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Abnormalities in Granule Formation in Acute Myelogenous Leukemia

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Granule formation was investigated in differentiating neutrophils of a patient with acute myelogenous leukemia (AML) by means of the combined techniques of electron microscopy and peroxidase cytochemistry. Two important pathologic features were observed: first, an abnormal concentration and packaging of peroxidase into Auer rods in leukemic promyelocytes, and second, the presence of Auer rods surrounded by single-unit membranes in some mature polymorphonuclear leukocytes (PMN). An additional unexpected finding was the discovery of two distinct populations of PMN circulating concurrently; a minor (<5%) normal one that contained both peroxidase-positive azurophilic and peroxidase-negative specific granules and a major abnormal one characterized by the absence of specific granules. None of these abnormalities was observed during the two remissions of this patient's disease. During relapse a "hiatus leukemicus" occurred, which also revealed two populations of cells, a majority population of leukemic blasts, and a minority population of a few normal PMN. These findings documented several developmental abnormalities in the differentiating cells of myelogenous leukemia and also suggested that concurrent normal and abnormal populations of PMN may be a helpful diagnostic feature of a leukemic process.

In the course of observing a 24-yr-old female patient with acute myelogenous leukemia (AML) before therapy and during 27 mo of remission and relapse, the following pathologic features were encountered: (1) the formation of Auer rods in leukemic myeloblasts and promyelocytes; (2) the presence of Auer rods in mature polymorphonuclear leukocytes (PMN); and (3) the phenomenon of "hiatus leukemicus." These findings were analyzed by the combined techniques of electron microscopy and peroxidase cytochemistry.

Auer rods are easily recognized on smears with Romanowsky-type stains because they are large cytoplasmic inclusions which stain reddish-purple (i.e., azurophilic); they also stain for peroxidase. The present study illustrates the fine-structural localization of peroxidase in early granulocytic cells that are producing Auer rods and documents several deviations from the usual secretory pathway by which this enzyme is packaged into azurophil granules.

Although Auer rods can be observed in myeloblasts and promyelocytes of 10%-15% of patients with AML, they have rarely been described in the mature PMN of such patients, and no attempt has yet been made to explain the significance of this finding. Initially, we speculated that perhaps the Auer rods had been phagocytosed by normal PMN, or, alternatively, that the leukemic cells had undergone significant maturation. By examining these cells by means of

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electron microscopy, which to our knowledge had not been done before, we were able to distinguish between these two possibilities.

Ultimately, late in the course of this patient’s illness “hiatus leukemicus” as described by Naegeli was observed, with the presence of immature forms (myeloblasts and a few promyelocytes) and mature PMN without many intermediate forms.

While pursuing these easily recognized pathologic features, we discovered that the PMN of this patient comprised two distinct populations, one normal and one abnormal. The normal PMN contained both peroxidase-positive azurophilic and peroxidase-negative specific granules, whereas the abnormal PMN contained only one granule population. Some of these abnormal PMN also contained Auer rods.

These findings, presented here in the form of a case report, document several developmental abnormalities in the differentiating cells of myelogenous leukemia. They also suggest that the presence of concurrent normal and abnormal populations of PMN in the circulation may be another helpful diagnostic feature of a leukemic process.

MATERIALS AND METHODS

Case Study

A 24-yr-old white female secretary was first admitted to the University of California San Francisco Medical Center on March 21, 1972 with a painful Bacteroides abscess of the buttock. The white blood cell count was 10.6 x 10^9/liter, and Wright’s-stained smear revealed 28%, myeloblasts, 14%, promyelocytes, 11%, myelocytes, 19%, metamyelocytes, 19%, PMN, 24%, lymphocytes, and 3%, monocytes. In addition, many myeloblasts and promyelocytes contained long, narrow azurophilic Auer rods, but none was observed in the later stages of PMN maturation. The leukocyte alkaline phosphatase (LAP) score was very low; only 11 cells contained any reaction product (normal range: 40–80). No clinical evidence of disseminated intravascular coagulation was found. The hemoglobin was 6.7 g/dl; platelets, 39 x 10^9/liter. A bone marrow aspirate revealed replacement of the granulocytic series (10%-15% myeloblasts and 25%-30% promyelocytes), about 10% of whose cells contained Auer rods (Fig. 1A). These azurophilic bodies were also visible in marrow granulocytes in all stages of maturation up to and including myelocytes and bands (Fig. 1B) as well as mature PMN (Fig. 1C). Careful reexamination of the peripheral blood smear showed no classic azurophilic Auer rods in circulating PMN, but indistinct lilac-staining rod shapes were detected. These structures were most easily visualized by phase-contrast microscopy. The diagnosis of acute myelocytic leukemia was made. Bone marrow and blood were processed for fine-structural examination by methods that have been previously reported.

Complete remission was achieved for 13 mo by the administration of cytosine arabinoside and 6-thioguanine, with consolidation therapy in June, July, and August 1972. The patient suffered recurrent relapses with partial remissions in April, July, and November 1973, as well as in January and February of 1974. Auer rods were consistently present in early myeloblasts of both the marrow and peripheral blood until March 1974, after which they disappeared and were not observed again. Tissues were obtained for electron-microscopic examination during remissions in August 1972 and in August 1973. In November 1973 and February 1974 a definite “hiatus leukemicus” was noted in smears of bone marrow (Fig. 2); that is, the presence of myeloblasts, a few promyelocytes, and PMN without many intermediate forms. This tissue was analyzed by electron microscopy. No further specimens were examined by electron microscopy after this time.

After treatment with whole-body irradiation and Cytoxan (Mead Johnson, Evansville, Ind.), a successful bone marrow transplantation from an HLA-matched brother was performed in April 1974, resulting in restoration of normal hematologic parameters and apparent remission of leu-

*We believe them to be Auer rods that had lost their metachromasia and no longer stained azurophilic. Normal azurophil granules also lose their metachromasia during maturation.
Fig. 1. Light micrographs of Wright's-stained smears of bone marrow and blood obtained before institution of therapy for AML. Large, elongated azurophilic Auer rods (arrows) were seen in (1A) numerous blasts and promyelocytes (P), (1B) myelocytes (M), metamyelocytes, and bands (B); and (1C) in mature PMN. A and B, × 1230; C, × 1600.

The patient felt well until late June, when she acquired a disseminated herpesvirus infection, and she died on July 4, 1974. At autopsy, scattered foci of leukemic cells were found in the ovaries, kidneys, and bone marrow.

RESULTS

Electron-microscopic Studies

Previous studies have identified Auer rods as large, membrane-bound organelles containing crystalline matrices. These structures are very dense (electron opaque) because of their content of peroxidase, and they are formed...
Fig. 3. Diagrammatic representation of the hypothetical steps involved in the formation of azurophilic granules in normal neutrophilic promyelocytes. Peroxidase reaction product has been observed in increasing concentrations within the RER, Golgi cisternae, and azurophilic granules, indicating that in general the pathway of secretion and condensation of this enzyme conforms to that of secretory proteins in the pancreas and in other cell types. These steps include the following: (1) synthesis on bound ribosomes, (2) segregation within RER cisternae, (3) pinching off of vesicles from transitional elements of the RER and their transfer to the Golgi complex via junctional vesicles, (4) packing and concentration of the enzyme within the Golgi cisternae and the formation of Golgi-derived vesicles, (5) aggregation of smaller vesicles into large, immature azurophilic granules, and (6) condensation to produce the azurophilic granules of relatively uniform size and shape. Azurophilic granules occur in two main forms: the majority are spherical, with dense homogeneous matrices; others are ellipsoid, with crystalline substructures. Round granule profiles with a central periodicity are presumed to represent ellipsoids cut perpendicular to the crystal axis (right lower corner).

in the early stages of maturation during the promyelocyte stage. For full appreciation of the aberrations which occur in their formation, the pathway of normal azurophil granule formation (RER → Golgi complex via vesicles → azurophil granules) is reviewed briefly in Fig. 3.

Observations on Bone Marrow and Blood Before Therapy

In the abnormal promyelocytes of our patient (Figs. 4 and 5), definite deviations from the normal process of enzyme concentration and granule formation were observed as follows:

(1) A smaller concentration of peroxidase within the Golgi cisternae (Figs. 4 and 5A). This finding was in accord with the earlier report of Breton-Gorius and Houssay.

(2) Numerous small vesicles with high peroxidase activity (Figs. 5A and 5B). These could have been junctional vesicles (see diagram, Fig. 3, step 3) that had pinched off the RER, bypassed the Golgi cisternae, and aggregated to form the larger Auer rods (Fig. 4), or perhaps they were derived from the Golgi vesicles, concentration having occurred after this point (see diagram, Fig. 3, step 5). Our data did not permit a distinction between the two possibilities.
Fig. 4. Electron micrograph of an abnormal promyelocyte containing Auer rods from the initial bone marrow specimen, staining for peroxidase. This cell has a large, immature nucleus (N) with dispersed chromatin and a nucleolus (Nu). The most striking abnormality of the cytoplasm, when compared to that of a normal promyelocyte, is the marked heterogeneity of granule size, shape, and matrix morphology. Auer rods are intensely reactive for peroxidase and may appear oblong (Au) or round (Au'), depending on the plane of sectioning. In addition to the Auer rods, numerous small vesicles located either in the Golgi region (Ve') or in the peripheral cytoplasm (Ve") contain dense reaction product. It appears that Auer rods are formed by the aggregation and fusion of small, reactive vesicles, such as those marked Ve". Images such as those marked Ve" may represent the product of fusion of numerous smaller peroxidase-positive vesicles. Very faint reaction product for peroxidase can be seen in the RER (rer) and perinuclear cisterna (pn). Note that the Golgi cisternae (Gc) contain little or no reaction product; m, mitochondria. Tissue fixed for 10 min at 4°C in 1.5% glutaraldehyde, reacted in 3,3'-diaminobenzidine and H₂O₂ at pH 7.6 for 1 hr, and postfixed in OsO₄. × 15,650.
Fig. 5. A, Golgi region of another abnormal promyelocyte, reacted for peroxidase. The reaction product in the RER (rer) and Golgi cisternae (Gc) is better visualized here than in Fig. 4 because of a difference in tissue preparation. The cell shown in Fig. 4 was stained en bloc with uranyl acetate, which tends to obscure this reaction product, whereas this staining procedure was omitted in Fig. 5. Otherwise, the two specimens were prepared identically. This micrograph also illustrates an abnormality: the Golgi cisternae (Gc) are much less reactive than the RER, vesicles (Ve), and forming granules (g). In normal promyelocytes, Golgi cisternae contain moderately heavy reaction product, usually more than the RER. × 12,200. B. A portion of cytoplasm from another abnormal promyelocyte, illustrating the accumulation of many small vesicles (Ve), which aggregate to form the large granules (g) and Auer rods. × 26,100. C. Auer rods with crystalline cores (arrow). × 87,000. Periodicity of these rods was difficult to visualize in this type of preparation because the reaction product partially obscured their fine structure. No precise measurements were attempted. pn, perinuclear cisternae.

(3) A disturbance in the process of aggregation and concentration of some of the azurophil granules, resulting in abnormally large, elongated granules: the Auer rods (Fig. 4), with crystalline cores (Fig. 5C).

In mature PMN, two populations of cells could be distinguished: (a) a minor population (<5%<sub>n</sub>) of mature PMN, which appeared to be normal, i.e., containing normal numbers of both peroxidase-positive azurophilic and peroxidase-negative specific granules (Fig. 6); and (b) a major abnormal population, i.e.,
GRANULE FORMATION IN AML

Fig. 6. Normal mature PMN, reacted for peroxidase, obtained from the initial bone marrow specimen. The cytoplasm is filled with both light and dark granules. The presence of peroxidase within the azurophilic granules (ag) renders them electron opaque and very dense, in contrast to the peroxidase-negative specific granules (sg), which are pale. Many small β particles of glycogen (β) are also present, as well as three lobes of the nucleus (N1–N3), and a small Golgi complex (Gc). × 16,500. Inset: specific granules (sg) and azurophils (ag) are better seen at higher magnification. × 43,500.

containing only peroxidase-positive azurophilic granules and lacking a specific granule population (Fig. 7). This finding indicated that cytoplasmic maturation had ceased at the postpromyelocyte stage and may have accounted for the very low leukocyte alkaline phosphatase activity seen at this time. Some abnormal PMN also revealed Auer rods (Figs. 7A and 7B). These peroxidase-positive structures were surrounded by single unit membranes (Fig. 7B) and were not contained within phagocytic vacuoles.

Observations on Bone Marrow and Blood During Remission and Relapse

Blood and bone marrow were obtained for fine-structural examination at the time of two of the chemotherapeutically induced remissions, and no abnormalities could be detected. All of the circulating PMN contained both peroxidase-positive azurophilic and peroxidase-negative specific granules similar to those illustrated in Fig. 6.

In November 1973 and February 1974 a true “hiatus leukemicus” was
Fig. 7. Mature PMN obtained before initial therapy and reacted for peroxidase. A. Nucleus shows the hallmarks of a mature cell in that five lobes (N1-N5) with markedly condensed chromatin are present. It is stained less intensely than the nuclear material in Fig. 6 because of the omission of uranyl acetate staining on the grid. Also, the cytoplasm contains numerous small β particles of glycogen (β) typical of the normal mature PMN. However, in contrast to normal PMN, this cell contains no peroxidase-negative (or specific) granules, which usually comprise 67% of the total granule population (cf. Fig. 6). In addition, there is considerable variation in the size and shape of normal azurophils (ag); a few are very small (little arrows), and others are abnormally large (long arrows). One obvious elongated Auer rod (Au) is present. × 16,500. B. Enlargement of A. The Auer rod (Au) is contained within a single membrane. × 42,500.
demonstrated in smears from bone marrow (Fig. 2). When this tissue was analyzed by electron microscopy, we found many myeloblasts and some promyelocytes including Auer rods (as in the cells depicted in Figs. 4 and 5), but the few PMN which were present were normal (i.e., similar to the cell in Fig. 6).

DISCUSSION

Formation of Auer Rods in Promyelocytes

Our findings confirm and extend those of earlier investigators regarding the morphology, time of appearance, and peroxidase content of Auer rods and document several aberrations in the usual secretory process by which peroxidase is segregated and stored. As in normal promyelocytes, the enzyme is synthesized near the beginning of neutrophil differentiation and can be visualized within the RER cisternae. In normal promyelocytes, however, the Golgi cisternae usually contain considerable amounts of peroxidase reaction product (at least comparable in quantity to that in the RER), and frequently enzyme deposits are more concentrated in cisternae along the concave side of the Golgi complex. However, in the Golgi cisternae of these leukemic cells containing Auer rods, reaction product generally appeared fainter (as originally noted by Breton-Gorius and Houssay) and the enzyme deposits were highly concentrated in numerous small vesicles. At present, it is impossible to determine the origin of these multiple vesicles containing concentrated peroxidase. They could be junctional vesicles (Fig. 3, step 3) derived from the transitional elements of the RER, which bypass the Golgi cisternae. Alternatively, the Golgi cisternae may fail to concentrate well (Fig. 3, step 4), so that most of the concentration is accomplished in the vesicles which “pinch off” from Golgi cisternae.

Control of aggregation and concentration within the forming granules is also abnormal, since the granules vary in size and are heterogeneous in content, while the crystals can be huge. Although the factors responsible for the concentration and packaging of enzymes into azurophil granules are completely obscure, Palade has explored the phenomenon of concentration of enzymes in zymogen granules of the pancreatic exocrine cell, and we believe that the events may be similar. Palade has proposed that concentration in the pancreatic exocrine cell is not achieved by membrane ion pumps in the condensing vacuoles but possibly occurs as a result of the osmotic effects of a sulfated polyanion, presumably a peptidoglycan. Hypothetically, this polyanion, together with secretory proteins that are predominantly cationic, can cause a reduction in the osmotic activity within the condensing vacuoles; the resultant efflux of water would thereby bring about concentration. Young has demonstrated by autoradiography the rapid incorporation of inorganic sulfate into the complex of immature granulocytes of rats and mice. Additional data on normal human leukocytes are needed before abnormalities in the condensation of azurophil granules in leukemic cells can be analyzed further.

Other workers have demonstrated that in addition to peroxidase, acid phosphatase and esterase are contained within Auer rods. In content, then, these structures resemble the azurophilic granules of normal neutrophilic promyelocytes as proposed by Ackerman in 1950 and confirmed by many
others, but the method of concentration and packaging of their constituents into storage organelles via Golgi cisternae may differ.

Auer Rods in PMN

As previously mentioned, we initially speculated that the presence of Auer rods in mature cells might indicate that these structures had been phagocytized by normal mature PMN or that they were present in leukemic cells that had undergone nuclear maturation. The first possibility was ruled out by the fact that these bodies were surrounded by single limiting membranes, whereas a phagocytic vacuole containing a phagocytized granule would be encircled by two membranes, the membrane of the vacuole itself and that of the granule. Thus we believe that these "mature" PMN with Auer rods were in fact leukemic cells in which the nuclei had undergone relatively normal maturation. This possibility of leukemic cell differentiation has been previously suggested by Rohr, Bessis, and Leder.

"Hiatus Leukemicus"

Naegeli described "hiatus leukemicus" as a phenomenon indicated by the appearance of myeloblasts and mature granulocytes without many intermediate forms. Bessis, noting Naegeli's observations, explained this finding as the simultaneous presence of leukemic cells which failed to mature and granulocytes which were derived from the maturation of the few remaining normal granulocytic precursors. The results of our fine-structural studies were in agreement with Bessis's hypothesis, since all the PMN we located were normal.

Concurrent Presence of Two PMN Populations: Normal and Abnormal

The combined techniques of electron microscopy and peroxidase cytochemistry have permitted us to identify at least two different populations of differentiating neutrophilic granulocytes—normal and abnormal—in this patient with AML. The abnormal PMN were identified by the absence of specific granules. It appeared that cytoplasmic development ceased after the promyelocyte stage, whereas nuclear maturation had progressed in a normal fashion, a phenomenon called "maturation anarchy" by Bessis. Before therapy, a minority (<5%) population of normal neutrophilic precursors and mature PMN could also be seen in the bone marrow. This observation, which was based on morphological data, was in keeping with the conclusions of Greenberg et al. and Metcalf et al., who analyzed the colony-forming capacity of bone marrow in cases of AML and also judged that coexistent normal and leukemic clones were present. Metcalf et al., in studying cultured marrow cells from patients with AML in remission, observed that clones of normal granulopoietic cells could appear and displace preexisting leukemic populations. The fact that the abnormal neutrophil population was not detectable during two complete remissions of disease in our patient agrees with this observation.

Since the recognition of this signal case, we have collected material from five other cases of AML that show abnormal, mature PMN intermixed with normal PMN as follows: (1) PMN containing only azurophils and lacking specific granules as reported in detail here; (2) PMN containing only specific granules and lacking azurophils; and (3) PMN containing both types of granules but
lacking the characteristic enzyme peroxidase. Similar abnormal PMN have been observed by Ullyot and Bainton in blastic crises of chronic myelogenous leukemia. These abnormal PMN probably account for the scattered observations of enzyme deficiencies made by conventional histochemical methods.

Of the 28 cases of AML studied by Catovsky's group, the PMN in 12 (43%) lacked peroxidase, an abnormality which affected 8%–70% of the circulating PMN. In our experience, these peroxidase-negative PMN would correspond in fine-structure to groups 2 and 3 mentioned above; i.e., they may be missing the azurophil granule population or simply missing the enzyme peroxidase.

Abnormalities of PMN granules have also been observed in a few nonleukemic disease states; only one population of PMN is mentioned. In this connection, it is noteworthy that in several instances of acquired refractory anemia, a disorder which frequently transforms into AML, two populations of PMN—one normal, the other with peroxidase deficiency—have been observed. One wonders now whether these patients could not be considered preleukemic.

Thus far, a survey of currently available information reveals that while an abnormally homogeneous PMN population can be present in nonmalignant disorders, heterogeneous (abnormal and normal) populations are strongly suggestive, if not diagnostic, of a leukemic process. Work in the future should be directed toward correlating the presence of these two populations with prognosis and response to therapy.

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