Crystalloid-Containing Giant Granules in Leukemic Monoblasts

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Giant granules (≥ 1 μm in diameter), Auer rods, and unique giant granules that contained crystalloid material were investigated in monoblasts from a patient with acute monocytic leukemia using ultrastructural and cytochemical techniques. The latter granule type differed from abnormal granules previously described in leukocytes. In Wright's stained preparations, these granules appeared to be crystalloid-containing vacuoles and were observed in approximately 10% of the blasts. Ultrastructural examination of this granule type revealed a limiting membrane enclosing a variably dense matrix and a central crystalloid. Some giant granules contained multiple crystalloids. Giant granules, Auer rods, and both the matrix and the crystalloid of the crystalloid-containing giant granule possessed heavy acid phosphatase and peroxidase reactivity. At the electron microscope level, 45% of the monoblasts contained abnormal granules. Auer rods were observed in 1% of monoblasts, giant granules in 26% of monoblasts, giant crystalloid-containing granules in 10% of monoblasts, and 8% of monoblasts demonstrated both giant granules and crystalloid-containing giant granules. Auer rods were not observed in cells containing giant granules or crystalloid-containing giant granules. Neither phagocytosis of Auer rods nor autophagy was observed, and the transition of Auer rods to giant granules or crystalloid-containing giant granules could not be demonstrated. Genesis of giant granules appeared to result from fusion of small Golgi-derived precursor granules, azurophilic granules, and giant granules with each other. Crystalloid formation was only observed after substantial granule enlargement. These findings detail multiple abnormalities of granule genesis in leukemic monoblasts, which are comparable but distinctly different from abnormalities previously reported in leukocytes from patients with the Chediak-Higashi syndrome or in leukemic cells.

ABNORMAL GRANULOGENESIS is a common finding in patients with nonlymphocytic leukemia. Previously, two distinct populations of aberrant granules were reported in the leukocytes of patients with myelocytic or monocytic leukemia and represent pathologic formation of primary or azurophilic granules. The more frequently observed type consists of Auer rods that contain a periodic crystalloid structure and exhibit cytochemical staining for peroxidase, acid phosphatase, esterase, and phospholipids. A second and less frequent type of abnormal granule in leukemic myeloid cells is a giant granule, which is morphologically and cytochemically similar to the giant granules seen in the Chediak-Higashi syndrome. Previous studies of Auer rods and giant granules in myeloid cells indicated an origin from Golgi-derived organelles and in the latter granule type, from fusion of the primary granules and autophagy. In addition, rarely did these two types of abnormal granules occur in the same cell, presumably as a result of their different mechanisms of granulogenesis.

The present report extends previous morphological and cytochemical investigations of abnormal granules to the monocytic series and describes an additional unique granule population in the monoblasts of a patient with monocytic leukemia.

MATERIALS AND METHODS

Patients

The proposita is a 15-yr-old girl who developed fever, respiratory distress, and bleeding from her gums, which progressed over the previous week. Positive physical findings included pallor, petechiae, and hepatosplenomegaly extending 3 cm beyond the costal margin.

Admission lab work included a CBC, which showed a Hb of 6.2 g/dl, a Hct of 21.1%, 194,000 RBC/cumm. 210,000 WBC/cumm, and 8000 platelets/cumm. The leukocyte differential demonstrated 98% blasts, 1% segmented neutrophils, and 1% lymphocytes. Coagulation screen (PT, PTT, fibrinogen) was normal. The bone marrow aspiration was consistent with acute monocytic leukemia (see Results, Light Microscopy), and the chromosomal preparations of marrow and blood cells revealed no abnormalities.

The patient was initially treated with a drug regimen consisting of vincristine, adriamycin and prednisone. Complete response was not obtained after 4 wk, and therapy was changed to cytosine arabinoside and 6-thioguanine. Her course was further complicated by severe Candida albicans gingivitis and gastroenteritis requiring therapy with intravenous amphotericin B. No bacterial pathogens could be identified. She achieved remission 8 wk after initial diagnosis, and after maintaining a remission an additional 12 wk, she was referred to Seattle, Wash., for bone marrow transplant. Engraftment was successful, and she remains in remission 44 wk post-transplant.

Light Microscopy

In addition to routine Wright's stained preparations of blood and marrow smears, the following cytochemical procedures were performed. (1) Peroxidase reactivity was demonstrated by staining ethanol-formalin-fixed smears according to the method of Kaplow. (2) Acid phosphatase reactivity was shown by utilizing a naphthol AS-B1 phosphoric acid solution (Sigma Chemical Co., St. Louis, Mo.). (3) Combined esterase staining was demonstrated on citrate-acetone-methanol-fixed smears with α-naphthyl
stained blood smear (A: granules are present in monoblasts of this Wright's large vacuoles (v) or lucent containing giant granule in B) and (arrow, enlarged x1600; B: x2500).

Electron Microscopy

At the time of initial diagnosis and after receiving informed consent, marrow and blood samples for electron microscopy were obtained in heparinized syringes by aspiration of the posterior superior iliac crest and routine venipuncture, respectively. Buffy coats obtained by centrifugation at 1000 g for 3 min were finely minced in 3% glutaraldehyde 0.1 M cacodylate buffer, pH 7.35, at 4°C. After 1 hr fixation, the tissue fragments were rinsed 3 times in 0.1 M cacodylate, 7 g/dl sucrose buffer, pH 7.35.

In addition to preparation of the tissue for morphological studies, various cytochemical procedures were performed. (1) Intrinsic peroxidase reactivity was demonstrated by the method of Graham and Karnovsky.22,23 Tissue was incubated in a substrate medium consisting of 10 ml of 0.05M Tris HCl, pH 7.6, and saturated with 3 mg of 3,3'-diaminobenzidine (DAB, Sigma Chemical) and 0.01% H2O2 (3 drops of 3% H2O2 added immediately prior to use). Some specimens were pretreated by overnight incubation at 21°C in substrate medium without H2O2. Control specimens were processed simultaneously in medium lacking DAB or H2O2. (2) Acid phosphatase reactivity was accomplished by incubation of the specimen for 45 min at 37°C in Gomori's medium as modified by Barka using β-glycerophosphate (Sigma Chemical) as the substrate.24 Control specimens were processed similarly except that the substrate was omitted. (3) Spicer's high iron diamine staining was accomplished by overnight incubation of the tissue in a phenylenediamine (Eastman Kodak Co., Rochester, N.Y.) and ferric chloride solution.25 Intrinsic density of a simultaneously processed specimen without the incubation medium served as the control specimen.

All tissues were then rinsed 3 times in cacodylate sucrose buffer and postfixed in 2% OsO4 in 0.1 M cacodylate buffer. Dehydration through graded alcohols and propylene oxide was followed by embedding in Spurr low viscosity medium (Ladd Research Industries, Inc. Burlington, Vt.). Thin sections (60 nm thick) of morphological preparations were counterstained with uranyl acetate and lead citrate, whereas those for cytochemical preparations were not counterstained. All cytochemical and morphological specimens were examined with a Philips 300 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Light Microscopy

Wright's stained blood and marrow smears demonstrated a sheet of leukemic blasts that contained folded nuclei, prominent nucleoli, and a moderate amount of cytoplasm (Fig. 1). Giant granules, Auer rods, and crystalloid-containing giant granules were found in 25%-27%, 14%-18%, and 9%-10% of the leukemic monoblasts, respectively (Table I). A few myeloid cells were observed but lacked giant granules or Auer rods. Crystalloid-containing giant granules varied from 1 to 5 μm in diameter and contained a reddish metachromatic crystalloid in a lucent matrix. Vacuoles were noted in many of the leukemic monoblasts and could not be distinguished from some lucent or extracted giant granules. Moderate to strong α-naphthyl esterase reactivity was present in 36% of cells in marrow specimens but was confined to smaller granules, and little or no such reactivity was observed in abnormal granules of the same cells. Weak α-naphthyl esterase staining was present in almost all leukemic cells. Naphthol AS-D chloroacetate esterase staining was present in only 6% of cells, and none of these cells contained giant granules or Auer rods.

Peroxidase reaction product was present in the granules of almost all monoblasts (Fig. 2). Unfortunately, the ability to distinguish giant granules from crystalloid-containing giant granules in these monoblasts was obscured by the density of the reaction product. Unlike Wright's stained preparations, few vacuoles were observed in monoblasts of cytochemical preparations. Consequently, the vacuoles in Wright's stained preparations appeared to contain peroxidase. The distribution and intensity of Sudan black B staining was similar to that observed for peroxidase.

Acid phosphatase reactivity in the granules was

Fig. 1. A crystalloid-containing giant granule (arrow, enlarged in B) and large vacuoles (v) or lucent granules are present in monoblasts of this Wright's stained blood smear (A: x1600; B: x2500).
### Table 1

<table>
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<tr>
<th>Method of Evaluation</th>
<th>Distribution of Granule Populations in Leukemic Cells</th>
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<td>No Abnormal Granules (%), Giant Granules Only (%), Crystalloid-Containing Giant Granules Only (%), Auer Rods Only (%), Giant Granules and Crystalloid-Containing Giant Granules (%)</td>
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<td>Light microscope (Wright’s stain) Blood (n=200) Marrow (n=200)</td>
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<td></td>
<td>46 47 25 27 9 11</td>
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<td></td>
<td>Electron microscopy* Blood (n=458)</td>
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<td>55 26 10 1 8 1 0 1 0</td>
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*Entire cell scored.
T Volunteers were observed in several cells.
*Mid-profile of cell (60-nm thick) scored.

The discrepancy between light and electron microscope results suggests that the vacuoles appearing in a larger percentage of cells at the light microscope level were actually giant granules, as evidenced by the relative infrequency of vacuoles at the electron microscope level and the apparent enzymatic reactivity of these vacuoles in cytochemical preparations.

Approximately 10% of the monoblasts displayed PAS block positivity. The reaction product could not be clearly localized to giant granules, Auer rods, or giant crystalloid-containing granules.

Electron Microscopy

Morphology

The majority of leukemic cells demonstrated typical features of leukemic monoblasts. These cells contained a single folded nucleus with dispersed chromatin and one or two large nucleoli per cell profile (Fig. 3). The cytoplasm enclosed a moderate amount of single ribosomes, a variable number of polyribosomes and mitochondria, and sparse rough endoplasmic reticulum. The majority of cell profiles contained prominent Golgi cisternae and a few to several small pleomorphic granules. No autophagy or heterophagy was observed. Abundant microfilaments were observed in several cell profiles. Microtubules were less frequent but were present and appeared morphologically normal.

At least three distinct abnormal granule types were identified with variable frequency in the leukemic monoblasts. The content of giant granules (1 μm in diameter) was generally homogeneous and varied from loose floculent material to moderately dense material (Figs. 4 and 5). Some of these granules contained myelin figures (Fig. 3). A distinct subpopulation of giant granules contained 1-10 crystalloids. (Fig. 2. This light microscopic cytochemical preparation demonstrates peroxidase reactivity in the abnormal granules and apparent "vacuoles" (cf. Fig. 1). Although some giant granules (arrows) are apparent, crystalloids are obscured by reaction product (x 1600).)
per profile (Fig. 5). Many of the crystalloids were rod-shaped and extended over the diameter of the granule (Fig. 3). Periodic dense material (6.5–7 nm apart) was observed in a few crystalloids (Fig. 3). The matrix of these granules was similar to giant granules lacking crystalloids. Auer rods up to 5 µm in length were also observed and appeared electron dense (Fig. 6). Rare Auer rods contained less dense material located at either pole of the crystalloid (Fig. 7).

Abnormal granules, ≥1 µm in diameter, were observed in 45% of 458 consecutive and randomly scored mid-profile leukemic cells. Only giant granules were observed in 26% of the cells, only crystalloid-containing giant granules in 10%, and only Auer rods in 1% of the cells. Giant granules and crystalloid-containing giant granules were not observed in cells with Auer rods. Giant granules and crystalloid-containing giant granules were observed in 8% of cells.

Fusion of Golgi-derived precursor granules and intact primary granules was frequently observed in leukemic monoblasts. Auer rods were observed fusing with small Golgi-derived precursor granules only, while giant granule enlargement occurred by fusion of small pleomorphic granules in addition to fusion with precursor granules. Giant granules were also observed fusing with other giant granules and with crystalloid-containing granules.
Fig. 4. A giant granule and a crystalloid-containing giant granule (arrow) are located adjacent to Golgi cisternae (G) in this portion of a monoblast. The centriole (C) appears morphologically normal. Thin section stained with uranyl acetate and lead citrate (×25,000).

Fig. 5. The majority of giant granules in this leukemic monoblast contain moderately dense flocculent material. One granule contains multiple small electron-dense crystalloids (CG), while another granule contains a myelin figure (MF). Apparent fusion (arrows) is occurring between both granule types. Thin section stained with uranyl acetate and lead citrate (×22,500).

Fig. 6. This monoblast contains two closely adjacent Auer rods. Thin section stained with uranyl acetate and lead citrate (×15,000).

Fig. 7. This rarely observed granule contains an Auer rod and moderately dense material located at one or both poles (arrows). Thin section stained with uranyl acetate and lead citrate (×22,500).
Cytochemistry

Giant granules, Auer rods, and giant crystalloid-containing granules in leukemic monoblasts demonstrated dense reaction product indicative of the presence of peroxidase (Figs. 8–11). Both the crystalloid and matrix of the crystalloid-containing giant granule demonstrated variable reactivity (Fig. 8). In some giant granules, the crystalloid appeared obscured by the intense staining of both the crystalloid and the matrix. The reaction product was also localized to small pleomorphic granules, Golgi cisternae, and endoplasmic reticulum (Fig. 9). Occasionally, weak reaction product was observed in mitochondrial cristae. Similar staining was observed after substrate incubation for 18 hr or 30 min. Control specimens

Fig. 8. Dense DAB reaction product localizes peroxidase in giant granules (GG) and crystalloid-containing giant granules (CG). Small pleomorphic granules are also reactive. Thin section unstained (x22.500).

Fig. 9. This micrograph of a DAB-stained specimen demonstrates coalescence of two giant granules as well as numerous small pleomorphic granules. Some profiles are suggestive of granule fusion (arrows). DAB reaction product is also present in endoplasmic reticulum (ER). Thin section unstained (x21,000).

Fig. 10. This crystalloid-containing giant granule possesses a flocculent matrix and a single crystalloid (arrow) that reacts strongly with DAB. Thin section unstained (x23,000).
Fig. 11. Dense DAB reaction product is present in an Auer rod and azurophilic granules of this monoblast. A small granule appears to have fused with the Auer rod (arrow). Thin section unstained (x32,500).

without DAB lacked the electron density observed in the experimental specimen. Specimens incubated 30 min without H₂O₂ also lacked the staining.

Acid phosphatase reactivity was observed in variable amounts in the normal and abnormal granules of monoblasts (Fig. 12). Some reactive giant granules and smaller pleomorphic granules evidenced stronger staining in the periphery. Both the matrix and the crystalloid of crystalloid-containing giant granules contained reaction product (Fig. 13). These stained sites lacked similar electron density in control specimens processed without substrate incubation.

Staining with high iron diamine localized sulfated glycosaminoglycans in some neutrophil primary granules. Granules within leukemic monoblasts possessed no reaction product.

DISCUSSION

Morphological and cytochemical examinations of abnormal granules in leukemic monoblasts from a patient with acute monocytic leukemia have identified giant granules, Auer rods, and a unique population of crystalloid-containing giant granules. Acid phosphatase and peroxidase reactivity of these latter granules, and their apparent origin from Golgi-derived organelles, indicated their lysosomal nature. Autophagic and heterophagic processes did not appear to contribute to the genesis of these abnormal granules. Although previous studies have described both Auer rods and giant granules in leukemic myeloblasts, crystalloid-containing giant granules similar to those seen in our patient have not been reported in leukemic cells, nor has the formation of giant granules been examined in monoblasts. Ultrastructural examination of giant granules from myeloblasts of a previously reported patient did, however, demonstrate microcrystalline structures distinct from Auer rods and not evident at the light microscope level. The presence of large crystalloids in some giant granules appears to be unique to the leukemic monoblasts of our patient and is distinct from previous observations in leukemic myeloblasts and leukocytes from patients or animals with the Chediak-Higashi anomaly.

The observation of giant granules with and without crystalloids in the same cell suggested a common mechanism in the formation of these two granule types. The lack of crystalloids in smaller granules indicated that crystalloid formation occurred in a late stage of granule enlargement. Consequently, some giant granules may represent a precursor stage of crystalloid-containing giant granules. The origin of multiple crystalloids in some giant granules is not clear, but conceivably could represent a precursor stage of crystalloid assembly, fragmentation of a single crystalloid, or a separate end-stage giant granule with multiple crystalloids (Fig. 14).

Previous ultrastructural studies of myeloid cells from patients with the Chediak-Higashi syndrome have demonstrated at least two mechanisms of abnormal granule genesis. The first mechanism involved fusion of Golgi-derived granule precursors and intact primary granules to form a giant primary granule with homogeneous content. This mechanism appeared to represent an exaggeration of the normal process of fusion of small Golgi-derived precursor granules to form primary granules. A second mechanism of
giant granule formation implies a broader range of cellular dysfunction, in that primary granules and their precursors fuse with other cell organelles, resulting in a giant autophagic granule or vacuole containing heterogeneous material. In our previous studies of giant granules in leukemic myeloblasts from two patients, both mechanisms of giant granule formation were observed. The present case differed in that giant granule formation was not accompanied by evidence of autophagy. Furthermore, ring-shaped lysosomes seen in Chediak-Higashi monocytes and some leukemic myeloblasts were not encountered in this study. Thus, giant granule formation in the monoblasts from this patient appeared to result only from fusion of enlarged granules, azurophilic granules, and their precursors without entrapment of cytoplasmic organelles.

The lack of giant granules and crystalloid-containing giant granules in cells with Auer rods, indicated that formation of the latter abnormal granule type (Fig. 15) was distinct from that observed for the former granule type (Fig. 14). Similarly, in previous studies of two patients, only very rarely were giant granules and Auer rods in the same cells. Also, in these earlier studies, fusion of small Golgi-derived granule precursors appeared to contribute to the formation of both abnormal granule types, whereas fusion of larger granules and autophagy appeared to occur only during giant granule formation. Although autophagy was not a prominent feature in the leukemic cells from the patient in the present study, fusion of Golgi-derived precursor granules and/or azurophilic granules with giant granules or with each other, appeared to contribute to giant granule genesis, whereas genesis of Auer rods involved only fusion of small Golgi-derived precursor granules. This observation implies that giant granules result from a defect that persists throughout granule formation, whereas granules in cells containing Auer rods demonstrate a defect only at an early stage of granule assembly, i.e., the Golgi-derived precursor.

Our patient never demonstrated laboratory or clinical evidence of disseminated intravascular coagulation. This syndrome has been observed in some other cases of myelocytic leukemia in which hypergranular blasts contained giant granules. In other reported cases without evidence of coagulopathy, most leukemic cells did not contain an excessive number of granules. This preliminary analysis indicates that it is the quantity of granules, rather than the presence of giant granules or Auer rods, which results in the coagulopathy observed in previous patients.

In summary, we have demonstrated multiple abnormal granule types in leukemic monoblasts. Auer rod formation appears similar to that observed in some cases of myelocytic leukemia. Similarly, some aspects of giant granule formation resemble those observed for giant granules in the Chediak-Higashi syndrome and myelocytic leukemia. The formation of crystalloid-containing giant granules appears to be an unique finding in the monoblasts of our patient and may represent a variant of the giant granules observed in the leukemic monoblasts. The observation of at least two exclusive populations of cells—one containing giant granules and the other containing Auer rods—suggests the presence of two or more biologically different leukemic monoblasts. The presence of several metabolically different cell types may account for the poor response to chemotherapy in this patient and previous patients with leukemic cells containing multiple abnormal granule populations.

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