Differential Chromatin Structure-dependent Binding of 7-Aminoactinomycin D in Normal and Malignant Bone Marrow Hematopoietic Cells

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ABSTRACT

Chromatin structure-dependent binding of the DNA-specific dye 7-aminoactinomycin D (7-AMD) in leukemic and normal cells in bone marrow aspirates from childhood acute leukemia patients and patients without bone marrow neoplasia was assessed by multiparameter flow cytometry. Simultaneous staining with fluorescein isothiocyanate-labeled antibodies was needed in many cases for determination of the immunophenotype of the cells that exhibited differential binding of 7-AMD. 7-AMD binding was enhanced in normal (4 patients) and malignant (8 patients) myeloid cells, and was generally low in normal and leukemic lymphocytes and normoblasts. Four of 18 aspirates from 16 patients with acute lymphoblastic leukemia contained neoplastic cells with increased 7-AMD binding capability.

The 7-AMD binding of the leukemic cells was not correlated to S-phase fraction (P = 0.87), but was significantly correlated to cell size as measured by forward angle light scattering (r = 0.49, P = 0.007). Patients with tumor cells exhibiting low 7-AMD binding at last aspirate survived significantly longer than the patients with leukemic cells binding high amounts of 7-AMD (P = 0.03). Neither cell size, S-phase fraction, nor ploidy status predicted patient survival in this small scale study.

INTRODUCTION

The heterogeneity of acute leukemias is thought to reflect the diversity of normal hematopoietic precursors in the bone marrow (1). Acute leukemia is the most common pediatric neoplasm, and many studies have been carried out to characterize the phenotypes of the malignant cells and to establish reliable prognostic criteria. Acute leukemias are classified into 2 groups: AML and ALL. AMLs and ALLs can be further subdivided into the M1-M6 and L1-L3 subgroups, respectively, according to the morphology of the malignant cells (French-American-British classification). The prognosis of untreated acute leukemia patients is poor. With multidrug regimens, however, remission and long-term survival approach 80% among pediatric ALL patients, while chemotherapy at best results in a long-term survival of 50% of the AML patients.

The heterogeneous composition of the bone marrow makes studies of normal differentiation and neoplasia difficult in this organ. Additionally, some cell types are present in such low numbers that they would be easily missed by the hematologist even if examined after immunolabeling. Both these problems may be dealt with by the use of multiparameter flow cytometry. Andreff et al. (2) measured the green (DNA) and red (RNA) fluorescence intensities of acridine orange-stained bone marrow cells as well as the green fluorescence pulsewidth. Loken et al. (3–5) used multiparameter flow cytometry to study differentiation, proliferation, and other cellular characteristics in human bone marrow. The expression of up to 4 different antigens has been assessed simultaneously in bone marrow and peripheral blood cells (6).

The packing of DNA into chromatin in situ occurs at many levels in mammalian cells (7, 8). The binding of DNA-specific dyes to chromatin is restricted by histones, compared to the binding to free DNA (9–14). Active chromatin is believed to have a more open structure than nontranscribed chromatin; phenotype-specific differences of the binding of some dyes to DNA are attributed to differences in higher order chromatin structure (12, 13, 15, 16). The DNA-specific, fluorescent dye 7-AMD appears to be especially sensitive to chromatin structure (12, 13, 15–17). In contrast, the binding of H33258 to DNA is almost completely independent of chromatin structure. Hence, we have used this dye to determine cellular DNA content (15, 16).

The binding of 7-AMD is correlated with overall transcriptional activity and overall DNase1 sensitivity (15, 16), and is often, but not always, increased in proliferating cells. Myeloid cells in peripheral blood exhibit increased 7-AMD binding [7-AMD+ phenotype (15)] compared to normal peripheral blood lymphocytes. Lymph node lymphocytes, including malignant lymphocytes from patients with low grade non-Hodgkins lymphoma, bind low amounts of 7-AMD (7-AMD− phenotype), while malignant cells from high grade non-Hodgkins lymphoma patients are 7-AMD+ (16).

In this work, we have measured the binding of 7-AMD in normal and neoplastic bone marrow cells. The prognostic and/or diagnostic value of this parameter, as well as of S-phase fraction, ploidy, and cell size in ALL and AML were also investigated. 7-AMD binding was the only parameter of prognostic significance.

MATERIALS AND METHODS

Patients. Patients were selected from among those hospitalized at the Department of Pediatrics, The Norwegian State Hospital, during the period 1982–1985 according to the following criteria: (a) hematological and immunological (18) diagnosis of ALL or AML; and (b) representative cell suspension of bone marrow aspirate available in liquid nitrogen. Altogether 24 patients (16 ALLs and 8 AMLs) were selected. (Cell suspensions were also prepared from 2 of the 4 patients with ALL who relapsed after induction therapy.) The average age of the AML and ALL patients was 4.3 years (SD = 4.7 years) and 5.3 years (SD = 2.8 years), respectively. Aspirates were also obtained from 6 patients without signs of bone marrow neoplasia.

The ALL and AML patients were given chemotherapy as described previously (19, 20). Due to the selection criteria, not all patients seen at the Hospital during the study period are included, but the selected group is considered representative with respect to age and survival (19, 20).

Cells. The precursor B-cell line Reh was grown in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum at 37°C in 5% CO2. Peripheral blood lymphocytes and monocytes were prepared by density gradient separation of blood using Lymphoprep (Nyegaard, Oslo, Norway).
Bone marrow aspirates from patients without bone marrow neoplasia, as well as bone marrow aspirates and peripheral blood from pediatric patients with acute leukemia, were prepared as described elsewhere (21).

Antibody Labeling. Cells measured for 7-AMD fluorescence and surface antigen expression were first labeled by the indirect immunofluorescence method. Fifty μl of solutions of monoclonal antibodies against the following surface antigens comprised the first layer: glycoporphin A (R10 antibody); MHC class II (D54-2 antibody); the antigen recognized by the 4F2 antibody; CD2; CD4; CD7 (Wt1 antibody); CD8; CD10 (J5 antibody); CD11b (Mo1 antibody); CD13 (My7 antibody); CD14 (My4 antibody); CD15 (1G10 antibody); CD19 (AB1 antibody); CD20 (B1 antibody); CD33 (My9 antibody); CD34 (B1-3C5 antibody); CD36 (5F1 antibody); CD37 (HH1 antibody); CD71 (B3/25 antibody); or a sarcoma cell-associated antigen for control staining (TP3 antibody, kindly provided by Dr. Ø. Bruland). FITC-conjugated goat-anti-mouse antibodies (50 μl, 1/50 dilution; Tago, Burlingame, CA) were used as the second layer.

Fixation and DNA Staining. Cells were washed once in phosphate-buffered saline, resuspended in phosphate-buffered saline, and fixed by the addition of freshly prepared 4% phosphate-buffered formaldehyde to a final concentration of 3%. Cells were fixed for 2 days at 0°C. The fixation time is not critical for 7-AMD staining (22).

Fixed cells were washed once in 0.15 M NaCl, 0.5 mivM MgCl2, 10 miv phosphate-buffered saline, and reconstituted in the same buffer with 2 μg/ml H33258 (Riedel de Haen, Hannover, Germany). The H33258 fluorescence was measured as described below. Thereafter, 7-AMD (Calbiochem, La Jolla, CA) was added to a final concentration of 25 μg/ml, and the cells were measured for 7-AMD fluorescence or 7-AMD and FITC fluorescence.

Flow Cytometry. H33258, 7-AMD, and FITC fluorescence of single cells were measured simultaneously with FALS and large angle light scattering in an Argus flow cytometer (Skatron, Tranby, Norway). H33258 fluorescence (≥420 nm) and 7-AMD fluorescence (≥640 nm) were measured with excitation at 365 and 578 nm, respectively. For the simultaneous measurement of FITC fluorescence (510-550 nm) and 7-AMD fluorescence (≥640 nm) as well as FALS, the excitation wavelength was 470-490 nm. H33258 is not excited at 578 nm or 470-490 nm, and DNA-bound H33258 does not interfere with the binding of 7-AMD (15, 23). Paraformaldehyde-fixed rat thymocyte nuclei and 7-AMD (15, 23). Paraformaldehyde-fixed rat thymocyte nuclei and DNA-bound H33258 does not interfere with the binding of 7-AMD (15, 23). Paraformaldehyde-fixed rat thymocyte nuclei and DNA-bound H33258 does not interfere with the binding of 7-AMD (15, 23). Paraformaldehyde-fixed rat thymocyte nuclei and DNA-bound H33258 does not interfere with the binding of 7-AMD (15, 23). Paraformaldehyde-fixed rat thymocyte nuclei and DNA-bound H33258 does not interfere with the binding of 7-AMD (15, 23).

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Data Treatment. Median 7-AMD fluorescence values are given in units of the fluorescence of rat thymocyte nuclei. The DI, which is the DNA content relative to that of diploid cells, was calculated from the peak channel numbers in the H33258 fluorescence histograms. Median FALS of G0/G1 cells is given in units of the FALS of 1.33-μm fluorescent microspheres. Rat thymocyte nuclei and dead cells scatter less light than intact cells; 7-AMD binding is also increased in dead cells (16). The data were collected in list mode; rat thymocyte nuclei and dead cells could thereby be gated out retrospectively.

The percentage of cells in S-phase was calculated by computer simulation of the data (24). In some cases, the S-phase fraction in the tumor cell population was obtained by gating the DNA histogram on DNA content (aneuploid cases), FALS (AML cases), or abnormal H33258 fluorescence (to eliminate normoblasts (25)). In the remaining cases, except for sample 409/85, the fraction of leukemic cells was so high that the S-phase fraction was representative of the tumor cells (Table 1).

The parameter 7-AMD fluorescence divided by DI (7-AMD fluorescence/DI) was used as an indicator of the chromatin structure of the cells. The border between the "7-AMD+" and the "7-AMD-" phenotypes (7-AMD fluorescence/DI = 0.90) was chosen such that all normal lymphocytes/normoblasts and myeloid cells were 7-AMD- and 7-AMD+, respectively. Additionally, in all the diploid leukemia cases in which 2 G0/G1 peaks were evident in the 7-AMD histograms, it was required that the cells with lower and higher fluorescence should be 7-AMD- and 7-AMD+, respectively (16).

Correlations between the different parameters were estimated by linear regression analysis. Survival was calculated from the date of diagnosis or from the date of the second bone marrow aspirate by the Kaplan-Meyer method. Differences in survival were estimated by the log rank test (26).

RESULTS

Table 1 shows the S-phase fraction and the DI, 7-AMD fluorescence/DI, and FALS of the G0/G1 cells in bone marrow from ALL and AML patients. The hematological and immunohematological classifications of the different samples are also given. Fig. 1A shows 2 typical examples of the DNA histograms. The mean coefficient of variation of the G0/G1 peaks in the H33258 histograms was 2.3% (SD = 0.5%) for the 18 ALL samples and 3.2% (SD = 0.6%) for the 8 AML samples. The cells in case 165/83 were diploid, while the majority of the cells in case 59/83 was aneuploid (Fig. 1A). Two peaks are seen in the corresponding 7-AMD fluorescence histograms of both of these samples (Fig. 1B). Since the cells in case 165/83 were diploid,
In most of the diploid ALL cases, the 7-AMD fluorescence histograms exhibited only one G0/G1 peak. The median channel number of the 7-AMD fluorescence peak was therefore representative of the binding of 7-AMD in both the normal and neoplastic G0/G1 cells in the sample.

In the cases in which 2 peaks appeared in the 7-AMD fluorescence histograms, the phenotypes of the cells with different 7-AMD fluorescence were studied by simultaneous measurement of FITC-labeled antibodies against defined surface antigens. The 7-AMD* peak in case 165/83 was found to represent mostly T- and B-lymphocytes and a small amount of other unidentified cells (Fig. 2A). In contrast, none of the 7-AMD* cells expressed B- or T-cell markers, whereas all 7-AMD* cells expressed glycoprotein A and low amounts of CD71 (Fig. 2B), indicating a monoclonal nature of this population. The 7-AMD* cells were not hemoglobin-containing normoblasts, as no cells with decreased H33258 fluorescence were observed (25). The findings are compatible with the hematological description of this case as an erythroblastic leukemia or AML M6, and demonstrated that the 7-AMD* cells were the tumor cells. Although it was obvious that the cells with increased 7-AMD binding in ALL 59/83 were the tumor cells (Fig. 1A), a similar analysis of the phenotypes of the different subpopulations defined by 7-AMD fluorescence and FALS was performed. The diploid subpopulation consisted of more than 50% T-cells, some mature B-cells, and a small fraction of unidentified cells (Fig. 3A). The aneuploid cells all expressed CD10 and CD19, suggesting a monoclonal B-precursor phenotype of this population (Fig. 3B). Interestingly, CD20 expression was heterogeneous in the leukemic cells in cases 59/83 (Fig. 3B) and 152/83 (data not shown), indicating that differentiation was not completely blocked in these tumors.

The 7-AMD fluorescence histograms of nonmalignant bone marrow samples were characterized by the presence of 2 peaks corresponding to the 7-AMD* and 7-AMD* phenotypes in 4 of 6 cases (Fig. 4). Only 7-AMD* cells could be identified in cases 183/85 and 205/85 (20). The 7-AMD phenotype of different subpopulations of cells was studied in the same way as for the

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**Fig. 1.** DNA content and 7-AMD binding in acute leukemias. Fixed cells from cases 165/83 and 59/83 were stained with H33258 and measured for DNA content with UV excitation (A). Subsequently, 7-AMD was added and 7-AMD binding was measured with excitation at 578 nm (B). Rat thymocyte nuclei (included as internal standard) were gated out and are not shown.

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**Fig. 2.** Immunophenotyping of cells with different 7-AMD binding. Cells from case 165/83 were indirectly FITC-stained for the indicated surface antigens, fixed, and stained with 7-AMD. 7-AMD fluorescence, FITC fluorescence, and FALS were measured with 470–490-nm excitation. The FITC fluorescence histograms of the 7-AMD* cells (A) and the 7-AMD* cells (B) are shown together with the corresponding gates in the dual parameter 7-AMD fluorescence versus FALS histograms.
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Fig. 3. Immunophenotyping of cells with different DNA content. Cells from case 59/83 were indirectly FITC-stained for the indicated surface antigens, fixed, and stained with 7-AMD. 7-AMD fluorescence, FITC fluorescence, and FALS were measured with 470-490-nm excitation. The FITC fluorescence histograms of the diploid cells (A) and the aneuploid cells (B) are shown together with the corresponding gates in the dual parameter 7-AMD fluorescence versus FALS histograms.

Fig. 4. 7-AMD fluorescence/DI in G0/G1 phase bone marrow cells. 7-AMD binding was measured as in Fig. 1 and the phenotype of the different 7-AMD binding subpopulations was determined as in Figs. 2 and 3. PBL, peripheral blood lymphocyte; BM, blood monocyte.

leukemias. As shown in Table 2, T-cells and erythroid precursors had the 7-AMD" phenotype. Myeloid (CD15+) and monocytoid (CD14+) bone marrow cells were 7-AMD". CD36 is expressed on both erythroid cells and monocytes/monoblasts. In the cases 183/85 and 205/85, myelopoiesis was almost completely suppressed and nearly all the CD36"+ cells were also positive for glycophorin A (25). The CD36"+ cells were 7-AMD" in these 2 cases, suggesting that the CD36"/7-AMD" cells were of erythroid origin and that the CD36"/7-AMD" cells identified in the cases 100/89, 17/90, and 223/89 were of monocytoid origin.

The different characteristics of the tumor and normal cells in the leukemic and nonmalignant samples are summarized in Tables 1 and 2, and the chromatin structure parameter 7-AMD fluorescence/DI is graphically displayed in Fig. 4. Fig. 4 also shows the results obtained with the B-precursor cell line Reh, peripheral blood lymphocytes, and monocytes. It appears from Tables 1 and 2 and Fig. 4 that normal lymphocytes and normoblasts were consistently 7-AMD", whether found in normal or leukemic samples. Six of 18 ALL samples contained aneuploid cells, while none was found in the AML cases. There were no significant differences between the fraction of cells in S-phase in AML (7.2%, SD = 3.0%) and ALL (9.9%, SD = 5.9%, P = 0.14). The AML blasts generally scattered more light (FALS: 3.01, SD = 0.58) than the ALL blasts (FALS: 2.30, SD = 0.35; P = 0.01); the blasts in most ALL cases scattered the same amount of light as normal lymphocytes. Chromatin structure (7-AMD fluorescence/DI) was the parameter that discriminated best between the AML cells (1.01, SD = 0.06) and ALL cells (0.84, SD = 0.13; P = 0.0001), despite the inclusion of 4 ALL cases with clearly enhanced 7-AMD binding (Fig. 4). 7-AMD binding was positively correlated to the FALS of the leukemic cells (r = 0.49, P = 0.007), but there was no significant correlation between 7-AMD binding and the fraction of cells in S-phase (P = 0.07).

With the limited number of cases studied, no significant difference between the survival in the AML and the ALL groups was observed (P = 0.06), although the survival was higher among the ALL patients. The survival of patients presenting with high and low 7-AMD binding tumor cells at first and last bone marrow aspirate is shown in Fig. 5, A and B, respectively. (Two cases were examined at diagnosis and at relapse.) Low 7-AMD binding of the tumor cells in the last aspirate was significantly correlated with a good prognosis (P = 0.03). The
7-AMINOACTINOMYCIN D BINDING IN BONE MARROW CELLS

Table 2 Distribution of the 7-AMD+ phenotype among subpopulations of bone marrow cells

<table>
<thead>
<tr>
<th>Source (phenotype)</th>
<th>CD7</th>
<th>CD10</th>
<th>CD14</th>
<th>CD15</th>
<th>CD19</th>
<th>CD20</th>
<th>CD33</th>
<th>CD34</th>
<th>CD36</th>
<th>Glycophorin A</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal bone marrow</td>
<td>0/5</td>
<td>1/1</td>
<td>4/4</td>
<td>1/1</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1/1</td>
<td>3/5*</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>AML (leukemic cells)</td>
<td>1/2</td>
<td>2/14</td>
<td>3/16</td>
<td>1/10</td>
<td>0/3</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
<td>3/16</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>ALL (leukemic cells)</td>
<td>1/2</td>
<td>2/14</td>
<td>3/16</td>
<td>1/10</td>
<td>0/3</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
<td>3/16</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>AL (normal cells)</td>
<td>0/3</td>
<td>0/2</td>
<td>0/1</td>
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* 7-AMD+ and 7-AMD− cells were found in cases 100/89, 17/90, and 223/85, while only 7-AMD− cells were found in cases 183/85 and 205/85.

Fig. 5. Survival of patients with different 7-AMD tumor cell phenotypes. Shown are survival of the patients with tumor cells of the 7-AMD− (---) and the 7-AMD+ (-----) phenotypes at first aspiration (A) and at last aspiration (B). The numbers of patients with 7-AMD+ tumor cells were 13 and 12 at primary and last aspiration, respectively. The total number of patients in the study was 24.

correlation between the 7-AMD binding of the tumor cells from the first diagnostic bone marrow aspirate and survival was not statistically significant (P = 0.10). The survival was also higher for the ALL patients with 7-AMD− tumor cells at last aspirate (11 of 12) compared to the ALL patients with 7-AMD+ tumor cells (2 of 4), but the difference between the survival curves was not significant (P = 0.09). Patients presenting with tumor cells with low FALS (≤2.4), low S-phase fraction (≤8%), or aneuploid tumor cells had a somewhat higher survival than the others, but the differences were not significant (P = 0.94, 0.60, and 0.09, respectively).

DISCUSSION

We report here on the use of a chromatin structure-related parameter, binding of 7-AMD, in order to study subpopulations of bone marrow cells. This parameter is correlated to transcriptional activity, and is expected to be related to the total RNA content of cells. RNA content can be measured together with DNA content by means of acridine orange. However, the broad emission spectrum of this dye makes it impossible to use in conjunction with the fluorophores most commonly used to label antibodies (FITC, phycoerythrin). It also appears that the DNA content is determined with higher precision by means of H33258 fluorescence than with the green fluorescence of acridine orange, as the average coefficients of variation observed in ALL and AML in the present study were approximately a factor of 2 lower than those reported by Andreeff et al. (2). In an earlier report (22), we showed that chromatin structure (7-AMD fluorescence), DNA content (H33258 fluorescence), cell size (FALS), and the expression of 2 antigens could be assessed simultaneously by dual wavelength excitation flow cytometry. Several normal and malignant cell types may exhibit the 7-AMD+ phenotype; simultaneous measurement of the immunophenotype is needed in many cases for determination of the 7-AMD phenotype of a given subpopulation of cells (e.g., the tumor cells). Chromatin structure and proliferative activity appear to be independent variables in bone marrow (described herein) and in lymph nodes (16). Hence, the simultaneous measurement of these 2 parameters together with cell size and up to 2 intra- and/or extracellular antigens should provide the hematologist with a powerful tool.

The present results show that all cells of the granulocytic and monocytic lineages were 7-AMD+, including malignant cells arrested at different stages of differentiation. This is in accordance with the 7-AMD+ phenotype of mature peripheral blood myeloid cells (15), and the high RNA content of myeloid cells (2). Our unpublished studies of CD34-expressing cells isolated by immunomagnetic beads indicate that early myeloid precursors (CD34+/CD33+) are also 7-AMD+. Normal proliferating normoblasts had the 7-AMD− phenotype, whereas erythroblastic cells were 7-AMD+. This may reflect either a peculiarity caused by the transforming events in erythroblasts or a shift from the 7-AMD+ to the 7-AMD− phenotype during the differentiation of immature erythroid precursors.

Normal marrow B- and T-lymphocytes, some of which may be derived from peripheral blood (4), were 7-AMD−. However, some of these were probably immature normal lymphocyte precursors, and the predominance of the 7-AMD− phenotype among the ALL lymphoblasts (described herein), non-Hodgkins lymphoma cells (16), and CD34+/CD19+ B-lymphoid precursors suggests that this may be the most common 7-AMD phenotype of lymphoid cells. A subset of ALL lymphoblasts (described herein), high grade non-Hodgkins lymphoma cells, and in vitro stimulated normal B-cells (16) had the 7-AMD+ phenotype. The finding that the neoplastic cells in some of the ALL samples were 7-AMD+ is apparently not in accordance with the low RNA content of the blasts in all ALL cases as measured by acidine orange (2). Increased RNA

4 E. B. Smeland and T. Stokke, unpublished observations.
turnover in 7-AMD+ ALL blasts may, however, explain the disparity between the data on RNA content and transcriptional activity.

As shown previously, 7-AMD binding is of prognostic value in lymphomas (16). Relapse and death occurred earlier and more often among the acute leukemia patients with tumor cells of the 7-AMD+ phenotype, indicating that this parameter is also of prognostic value in bone marrow-derived neoplasms. The number of patients in this study is too small, however, to draw any conclusion as to whether the ALL patients with 7-AMD+ tumor cells are at high risk and thus should be selected for more intensive therapy. Cell size, ploidy, and the fraction of cells in S-phase were not found to be of prognostic value in this study. A lack of correlation between the S-phase fraction and survival in pediatric ALL cases has also been reported by others (27–29).

More detailed studies must be performed to clarify the 7-AMD phenotype of normal early bone marrow precursors of different lineages as well as pluripotent stem cells. Further studies including more patients are also needed to determine whether 7-AMD binding is a reliable prognostic criterion in childhood ALL.

REFERENCES

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