Intracellular Lysozyme in Mature Neutrophils and Blast Cells in Acute Leukemia

By Hans Karle, Niels Ebbe Hansen, and Sven-Aage Killmann

Intracellular lysozyme (muramidase) activity was measured in leukemic blasts and mature neutrophilic granulocytes from 20 patients with acute myeloblastic and myelomonocytic leukemia and in 11 patients with acute lymphoblastic leukemia after differential centrifugation of cells in Ficoll and extraction of lysozyme with n-butanol. Considerable abnormalities in cellular lysozyme activity were found both in qualitative and quantitative terms. In contrast to normal myeloblasts, leukemic blasts of the myeloid series contained lysozyme in a considerable number of cases. Although no clear-cut distinction was seen, those patients with positive blast lysozyme reactivity tended to have the highest plasma lysozyme levels, whereas no good correlation was found between morphologic differentiation along myeloblastic or monocytoblastic lines of blasts and lysozyme reactivity. Calculations of the magnitude of lysozyme production in acute leukemias with high plasma lysozyme concentration was compatible with the hypothesis that in these cases lysozyme must be secreted by intact blasts and that, consequently, plasma lysozyme activity reflects the total leukemic cell mass. In mature neutrophilic granulocytes from patients with acute myeloblastic and myelomonocytic leukemia in relapse, the mean lysozyme activity was significantly decreased, although a great deal of variation was found. In remission, neutrophil lysozyme activity seemed to increase; among several possibilities this might be a reflection of different clones being operative in relapse and remission. In acute lymphoblastic leukemia, lysozyme activity in neutrophils was constantly low in relapse and increased to normal following induction of remission, which may be the main explanation of the low plasma lysozyme activity found in this type of acute leukemia. It is unexplained and puzzling why intraneutrophil lysozyme activity is low in a leukemic type where the myeloid cells are not believed to be primarily leukemic; one possible reason might be an effect of cell-to-cell interaction with the leukemic cell population.

Measurements of lysozyme activity in serum and urine have proven to be of some value in the classification of acute leukemia. Several reports have dealt with this diagnostic method; the subject has recently been reviewed by Perillie and Finch.

Lysozyme is found in cells of the myeloid series and in monocytes. It is primarily a granule bound enzyme which is present in both main types of neutrophilic granules, viz. the azurophils, which are formed at the promyelocyte stage, and the specifics which are formed in myelocytes. Consequently, whereas lysozyme is not present in normal myeloblasts, increasing lysozyme activity can be demonstrated from promyelocytes to myelocytes; in later myeloid cells...
Lysozyme activity is roughly the same as in myelocytes.

Modern pathogenetic concepts of acute leukemia imply a defect in cell differentiation which leads to an accumulation of immature cells with a retained potential for cell division. Due to the described orderly appearance of lysozyme activity in myeloid cells, measurements of lysozyme activity in leukemic cells may give information concerning cell differentiation.

Recently, a method of measuring intracellular lysozyme in neutrophilic cells, isolated by differential centrifugation, was outlined in our laboratory. The aim of the present work was to study lysozyme activity in blast cells and neutrophilic granulocytes from patients with acute leukemia of the myeloid/monocytic and lymphoblastic types, in remission and relapse.

MATERIALS AND METHODS

Patients

Lysozyme activity was measured in plasma, neutrophilic granulocytes, and blast cells from 20 patients with acute myeloid leukemia (AML), comprising myeloblastic and myelomonocytic types, and 11 patients with acute lymphoblastic leukemia (ALL). All the patients were adults, and the diagnoses were established on usual clinical, hematologic, and morphologic criteria. The induction of remission was ensured by bone marrow samples. When lysozyme studies were made, all the patients had normal serum creatinine levels, and infection was excluded on the basis of clinical evaluation, bacteriologic examination of blood and urine, and x-ray of the chest.

Morphologic Evaluation of Blast Cells

In the group of myeloblastic and myelomonocytic patients, a reevaluation of blast cell morphology in blood and bone marrow samples taken at the time of lysozyme studies was made by one of us (S.K.) without knowledge of lysozyme data. The blast cells were divided into three groups: typical myeloblasts, typical monocytoid blast cells, and intermediary cells, the main criteria for the distinction being features of nuclear morphology, especially the presence of indentations in or distortion of the cell nucleus. The nucleus was always leptochromatic. In each case 100 blasts were evaluated. The percentages of cells in the three groups in bone marrow and blood are presented in Table I.

Cell Separation and Measurement of Intracellular Lysozyme

Venous blood with EDTA as anticoagulant was used. The method of leukocyte separation and extraction of intracellular lysozyme has been described previously. The cell separation was made according to the Ficoll separation technique of Boyum, which effectively separates normal neutrophils from lymphocytes and monocytes. It has been shown that the "neutrophil layer" in normal individuals consists of 93% neutrophils, the remaining cells being eosinophilic and basophilic granulocytes and lymphocytes and only occasionally a few monocytes. This was true also for the leukemic patients, but in two cases of AML some blast cells were seen in this bottom layer. The blast cells were concentrated in the top layer. As, however, in some cases some mature neutrophils and monocytes were seen in this layer, an exact quantitation of blast cell lysozyme was not attempted, and, consequently, statements on blast lysozyme activity were limited to the qualitative terms "positive" and "negative." The total contamination from neutrophils and monocytes was in all cases below 15% except in one case (35%), and there was no difference in the degree of contamination between the groups classified as "positive" and "negative." In each patient, the amount of lysozyme contributed by neutrophils and monocytes was calculated using the values obtained from measurements on the bottom layer. Cases in which more lysozyme was present in the top layer than could be accounted for through this calculation were considered blast lysozyme positive. The assumptions for this calculation were that neutrophils in the top layer did not differ from those in the bottom layer with respect to lysozyme content and that neutrophils and monocytes have the same amount of lysozyme. Homogenization of the cell fractions was done by solubilization with n-butanol, and lysozyme concentration in plasma...
and cell material was measured with the turbidometric method of Litwack\(^7\) using a suspension of *Micrococcus lysodeikticus* (Boehringer and Soehne, Mannheim, Germany) and purified human lysozyme (kindly provided by Professor E. F. Osserman, Columbia University, New York, N.Y.) as standard. Lysozyme activity was expressed as micrograms per milliliter plasma and micrograms per 10^6 neutrophils.

Mixing experiments showed that neither potentiators nor inhibitors could be demonstrated in the cell extracts.

## RESULTS

*Lysozyme Activity in Blast Cells in AML in Relation to Plasma Lysozyme and Cell Morphology*

Tables 1A and B show lysozyme activity in blast cells in acute myeloblastic and myelomonocytic leukemia. Eighteen patients with blast cells in the periph-
eral blood were divided into two groups according to the plasma lysozyme concentration, one with values above normal (above mean + two times standard deviation, namely, 4.0 \(\mu g/ml\)), and one with normal or low values. For each patient the percentages of blast cells in the three morphologic groups are shown.

Nine of 11 patients in the high plasma lysozyme group showed positive blast lysozyme reaction, while only three of seven patients in the normal to low plasma lysozyme group were blast positive. The two blast-negative cases in the first group had only slight elevations in plasma lysozyme.

Although the patient with the highest plasma lysozyme value had the highest number of monocytoid blast cells, there was no good correlation between plasma lysozyme and cell morphology, and there was no significant difference in cell morphology between the two plasma lysozyme groups (Table 1). No correlation was found between the number of blast cells and the plasma lysozyme level in blast-positive cases.

As mentioned in Materials and Methods, exact quantitation of the blast cell content of lysozyme was not possible, but from the lysozyme levels in the blast-containing layer, it was evident that the amount of lysozyme in the blast cells must be of the same order of magnitude as in the mature neutrophils.

Lysozyme in Mature Neutrophils in AML

Determination of intraneutrophilic lysozyme content could be made in 18 cases of acute myeloblastic and myelomonocytic leukemia. The results are pre-

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Plasma Lysozyme ((\mu g/ml))</th>
<th>Neutrophils/(\mu l)</th>
<th>Neutrophil-Lysozyme ((\mu g/10^6) cells)</th>
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<tr>
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<td>4</td>
<td>69</td>
<td>8,730</td>
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<td>10</td>
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<td>19</td>
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<td>118</td>
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</tr>
<tr>
<td>20</td>
<td>1.0</td>
<td>386</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mean ± SD 1.4 ± 0.7
Normal 2.7 ± 0.8
\((n = 25)^2\)

Student's t test \(p < 0.001\)
presented in Table 2 and Fig. 1; it is seen that, although some variation was found, the mean intraneutrophilic lysozyme concentration was significantly decreased compared to normal (2.7 ± 0.8 (SD) µg/10⁶ neutrophils). In the group of patients with high plasma lysozyme, only low values were seen (Fig. 1). There was no correlation between neutrophilic lysozyme values and blast morphology.

Six patients had received cytotoxic agents (cytosine arabinoside, thioguanine, or daunomycin) for 1–4 days before the determination of neutrophilic lysozyme was made, but the values in these patients did not differ from the values in the remaining patients; the same was true for four additional patients who received only prednisone in doses of 15–40 mg/day.

Two patients were studied both at the time of diagnosis and during remission. As seen in Fig. 1, both showed an increase to normal values in remission.

Lysozyme in Mature Neutrophils in ALL

Table 3 shows plasma lysozyme, neutrophil counts, and neutrophilic lysozyme in ten patients with ALL in relapse. In all cases but one (early relapse) low values of intracellular lysozyme were seen. Three patients had received no treatment before the determination, four were on treatment with prednisone (15–80 mg/day), and three with both prednisone and cytotoxics (vincristine, 6-mercaptopurine, or thioguanine).

During remission (and still on continuous cytotoxic treatment), a remarkable change in intraneutrophilic lysozyme was seen in all cases. This is seen in Fig. 2, which shows the change from low values to normal or subnormal values in six cases. This phenomenon is furthermore illustrated in Fig. 3, which shows the changes in neutrophil lysozyme in relation to the hematologic course in a
patient with ALL. Initially, low values were seen simultaneously with lymphoblasts in the blood and low values of neutrophils and plasma lysozyme. In remission, concomitantly with a rise in neutrophil counts and plasma lysozyme, the amount of lysozyme in the neutrophilic granulocytes became normal. This pattern was repeated during successive periods of relapse and remission. As can be seen from the Fig. 3, there seems to be a further decrease in neutrophil lysozyme during the first period of treatment; this pattern was also seen in three other cases. One patient studied only during remission (on maintenance treat-

Table 3. Plasma and Intraneutrophilic Lysozyme Activity in ALL (Relapse)

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Plasma Lysozyme (pg/ml)</th>
<th>Neutrophils/μl</th>
<th>Neutrophil-Lysozyme (pg/10^6 cells)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>13,788</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1,730</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>580</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>1,740</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>1,900</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.8</td>
<td>169</td>
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</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>1,660</td>
<td>1.4</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>1,440</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>0.4</td>
<td>58</td>
<td>1.3</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>1,380</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Mean ± SD 0.9 ± 0.7 1.1 ± 0.5
Normal 2.4 ± 0.6 (2.7 ± 0.8)

Student's t test p < 0.001

Fig. 2. Intraneutrophilic lysozyme in patients with acute lymphoblastic leukemia in six cases examined both in relapse and in remission. The hatched area indicates normal neutrophil lysozyme (cf. Fig. 1).
ment with methotrexate) had a normal value of neutrophil lysozyme (2.4 μg/10⁶ cells).

Lysozyme activity could not be demonstrated in lymphoblasts.

DISCUSSION

This study has demonstrated profound abnormalities in leukemic cells in qualitative as well as in quantitative terms. Thus, contrary to normal, leukemic blasts of the AML variety may contain lysozyme activity, and mature neutrophilic granulocytes from patients with AML as well as with ALL showed significantly decreased lysozyme activity in relapse. Earlier studies on the quantitation of cellular lysozyme in leukemia have been made on total leukocyte extracts and give no information on lysozyme activity in the lysozyme-containing cells.⁴,⁸⁻¹¹

The present study showed no correlation between blast cell morphology in terms of differentiation along myeloid/monocytoïd lines and blast lysozyme reaction, since cases with obvious predominance of typical myeloblasts showed positive lysozyme reaction. Although Catovsky and Galton,¹² who used the method measuring bacterial lysis around individual cells, did find some correlation between blast lysozyme reactivity and morphology, there was a considerable overlapping between groups. On the other hand, in the present study, although there was some overlapping, the blast lysozyme-positive patients tended to have the highest plasma lysozyme levels. The presence of high plasma lyso-
zyme values seems thus to reflect total leukemic lysozyme-positive cell mass rather than a special cell type, and this might also explain the finding of normal plasma lysozyme in some cases with positive blast lysozyme reaction but few blast cells in the blood. That the plasma lysozyme level is related to cell mass of the leukemic population is also indicated from the studies of serial determinations of lysozyme in relation to therapy. 13,14

With knowledge about the rate of lysozyme turnover in man 15 one can easily calculate the turnover rate of cells giving rise to a certain plasma lysozyme level if the intracellular amount of lysozyme is known and if lysozyme stems only from disintegrating cells. Calculation along these lines on the basis of the highest plasma lysozyme value in this series (684 mg/ml) leads to a value for cell destruction amounting to about 15 kg cells per day (corresponding to about 45 g lysozyme per day), a result which seems quite unlikely. This would be compatible with the hypothesis that, at least in these cases, lysozyme is secreted from leukemic blasts. This possibility has been mentioned by Perillie and Finch 1 and experimentally supported by in vitro studies of Farhangi and Osserman. 6

The measurement of lysosomal enzymes makes it possible to evaluate neutrophil maturation. In the group of AML, the mean value of cellular lysozyme was significantly decreased in relapse, but some patients had normal values. The amount of lysozyme in the neutrophils did not correlate with either cell morphology of the blasts or plasma lysozyme. The finding of low neutrophil lysozyme is in agreement with the existence of agranularity or hypogranularity of the neutrophils in some cases of AML. 17 From the changes observed in relation to the induction of remission, the neutrophil lysozyme content seems to be related to the stage of the disease. This was in fact indicated by Asamer et al. 18 in an immunofluorescence study of lysozyme activity in leukemic cells. This pattern might have several explanations. In addition to those mentioned below, it could imply the operation of different clones of myeloid cells dependent on the phase of the leukemic disease.

More surprising was the constant finding of low neutrophil lysozyme in active ALL. In this group, all cases which were brought into remission showed a normalization of neutrophil lysozyme. The low plasma lysozyme levels found in ALL are generally explained as a result of a reduction in neutrophil turnover corresponding to the decrease in blood neutrophil counts, but low cellular content in the neutrophils would be a reasonable additional cause. From the parallel reduction in neutrophilic and plasma lysozyme values as shown in Table 3, it follows that the neutrophilic turnover rate in ALL in relapse should be normal.

The mechanism of the decrease in neutrophilic lysozyme in ALL is completely unknown and could be due to nonspecific factors or directly associated with the leukemic process. Thus, nonspecific factors such as bacterial infection and uremia have been shown to be associated with low neutrophil lysozyme contents, 2 and, although none of these were present in our patients, similar mechanisms might be operative (e.g., decreased protein synthesis in the cells). It is unlikely that the low neutrophil content resulted from treatment with cytotoxic agents or prednisone, since low lysozyme levels were found in both treated
and untreated patients, and since values were normalized when remission was induced with the patients still on treatment. If the low neutrophil lysozyme activity in ALL neutrophils was a direct result of the leukemic process, it could be due to an influence of the initiating leukemic factor on a very early pluripotential stem cell common to both lymphopoiesis and myelopoiesis or to a cell-to-cell interaction between leukemic lymphoblasts and neutrophilic granulocytes. Although at the present time these possibilities remain speculative, it has previously been shown that in both AML and ALL the frequency of infective episodes at every level of granulocyte count is greater in relapse than in remission, and recent evidence from cell culture studies in ALL indicates a defect in granulopoiesis.20

REFERENCES

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