Facilitated Light Microscopic Cytochemical Diagnosis of Acute Myelogenous Leukemia

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ABSTRACT

Hydroperoxidase-positive Phi bodies and rods are much more prominent and prevalent than rods visualized with a Romanovsky-type stain (Auer rods) in immature leukocytes of patients with active acute myelogenous leukemia (AML). They are readily observed with the light microscope in peripheral blood or marrow films of AML patients stained to show their peroxidatic activity. In many of these patients, Auer rods, which apparently constitute only a small subpopulation of the hydroperoxidase-positive Phi bodies and rods, were detected with difficulty, if at all. The hydroperoxidase-positive Phi bodies and rods were observed in 92% of 36 patients with active disease. They were never observed in leukocytes of patients with other hematopoietic disorders or of normal individuals. Thus, they facilitated the distinction of AML from chronic lymphocytic leukemia and chronic granulocytic leukemia in blast crisis. They were absent in full clinical remission after chemotherapy and were greatly diminished in partial remission. They were present in disease relapse and reappeared in five patients who had been in full remission. These results suggest that these hydroperoxidase-positive enlarged particles are pathognomonic of AML and that monitoring them with the light microscope may aid in guiding its clinical management.

INTRODUCTION

AML is the most rapidly progressive of the prevalent forms of adult leukemia and has the highest incidence of any of the acute forms (5). Since AML requires different treatment than other forms of leukemia, such as ALL and CGL/BC, its differentiation from these is highly desirable. Although immunological and biochemical markers are becoming increasingly useful in determining the cell type of origin, the classification of leukemia continues to depend primarily on morphology and cytochemistry (8). The rods or bodies described by John Auer in 1906 and which bear his name are visible with the Wright-Giemsa or Romanovsky procedures. This was facilitated by the insertion of a green filter between the light source and the eyepiece of the microscope (9).

MATERIALS AND METHODS

Patient selection, classification, clinical staging criteria, and chemotherapy protocols will be described in detail separately. Study patients and controls ranged between 15 and 78 years of age. Controls included normal individuals and patients with a variety of inflammatory and infectious conditions as well as granulocytic and lymphoid cancers. Auer rods were detected in blood films stained with classical Wright-Giemsa or Romanovsky procedures. This was facilitated by the insertion of a green filter between the light source and the eyepiece of the microscope (9).

Staining methods for the cytochemical demonstration of the hydroperoxidases in human leukocytes have been previously described (7, 18). The demonstration of these particles can be readily performed on smears of bone marrow aspirate, smears of peripheral blood if the patient has an elevated leukocyte count, smears from the lateral wall of the vagina obtained and

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3 The abbreviations used are: AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; CGL/BC, chronic granulocytic leukemia, blast crisis.

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4 J. O. Moore, J. S. Hanker, and J. Laszlo, manuscript in preparation.
fixed in the usual manner, or smears of leukocytes collected by lavage of the gingival sulcus with a small amount of 0.9% NaCl solution. Gingival leukocytes were collected in a tuberculin syringe fitted with a Becton-Dickinson Longwell Teflon catheter. Details of the staining methods, collection methods, and procedures for concentrating leukocytes in leukopenic patients used in this study have been described in another paper (9), but the following details are sufficient for studying bone marrow or peripheral blood films of patients who are not leukopenic.

Smears or films of bone marrow aspirate or of peripheral blood prepared in the usual manner on glass slides or coverslips are kept in the dark until ready for processing. They are fixed by treatment with a mixture containing 1.25% glutaraldehyde and 1% formaldehyde that is 0.1 M in phosphate buffer (pH 7.3) for 1 min. The slides or coverslips are then rinsed in 0.9% NaCl solution until they can be incubated for hydroperoxidase demonstration.

The films are incubated for 1 min in an incubation medium (7) prepared immediately before use. The medium consists of 5 mg 3,3'-diaminobenzidine tetrahydrochloride, 10 ml Tris-HCl buffer (0.05 M, pH 7.6), and 0.1 ml 1% H2O2. The films are then rinsed briefly in 3 changes of Tris-HCl buffer (0.05 M, pH 7.6). They are then immersed for 1 min in 0.5% Cu(NO3)2 or CuSO4 in 0.05 M Tris-HCl, pH 7.6. They are then transferred to a 0.9% NaCl solution rinse in which they are kept until counterstaining (optional) with a modification of the Papanicolaou method used by Gill (6) which uses special solutions, resulting in a counterstain which does not mask the Phi bodies or other hydroperoxidase-positive particles.

The counterstain is carried out as follows. Immerse the specimen in Gill's hematoxylin No. 2 for 1 min. Rinse in 2 changes of distilled water for 10 sec each with agitation. Immerse for 1 min in Scott's tap water substitute for bluing. Rinse through 2 changes of distilled water and 2 of 95% ethanol for 10 sec each with agitation. Immerse in Gill's OG-6 for 1 min. Rinse in 3 changes of 95% ethanol for 10 sec each with agitation. Place in Gill's EA-50 for 10 min. Rinse in 3 changes of 95% ethanol for 4 min, 2 min, and 1 min, successively. Take through 3 changes (for 1 min each) of absolute ethanol and xylene. Mount in Permount.

RESULTS

These hydroperoxidase-positive particles were exclusively observed in leukocytes of patients with AML (Figs. 2 to 14); they were observed in 33 (92%) of the 36 patients with active disease (Table 1). To date, they have not been observed in patients with other hematopoietic disorders or in normal leukocytes. Phi bodies were also absent from leukocytes of patients with ALL or CGL/BC.

They were absent from cells of 5 patients in full clinical remission after chemotherapy and were greatly diminished in patients in partial remission. Phi bodies and rods with peroxidatic activity were present, frequently in large numbers, in patients who were first studied during disease relapse. They reappeared, after disappearing in remission, in 5 patients in relapse. They were also observed in 2 patients who had not been studied prior to relapse. In these cases, they were usually more apparent in marrow than in peripheral blood films.

In Romanovsky-stained films from most AML patients, Auer rods could be observed with difficulty, if at all (Fig. 1). However, it was not unusual to go back to a "negative" Romanovsky film and find Auer rods, once hydroperoxidase-positive rods and Phi bodies had been observed on a replicate film. In marrow films of 2 patients undergoing chemotherapy and considered to be in full remission by other criteria, Phi bodies and rods were revealed by hydroperoxidase staining (Figs. 10 and 11). This proved to be the first indication of impending relapse. In several instances, the presence of these particles suggested AML where ALL had been suspected; later immunological or clinical evidence corroborated the diagnosis of AML which was first suspected by scrutiny of the hydroperoxidase-stained films.

Counterstaining with a modified Papanicolaou stain (6) that frequently permits ready visualization of these particles because of diminished nuclear staining helped to identify the types of neutrophil precursors containing the Phi bodies or rods. These were generally myeloblasts (Figs. 2 to 5, 13, and 14) or promyelocytes (Figs. 5, 6, and 10), although occasionally more mature granulocytic cells contained them (Fig. 6). These particles were also readily observed in immature leukocytes harvested from the vagina, gingival sulcus (Fig. 13), cerebrospinal fluid (Fig. 14), and marrow cultures of AML patients.

Comparison of replicate blood films stained with either a Romanovsky procedure to demonstrate Auer rods or bodies or, alternatively, a procedure to demonstrate hydroperoxidase-positive Phi bodies and rods showed that the latter were much more prevalent and prominent (compare Fig. 1 with Figs. 2 to 5). In some cases, only a very small percentage (~ 5 to 10%) of the immature leukocytes in blood or marrow films contained hydroperoxidase-positive Phi bodies and rods. In these cases, Auer rods rarely could be observed in the Wright-Giemsa- or Romanovsky-stained slides. In other cases, as many as 75%

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**Table 1**

<table>
<thead>
<tr>
<th>Patient type</th>
<th>Special status</th>
<th>No. studied</th>
<th>No. positive</th>
<th>%</th>
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<tr>
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<td></td>
<td></td>
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<td></td>
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<td>Remission</td>
<td>29</td>
<td>26</td>
<td>90*</td>
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<td>Relapse</td>
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<td>0</td>
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<td></td>
<td>&quot;Preleukemic&quot;</td>
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<td>7</td>
<td>100</td>
</tr>
<tr>
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<td></td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGL*</td>
<td>Blast crisis</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>18</td>
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</tr>
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</table>

* The overall incidence, 92%, was obtained from the ratio of the total number of patients positive for Phi bodies and rods, 33, to the total number with active disease diagnosed by other criteria, 36.  
* CGL, chronic granulocytic leukemia.
DISCUSSION

These results suggest that the light microscopic examination of blood films for hydroperoxidase-positive Phi bodies and rods is the easiest way to detect AML. This is because of the far greater prominence and prevalence of these particles than of Auer rods. In addition, these enlarged hydroperoxidase-positive particles are observed in a much higher percentage of patients with active AML (92% versus 5 to 20% reported for Auer rods). This disproportion could be further evidence that hydroperoxidase-positive Phi bodies and rods are not synonymous with Auer bodies or rods, a subject that is under further study. Bessis (3) suggested that many more rods are visible by phase contrast microscopy in leukocytes of AML patients than Auer rods, visible by virtue of their azurophilia demonstrated by the Romanovsky-type stain; this would suggest that Auer rods are only a small subpopulation of the rods present. On the other hand, our hydroperoxidase staining procedure may be demonstrating almost the entire population. The unique ellipsoidal or fusiform shape of Phi bodies and the fact that those of salivary gland cells are not visible with the Romanovsky stain would tend to support this conclusion. On the other hand, the staining properties of Auer rods may be atypical (2), and they could lose their azurophilia. A diminution in visibility of some Auer rods due to masking by the methylene blue-stained nuclei in leukocytes of Romanovsky preparations could be a factor. Our occasional observation of Phi bodies with cores negative for hydroperoxidase also is a consideration.

The much lower intrinsic visibility of Auer rods in the Romanovsky preparations appears, however, to be the paramount consideration. Whether or not Auer rods and Phi bodies are identical, they certainly appear related, and the light microscopic identification of these hydroperoxidase-positive Phi bodies or rods by our procedure offers the hematologist the easiest way of detecting active AML. The identification of Phi bodies and rods in immature cells during the leukopenia which generally occurs during the chemotherapy of the disease makes the detection of active disease much more unequivocal with our procedure than a search for Auer rods. The very large numbers of Phi bodies and rods observed with this procedure (Figs. 2 to 5), relative to only a very occasional Auer rod in corresponding Romanovsky-stained replicate films (Fig. 1) lend weight to the suggestion that Auer bodies and rods may be a subpopulation of the hydroperoxidase-positive Phi bodies and rods. This also receives support from their similar distribution profiles in diseased leukocytes and their similar electron microscopic appearances (4, 9, 10).

The relationship of the Phi bodies and rods having peroxidatic activity to the processes of leukemogenesis and carcinogenesis is not known, but it may be significant that they have also been observed in proximal convoluted tubular epithelial cells and hepatocytes of rats treated with large doses of clofibrate, in which they arise from transformation of peroxisomes (10, 21). In addition, they are present in tissues of rats treated with nafenopin (19), which is a potent carcinogen, and have been found in Leydig’s cell tumors of rat testis and other solid tumors (20). Increased catalase levels have been observed in hepatocytes of livers of animals treated with nafenopin and clofibrate (14, 21). This could be related to the increase in catalase observed in leukocytes of patients with AML (12, 13, 16, 17). It may be significant that Phi bodies and rods with peroxidatic activity are observed in the affected cells in each case; they could be involved in sequestering excess catalase for storage or elimination (9–11).

The ultrastructural and cytochemical features of the Phi bodies and rods and the tendency toward pleomorphism suggest a resemblance to cell wall-deficient bacterial L form variants, which may display much greater catalase activity than the normal forms from which they were derived (22). There is a possibility that bacteria, which have catalase-positive L forms, and azurophilic granules of leukocytes have evolved from some common ancestor. As a result of some specific biochemical derangement in AML, the ellipsoidal form of azurophilic or primary granule may “remember” its ancestry, enlarge due to proliferation of its axial crystalloid, become a Phi body (analogous to the large round body of L forms), and extrude the axial crystalloid, segments of which subsequently detach to become free rods.

The demonstration of rods and Phi bodies in gingival and vaginal leukocytes suggests that these readily accessible cells could be valuable in the study of remission status as well as the migration properties of leukemic leukocytes. Their presence in cerebrospinal fluid leukocytes suggests that the detection of the disease in sanctuary areas and the study of routes by which diseased cells migrate to these areas could be facilitated. The appearance of these particles in leukocytes cultured from the marrow of patients with AML may not only aid in predicting remission or relapse but could be useful in the study of factors that augment or inhibit leukemogenesis.

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REFERENCES

Fig. 1. Romanovsky-stained film of enriched leukocyte preparation from peripheral blood. Although Auer rods could be found upon microscopic scrutiny of the film, they are not apparent in this light micrograph. x 1200 unless otherwise noted.

Figs. 2 to 5. Hydroperoxidase-positive rods and Phi bodies typical of AML are readily observed in replicate films of this patient stained by our method and counterstained by the Gill modification (6) of the Papanicolaou method.°

Fig. 6. In this marrow film, a fusiform Phi body is observed in the leukocyte with intense basophilic granulation at the right. Note the smaller azurophilic granules and mitochondria with diaminobenzidine. J. Histochem. Cytochem., 20: 793–803, 1972.

Fig. 7. Phi bodies are frequently observed in the nuclear hof as seen in the myeloblast to the left.

Fig. 8. Three Phi bodies markedly different in size but all of the “potato-masher” variety, i.e., having only one axial projection from the ellipsoidal center, can be discerned in the myeloblast (arrow).

Fig. 9. This light micrograph shows that the Gill counterstain° not only provides a “window” for the visualization of particles stained by virtue of hydroperoxidase activity but aids materially in identification of leukocyte type and maturation stage.

Fig. 10. Area of marrow film of patient thought to be in full clinical remission by the usual criteria. Note rods and focal accumulation of hydroperoxidase in the disrupted myelocytic cell at the left.

Fig. 11. Myeloblast in marrow film of patient thought to be in complete remission from examination of Romanovsky-stained replicate film. This cell has apparently risen from a leukemic rather than a normal clone.

Fig. 12. A Phi body (arrow) is observed in the process of detaching a rod.

Fig. 13. A gingival myeloblast harvested by noninvasive lavage of the gingival margin. The enlarged football-shaped granule or Phi body (arrow) is similar to that discerned in the myeloblast at the left.

Fig. 14. A small Phi body (arrow) observed in a myeloblast obtained from a patient’s cerebrospinal fluid confirmed meningeal leukemic infiltration.
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