Varying Involvement of Peripheral Granulocytes in the Clonal Abnormality—7 in Bone Marrow Cells in Preleukemia Secondary to Treatment of Other Malignant Tumors: Cytogenetic Results Compared With Results of Flow Cytometric DNA Analysis and Neutrophil Chemotaxis

By Jens Pedersen-Bjergaard, Lars Vindeløv, Preben Philip, Petri Ruutu, Jens Elmgreen, Heikki Repo, Ib Jarl Christensen, Sven-Aage Killmann, and Grethe Jensen

DNA content of isolated peripheral granulocytes and mononuclear cells was determined by flow cytometry and the results correlated with neutrophil chemotaxis, in 10 patients with secondary preleukemia or overt acute leukemia and cytogenetic defects of chromosome no. 7 in bone marrow cells. Nine patients had a hypodiploid clone with a missing chromosome no. 7, one lacked the long arm of a chromosome no. 7. The DNA content of granulocytes was normal in 5 of these cases and significantly reduced in 4 cases. In one patient, two populations of granulocytes were observed, one with a normal, the other with a reduced DNA content. In vitro chemotaxis of washed peripheral granulocytes was normal in 3 patients, reduced in 7 patients. A relationship was observed between the cytogenetic results from the bone marrow, the DNA content of peripheral granulocytes, and the chemotactic response. Thus, 4 patients with a high percentage of abnormal mitoses in the marrow had a decreased DNA content in granulocytes and a reduced chemotactic response, possibly due to involvement of mature peripheral granulocytes in the leukemic clone. Conversely, 3 patients with a high percentage of normal bone marrow mitoses had a normal DNA content in granulocytes and a normal chemotactic response, suggesting a differentiation of granulocytes in these cases from normal bone marrow precursors. Two patients showed a decreased chemotactic response, despite a normal DNA content in all granulocytes, or in a major population of these cells. This indicates that mechanisms other than involvement of granulocytes in a leukemia clone with monosomy 7 may contribute to a decreased chemotaxis. The results confirm previous reports of decreased neutrophil chemotaxis in monosomy 7 in patients with granulocytes of a leukemic origin. We can now add a further case to two previously described cases of 7q— with decreased chemotaxis. This suggests that the long arm of chromosome no. 7 could be of special importance for neutrophil chemotaxis.

During recent years it has become evident that the majority of human malignant tumors have a clonal origin. This conclusion is a result of cytogenetic investigations and studies of cell markers such as monoclonal immunoglobulins and glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes. However, many questions remain unresolved, as these methods all have limitations. Cytogenetic studies are restricted to tumors with an abnormal karyotype, and until recently, it has only been possible to perform such studies on dividing cells. Immunoglobulin markers are only present in tumors derived from cells of the immune system, and G-6-PD isoenzymes can only be studied in heterozygous females.

In most patients with acute nonlymphocytic leukemia (ANLL) secondary to irradiation or chemotherapy for other malignant tumors, recent investigations have demonstrated karyotype abnormalities of the clonal type in bone marrow cells. The characteristic findings are a missing chromosome no. 7 observed in approximately 60% of the cases and/or numerical or structural defects of chromosome no. 5. Secondary ANLL is often preceded by a preleukemic phase with pancytopenia, most often observed in cases with the above specified cytogenetic abnormalities. During preleukemia, the chromosome abnormalities are already present in the bone marrow. Many preleukemic patients suffer from recurrent life-threatening bacterial infections, and 7 in bone marrow cells has recently been correlated with impaired neutrophil migration in vitro in patients with secondary, as well as in patients with de novo preleukemia and ANLL.

In a preliminary study in our departments, however, some patients with secondary preleukemia and 7 in bone marrow cells showed a normal chemotactic response. Furthermore, many patients with secondary ANLL, including preleukemic cases, present a mixture of normal and abnormal mitoses in the bone marrow.
This leads to the question of whether mature circulating neutrophil granulocytes differentiate from cytogenetically normal or abnormal bone marrow precursors. As the granulocyte is a non-dividing cell without specific immunoglobulin markers, and as we had no access to patients heterozygous at the G-6-PD locus, we searched for an alternative method for determining the origin of the peripheral granulocytes in patients with secondary ANLL.

Flow cytometric DNA analysis has been used by Barlogie et al. for estimating DNA abnormalities in leukemias and lymphomas, 8 and subsequent studies have confirmed the potentials of this new technique in leukemia. 9,10 Comparison with cytogenetic findings demonstrated an analysis sensitivity corresponding to a loss of at least 2 or a gain of at least 7 chromosomes. 9 In a recent study on DNA abnormalities in small-cell carcinoma of the lung, we have found that the detection limit was DNA changes corresponding to the loss or gain of 5 average-sized chromosomes. 11 These studies were performed with the use of an external standard (human granulocytes or lymphocytes). Since then we have increased the resolution by using a modified staining technique 12 and two internal standards: chicken red blood cells (CRBC) and rainbow trunk red blood cells (TRBC) mixed with the sample before staining. With these techniques it has been possible to detect the difference in DNA content between male and female granulocytes or lymphocytes caused by the different size of the X- and Y-chromosomes. 13 This difference has been estimated by scanning photometry of single chromosomes as amounting to 1.7%. 14 By the improved techniques it should be possible to detect the reduced cellular DNA content of 2.7% caused by a missing chromosome no. 7. 14

The present study was undertaken to explore the involvement of peripheral granulocytes in the clonal abnormality of 7 of bone marrow cells primarily in preleukemic cases of secondary ANLL, using the technique of flow cytometry. A further aim was to correlate these results with determinations of neutrophil chemotaxis as an indication of cell function, as well as to verify the reported association between -7 and defective neutrophil chemotaxis.

MATERIALS AND METHODS

Patients

Ten patients, 9 with secondary preleukemia and 1 with overt ANLL (case 9), were studied. Two of the preleukemic patients (cases 6 and 10) were in a transitory state to overt ANLL, but all except case 8 were aleukemic (Table 1). Cases 1, 4, 5, and 8 had previously been treated for Hodgkin’s disease, cases 2, 3, 7, and 9 for non-Hodgkin’s lymphomas, case 6 for mammary carcinoma, and case 10 for multiple myeloma. All 10 patients had received intensive chemotherapy including an alkylating agent for the primary tumor; cases 1, 4, 5, 6, 7, 8, and 9 had also received radiotherapy. Clinical and cytogenetic characteristics for cases 1, 3, and 5 have been described in a previous paper, 3 where these cases were designated as cases 10, 16, and 12, respectively. All patients were untransfused, in good performance status, and without infectious episodes for the last 2 wk before the present investigations. The interval between previous cytostatic or corticosteroid treatment and the present study was at least 6 mo except for cases 2, 8, and 9 for whom the intervals were 5, 2/3, and 4 wk, respectively. Nitrosourea preparations had not been administered during the last 6 mo.

Cytogenetic Investigations

Cytogenetic investigations of the bone marrow were carried out on aspirates from the iliac crest as previously described. 3 Cells were studied by a direct method on Giemsa-stained slides and further characterized by trypsin-Leishman banding technique, carried out either with directly prepared cells or after 1-4-day culture. Results were expressed in accordance with the ISCN (1978) nomenclature. 15

Flow Cytometry

Flow cytometry was performed on Ficoll-Isopaque fractionated cells 16 from peripheral blood. Two fractions were examined separately, the granulocytic fraction containing granulocytes and the mononuclear fraction containing lymphocytes, monocytes, and if present early granulocytic precursors. The cells were transferred to 400 µl ice-cold buffer (5% dimethyl sulfoxide, 250 mM sucrose, 40 mM sodium citrate, pH 7.6). One-half of this cell suspension was used for morphological examination of cyto centrifuge preparations, stained by May-Grünwald-Giemsa technique, the other half was used for DNA analysis.

Preparation for DNA analysis was done with the detergent/propidium iodide method 17 employing a newly developed modification. 12 The internal standards CRBC and TRBC were added to the sample before staining. The staining involved 3 steps. To the 200-µl suspension of cells in buffer was added 1800 µl of solution A [1.5 mM spermine tetrahydrochloride (Sigma, St. Louis, Mo.), 30 mg/liter of trypsin type IX (Sigma), 1 ml/liter of nonidet P40 (Shell, Carrington, England), 3.4 mM trisodium citrate, and 0.5 mM Tris, pH 7.6]. After 10 min at room temperature, 1500 µl of solution B was added [solution A without trypsin and with 500 mg/liter trypsin inhibitor type II-0 (Sigma) and 100 mg/liter of RNA-Sc type I-A (Sigma), pH 7.6]. After 10 min at room temperature, 1500 µl ice-cold solution C was added [solution A without trypsin, with 0.62 mM propidium iodide (Calbiochem) and made 4.8 mM with spermine tetrahydrochloride]. The samples were kept on ice until analysis. The flow cyrometer used was a FACS III cell sorter. The means of the peaks produced by the standards and the sample were estimated by fitting gaussians to the peaks. The DNA content was calculated as explained in the legend to Fig. 1 and expressed in percent of the means obtained by analyzing granulocytes and mononuclear cells from 6 men and 6 women, all proven to be cytogenetically normal. The final results are thus made up for a minor difference in the fluorescence of granulocytes and mononuclear cells.

Chemotaxis of Neutrophils

Chemotaxis of neutrophils was performed as described previously 18 on samples of fresh heparinized blood following removal of erythrocytes. Cells were washed and suspended in a buffered physiologic salt solution including 1% or 2% human serum albumin. Cell
Table 1. Differential Counts of Bone Marrow, Peripheral Blood, and Cell Fractions Examined by Flow Cytometry

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Bone Marrow</th>
<th>Peripheral Blood</th>
<th>Facit-Hiopaque Fractionated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Blasts + Promyelocytes</td>
<td>Percent Myelocytes + Metamyelocytes</td>
<td>Percent Erythroblasts</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>6</td>
<td>22</td>
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<tr>
<td>7</td>
<td>7</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>24</td>
<td>17</td>
</tr>
</tbody>
</table>
GRANULOCYTES IN SECONDARY PRELEUKEMIA

Fig. 1. The result of a typical DNA analysis showing a histogram for mononuclear cells. CRBC and TRBC indicate the peaks produced by the internal standards, chicken and trout red blood cells, respectively. Mo indicates the peak produced by mononuclear cells. A DNA ratio was calculated by first correcting the zero-point location so that the ratio TRBC:CRBC = 2.2800. The peak means were then corrected accordingly. The sample mean divided by the TRBC mean was used to indicate the DNA content.

Concentration was adjusted to 10^5 leukocytes/ml. Chemotaxis was measured by the Millipore filter method, allowing cells to migrate into 3 μm Millipore filters (Millipore Corp., Bedford, Mass.) in modified Boyden chambers for 55 or 60 min for quantitation of chemotaxis. Casein (Merck, Darmstadt, West Germany) 5 mg/ml was employed as the attractant. The distance that the leading front of the cells had migrated was determined in 5 fields of each of 2 or 6 filters. Determinations were made in parallel on the same day at the two independent laboratories (Helsinki, Finland and Herlev, Denmark). Normal controls were included in each experiment.

RESULTS

Cytology of Blood, Bone Marrow, and Cell Fractions

Results of the differential counts of bone marrow, peripheral blood, and fractions of granulocytes and of mononuclear cells obtained by the Ficoll-Isopaque technique are summarized in Table 1. Band forms never exceeded 9% of the granulocytes in blood or cell fractions. Circulating blasts were observed only in cases 8 and 9 (4% and 80%). One patient (case no. 6) presented a high percentage of monocytes (42%) in peripheral blood. Three patients (cases 2, 5, and 6) had an increased erythropoiesis with many immature and abnormal erythroid precursors in the bone marrow. On microscopy, Ficoll-Isopaque fractions of granulocytes showed 61%–100% of mature granulocytes and only 0%–39% mononuclear cells (Table 1). Correspondingly, the mononuclear fractions contained from 84% to 99% mononuclear cells, mostly lymphocytes, and 1%–16% granulocytes.

Table 2. Cytogenetic Examinations of Bone Marrow

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Stem Line</th>
<th>Number of Mitoses Examined</th>
<th>Mitoses (% Abnormal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45, XX, -7</td>
<td>33</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>45, XY, -7</td>
<td>43</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
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<td>6</td>
<td>45, XX, -7</td>
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<td>84</td>
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<tr>
<td>7</td>
<td>45, XY, -7</td>
<td>15</td>
<td>93</td>
</tr>
<tr>
<td>8</td>
<td>44, XY, -5, -6, -7, -8, -17, t (17; ?), + mar, + min.</td>
<td>46</td>
<td>98</td>
</tr>
<tr>
<td>9</td>
<td>44, XY, -7, -17, -20, -22, +2 mar</td>
<td>56</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>46, XY, -7, + t, (1; 7) (1q; 7p)</td>
<td>23</td>
<td>50</td>
</tr>
</tbody>
</table>

Cytogenetic Characteristics

Results of bone marrow cytogenetics are shown in Table 2. In 7 patients (cases 1–7), a missing chromosome no. 7 was the only clonal abnormality observed. Of bone marrow mitoses analyzed, 35%–95% showed this pattern, whereas 5%–65% were normal. Cases nos. 8 and 9 presented various other abnormalities in addition to -7, as shown in Table 2. In case no. 10, one chromosome no. 7 had lost the long arm and instead had received a long arm from a chromosome no. 1 by translocation.

Flow Cytometry

The DNA content, corrected according to sex of the granulocytic and mononuclear fractions of cells of all 10 cases studied, is shown in Figs. 2 and 3. The 95% confidence limits (U test) for both types of cells based on 240 samples from 12 normal controls are indicated on the figures.

Fig. 2. Results of flow cytometric DNA determinations of the granulocytic fraction of cells in 10 patients with secondary preleukemia and defects of chromosome number 7. All samples were analyzed in triplicate. Z = 19% of granulocytic cells; 95% confidence limits for granulocytic cells (•) based on normal controls are indicated on the figure.
The granulocyte fractions of cases 1, 2, 3, 4, 10, and a major population of cells in case 8 had normal DNA values (Fig. 2). A significantly reduced DNA content was observed in granulocytes from patients 5, 6, 7, 9, and in a minor population of cells in case 8. The decrease in DNA content was consistent with the loss of a chromosome no. 7 in cases 5–7 and the estimated loss of DNA for the more complex karyotypes of cases 8 and 9.

The mononuclear cell fractions from 6 patients (cases 1, 3, 4, 5, 7, and 10) had a normal DNA content (Fig. 3). A significantly reduced DNA value was observed in mononuclear cells of cases 6 and 9. Patients 2 and 8 presented major populations of mononuclear cells with a normal DNA content and minor populations with highly increased or decreased DNA values as shown on the figure. The decreased DNA content of mononuclear fractions of cells in cases 6, 8, and 9 was likewise consistent with the karyotype patterns observed in the bone marrow. The presence of two populations of cells with different DNA content is demonstrated by case no. 8, for which the histogram for granulocytes is seen on Fig. 4 and the corresponding dot-plot on Fig. 5.

**Neutrophil Chemotaxis**

Results of the determinations of neutrophil chemotaxis in vitro are given in Fig. 6. Assessed from the results from both laboratories, a normal chemotactic response was observed in three patients (cases 1–3), whereas a decreased response was observed in seven patients (cases 4–10) as compared with the results for normal controls determined simultaneously at the same institution. The 95% confidence limits (U test) for normal controls for each of the two laboratories are indicated in Fig. 6. The concordance between the results from the two laboratories was good.

**DISCUSSION**

In the present study of patients with secondary preleukemia or ANLL and a mixture of normal and

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*Fig. 3. Results of flow cytometric DNA determinations of the mononuclear fraction of cells in 10 patients with secondary preleukemia and defects of chromosome number 7. All samples were analyzed in triplicate. V = 2% of mononuclear cells, X = 25% of mononuclear cells; 95% confidence limits for mononuclear cells (---) based on normal controls are indicated on the figure.*

*Fig. 4. DNA histogram of the granulocytic fraction of cells in case 8. The sample peak was resolved by computer analysis into two overlapping peaks. The minor peak represents 19% of the cells and has a DNA index of 94.6%. The major peak, representing 81% of the cells, has a DNA index of 100.6%.*

*Fig. 5. Dot-plot corresponding to the DNA histogram shown in Fig. 3. Each dot represents one nucleus analysed for DNA content (abscissa) and light scattering (ordinate). The light scattering value is a measure of the size of the nucleus. The internal standards (C = CRBC and T = TRBC) and the two subpopulations (1 and 2) are seen.*
abnormal bone marrow mitoses with monosomy 7, we have sought by means of a sensitive method for DNA measurement to determine whether the mature peripheral neutrophils originate from the karyotypically normal or abnormal bone marrow precursors. As a strong association between monosomy 7 and defective migration has previously been reported, \(^6\) determination of neutrophil chemotaxis in vitro of cells from the same patients was performed simultaneously in Helsinki and Copenhagen, and the results were compared with the results of flow cytometry. The investigations revealed three patterns. The first pattern observed in cases 1, 2, and 3 was characterized by a high proportion of normal mitoses in the bone marrow, a normal DNA content of granulocytic cells, and a normal chemotactic response. These findings are best interpreted to indicate a differentiation of normal cells in the granulopoiesis. The second pattern observed in cases 5, 6, 7, and 9 was characterized by a high percentage of abnormal mitoses in bone marrow cells. These patients had a reduced DNA content of peripheral granulocytes and showed a decreased chemotactic response. Although their granulocytes were of a normal morphology, we suggest that these cells were leukemic in origin.

The findings in cases 4 and 8 need special consideration. In patient 8, 98% of the bone marrow mitoses were abnormal with modal numbers of 43–44, karyotype instability and the predominant clone 44, XY, −5, −6, −7, −8, −17,t (17;?) +mar, +min. However, only 19% of peripheral granulocytes showed a reduced DNA content equivalent to this karyotype (Fig. 2), whereas 81% had a normal DNA content. This indicates that even a reversed proportionality may occasionally be observed between the size of clones of aneuploid precursors in the bone marrow and the size of fractions of equivalent granulocytes in peripheral blood. In patient 4, 65% of bone marrow mitoses were normal, and correspondingly, the DNA content of peripheral granulocytes was normal. Despite these findings, patient 4 as well as patient 8 presented a decreased chemotactic response. These results seem to indicate that other mechanisms besides involvement of granulocytes in the cytogenetically abnormal clone demonstrated in the bone marrow may be responsible for a defective neutrophil migration in monosomy 7.

The results in patient 10 are further evidence in support of the hypothesis that the long arm of chromosome no. 7 is responsible for normal migration, previously based on similar observations in two cases.\(^7\) The combination of decreased chemotaxis and a normal DNA content in the granulocytes of this patient is not surprising, as no DNA was lost, because of substitution of the long arm of a chromosome no. 7 by a long arm of approximately the same size from a chromosome no. 1.

As expected, the DNA content of mononuclear cell fractions was in most cases normal. In patient no. 9, the decreased value was certainly caused by myeloblasts predominating in the peripheral blood as well as in the mononuclear fraction of cells (Table 1). In patient 6, the decreased content of DNA in mononuclear cells could possibly be related to a pronounced monocytosis in peripheral blood (Table 1). Involvement of monocytes in a leukemic clone has previously been suggested in secondary preleukemia, based on investigations of in vitro production of interferon by leukocytes stimulated with viral antigens,\(^19\) and in de novo ANLL, based on determination of G-6-PD isoenzymes.\(^20\) The decreased DNA content observed in a small population of mono-
nuclear cells in case 8 could, in conformity with the reasons adduced for case no. 9, be caused by the presence of leukemic cells. The presence of a tiny population of mononuclear cells with a highly increased DNA content, observed in case no. 2 with a nodular, poorly differentiated lymphocytic lymphoma, previously in leukemic phase, is at present unexplained.

The clinical significance of the involvement of peripheral granulocytes in the leukemic clone and of a reduced chemotactic response in patients with secondary preleukemia, is at the present time uncertain. A higher incidence of fever and infections in patients with monosomy 7 compared to a selected group of patients with ANLL and two normal chromosomes 7 was found in a recent study. In our study some patients (cases 1, 3, and 5) were free from bacterial infections for longer periods of time, whereas others (cases 4, 6, 8, and 9) had recurrent episodes of infection. This could indicate that the patients with a normal chemotactic response were less prone to infections than patients with abnormal granulocytes. However, other factors, such as granulocytopenia and immunosuppression, previously reported, could influence the risk of infection.

Leukemic involvement of mature cells in peripheral blood has previously been suggested in de novo preleukemia and ANLL. In preleukemia, morphological studies have demonstrated characteristic abnormalities of erythrocytes, platelets, monocytes, and granulocytes. These morphological abnormalities contribute to the diagnostic criteria for the disease. Erythroid precursors in the bone marrow in overt ANLL have been shown to be involved in the cytogenetically abnormal clone, and studies of G-6-PD isoenzymes in peripheral red cells in leukemia have occasionally demonstrated that these mature cells may also be of leukemic origin. It is thus possible that the morphological abnormalities of erythrocytes observed in preleukemia could indicate leukemic involvement. As regards platelets, studies of patients with preleukemia and ANLL have shown populations of thrombocytes with normal or abnormal ultrastructure and cytochemistry, suggesting a varying involvement of platelets in the leukemic clone. The leukemic origin of mature granulocytes in overt ANLL has been suspected, due to an occasional content of Auer rods in these cells. In preleukemia, other studies have demonstrated populations of granulocytes with varying content of myeloperoxidase, from normal to severely decreased values. These results have been taken as evidence for a possible differentiation of granulocytes from normal as well as from leukemic precursors in preleukemia. Finally, Koeffler and Golde have shown that cytogenetically abnormal bone marrow cells from patients with preleukemia may differentiate in vitro into mature-looking granulocytes. These previous reports are in agreement with the interpretation of the results in the present study, as indicating a varying involvement of peripheral granulocytes in the leukemic clone in secondary preleukemia and ANLL.

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