional studies are required to define underlying defects leading to the abnormal granule fusion in leukemic and Chediak-Higashi leukocytes.

The nature of the increased small dense granules in these specimens is not known. A similar increase in small granules has been observed in neutrophils of mink with Chediak-Higashi disease (15). The reactivity of these small granules with diaminobenzidine, pH 7.4, suggests a Golgi-derived origin similar to that of peroxidase-containing primary granules rather than an origin similar to that of catalase-containing peroxisomes that demonstrate similar staining at higher pH levels (24).

The distribution of the diaminobenzidine reaction product in the abnormal granules is similar to previous observations localizing peroxidase in Auer rods of leukemic cells (1, 4, 11) and giant granules in Chediak-Higashi leukocytes (22). The diaminobenzidine reaction product presumably localizes peroxidase in neutrophil primary granules (16); however, this reagent also reacts with hemoglobin and other heme proteins under similar conditions (21). The interpretation of the reaction is more specific for enzyme activity after a 30-min incubation with a negative control solution lacking H2O2, as done for some specimens in this study. Although the overnight incubation with the diaminobenzidine improves stain penetration in the specimen, the “control” specimen lacking H2O2 also demonstrates some staining. This may result from localization of a broader range of heme proteins or may indicate the presence of some H2O2 intrinsic to the specimen. The lack of peroxidase staining in a few Auer rods and giant granules with both incubation times is consistent with the peroxidase deficiency reported for abnormal primary granules in some cases of myelocytic leukemia (14). Alternatively, the finding could result from masking of the peroxidase by another component of the granule or extraction of the enzyme during tissue processing. The possibility that some of the peroxidase-negative giant granules originate from secondary granules similar to those recently observed in the Chediak-Higashi syndrome (30) would seem unlikely in these patients, since secondary granulogenesis was not observed in the leukemic blasts.

The findings of numerous giant granules and few Auer rods in one patient, numerous Auer rods and few giant granules in another patient, and the infrequent occurrence of both granule types in the same cell suggest that the 2 granules differ and that one is not a precursor of the other. However, the presence of moderately enlarged granules (1 µm or less in diameter) with homogeneous content in cells with Auer rods observed here and reported previously (6, 11, 32, 38) indicates that some granule enlargement, similar or possibly identical to that of giant granule precursors, does occur prior to condensation of the granule contents to form the Auer rod. The lack of morphological or quantitative evidence for a transition from giant granules to Auer rods in this study suggests that, although these granules share some abnormalities in granulogenesis, they mature to form 2 distinct abnormal granule types. Alternatively, a transition of Auer rods to giant granules could occur with progressive cellular dysfunction and autophagy of Auer rods. However, the rare occurrence of pseudo-Chediak-Higashi granules in myelocytic leukemia, despite the relatively frequent observation of Auer rods, would suggest that, if this process occurs, it is very infrequent.

The presence of cytokinetic differences in these cells is indicated by their difference in nucleoside incorporation, which persisted even under the influence of chemotherapy. The marked difference between the [3H]dThd labeling of leukemic blasts with and without giant granules in Patient 1 may indicate that a relatively larger proportion of the cells with abnormal granules was undergoing DNA synthesis. The high labeling index could result from rapid cell division with few resting cells or from a slowing of the DNA synthesis time with accumulation of cells in the S phase of the cell cycle. Alternatively, there may be accelerated production of giant granules during the later part of interphase which is subsequently related to death of these cells as they pass into the early G1 phase. This would result in a decrease in G0 and G1 cells and a relative increase in S-phase cells. Accelerated death of myeloid cells in Chediak-Higashi disease (8) is consistent with this hypothesis.

Recent studies of Chediak-Higashi leukocytes (10, 27) have shown both excessive amounts of cyclic adenosine 3':5'-monophosphate, which is known to stimulate the flow of cells into S phase (2, 35), and simultaneous dysfunction of microtubules (23, 27, 29), which may prevent cells from completing a normal mitosis and further accentuate an accumulation in late interphase. Similar abnormalities may exist in leukemic cells with giant granules and result in perturbations of both granulogenesis and the cell cycle. The additional observation of malignant degeneration of leukocytes in the Chediak-Higashi syndrome (9, 33) further suggests an underlying metabolic defect which could alter normal cell growth and division. Conceivably, the presence of giant granules in leukemic cells could herald the emergence of a second rapidly dividing cell clone; however, the consistently low percentage of affected cells despite progression of the disease would argue against this possibility. Nevertheless, the findings in this study of defective growth and division evidenced by giant granule formation and increased [3H]dThd labeling in the same cell population suggest a unique deviation of these cells from normal control mechanisms.

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Fig. 4. A giant inclusion in this cell contains heterogeneous membrane-limited material. × 40,000.

Fig. 5. Rare "leukemic" cells, such as this, demonstrate apparent condensation of nuclear chromatin and segmentation of nuclear lobes. Granule maturation is, however, grossly abnormal in that most primary granules appear degenerated and contain myelin figures (arrows). × 19,000.

Fig. 6. This leukemic cell contains several enlarged and elongated granules that correspond to Auer rods sectioned longitudinally (L), perpendicularly (P), or tangentially (T). The central portions of the granules appear less dense and may have been extracted during tissue processing. × 20,000. Inset, an almost circular (possibly spherical) profile that appears to result from fusion of small dense granules. × 51,000.
Fig. 7: This leukemic cell contains a number of abnormal granules, some of which resemble Auer rods and one of which contains heterogeneous material similar to that observed in some giant granules (inset). A few granules (arrows) are tangentially sectioned and may represent branched or Y-shaped variants of the Auer rod. × 26,000; inset, 65,000.
Fig. 8. Golgi lamellae (G) adjacent to a giant granule containing a central nucleoid (thick arrow) and heterogeneous material. The inclusion is contacted by a primary granule (P) below and several Golgi vesicles (thin arrows) above. Adequate fixation of this cell is evidenced by the preservation of the nuclear envelope and mitochondria (M). × 40,000.

Fig. 9. Concentric (possibly spherical) profiles of membrane-bound dense material are present in this leukemic cell. Additional circular profiles are in the periphery of the cell and are associated with coalescence of small dense granules (arrows). × 42,400.

Fig. 10. The circular profiles of rough endoplasmic reticulum (arrows) closely approximate each other, forming a lamellated structure. × 28,100.
Fig. 11. Dense diaminobenzidine reaction product stains primary granules as well as a giant granule (arrow). The nucleoid of the megagranule contains fine dense reaction product and is surrounded by a more heterogeneous flocculent reaction product. × 22,600.

Fig. 12. A diaminobenzidine-reactive megagranule (arrow) is present in this mitotic leukemic cell with an anaphase distribution of chromosomes (C). × 17,000.

Fig. 13. Several diaminobenzidine-reactive small granules appear to be fusing with this Auer rod. × 4200.
Fig. 14. Periodic arrays of dense diaminobenzidine reaction product are present in perpendicular (P) and longitudinal (L) sections of Auer rods in this leukemic cell. × 28,100. Inset, a small granule fusing with one of these structures. × 70,000.

Fig. 15. Dense diaminobenzidine reaction product is present in these circular profiles (cf. Fig. 9), suggesting a relationship to aberrant primary granulogenesis. One structure encloses a mitochondrion (arrow). Dilated segments of endoplasmic reticulum (ER) lack enzymatic reaction product. × 69,300.

Fig. 16. In this control specimen incubated for 30 min in a diaminobenzidine solution without added H$_2$O$_2$, megagranules (arrows) lack the density observed in cytochemical preparations (cf. Figs. 11 to 15). × 20,500.
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