Karyotypically Distinct Subpopulations in Acute Leukemia With Specific Growth Requirements

By B. Löwenberg, A. Hagemeijer, and K. Swart

Bone marrow and blood cells of a patient with acute myeloid leukemia frequently show acquired cytogenetic abnormalities and, when present, these karyotypic changes provide a tool to follow the evolution of the disease. New clones of cells derived from the original one, may present themselves with disease relapse but in some cases karyotypic evolution has taken place before diagnosis, as indicated by the presence of multiple clones at first presentation. In chronic myeloid leukemia the emergence of additional cytogenetic abnormalities is a frequent phenomenon and may herald the transformation to blastic phase. The clinical role of a new clone is usually evident only from retrospective evaluation, and therefore in vitro tests have not been shown to successfully discriminate subclones of individual tumors.

In vitro colony assays are utilized to investigate the proliferative properties of leukemic progenitors. The leukocyte feeder colony cultures, initially employed for nonleukemic myeloid precursor cells, did usually result in only abortive (so called cluster) growth when cells from patients with acute myeloid leukemia were cultured. Culture methods supplemented with phytohaemagglutinin (PHA) or medium conditioned by PHA stimulated lymphocytes did not stimulate normal myeloid colony growth but proved successful in supporting the proliferation of acute leukemia stem cells. The application of more than one colony assay to bone marrow and blood cell specimens from patients with hematological malignancies, coupled to cytogenetic analysis, may permit the differentiation of coexisting clones in single patients.

Here we report the cytogenetic and colony culture results in a newly diagnosed patient with acute monocytic leukemia who carried a mixture of related subclones with distinguishable karyotypes. By using two culture systems and leukemic purified cell fractions, it could be demonstrated that leukemic cells belonging to cytogenetically different populations and with different roles in the clinical course of the disease, had different growth requirements in vitro.

MATERIALS AND METHODS

Colony Assays

Nucleated cells from bone marrow and blood were harvested following sedimentation of the erythrocytes in 0.1% methylcellulose. Our techniques for cell collection, the leukocyte feeder method of colony formation in culture and the PHA-leucocyte feeder assay (PHA-L.F. assay) have all been described. Leukemic colony formation in the leucocyte feeder and in the PHA-L.F. assays were estimated in triplicate cultures for unfractionated marrow and blood cells and for different density fractions of cells obtained as described below. Morphological examination and cytogenetic analysis of colony cells were also performed.

Cell Fractionation Procedure

Blood or bone marrow cells were separated by a combination of E-rosette sedimentation and discontinuous albumin gradient fractionation. Nucleated cells (10 x 10^6/ml) were incubated with an equal volume of a 1% (v/v) neuraminidase treated sheep red blood cells suspension as described. The sedimented cells were carefully resuspended in bovine serum albumin (BSA) of density 1.050 g/ml, and pipetted on top of the gradient. The gradient was prepared of the following layers from top to bottom: 1.056 g/ml, 1.059 g/ml, 1.062 g/ml.
g/ml, and 1.083 g/ml. BSA processing and preparation of the fractions have been reported.17

**Cytogenetic Analysis**

Cytogenetic analysis was done according to standard procedures, slightly adapted to the different materials which were examined. Total bone marrow, blood, and the different density fractions were cultured for 24 or 48 hr in the absence of PHA stimulation; the technique using methotrexate treatment and the harvesting procedure have been reported earlier.18 Remission blood was cultured once for 72 hr with PHA stimulation in order to verify the karyotype of (nonleukemic) lymphocytes. Colony cells were derived from 7 day PHA-IF cultures and from 12 to 14 day leucocyte feeder cultures. The night before harvesting, medium containing methotrexate (10−7M) or BrdU (10−7M) was added to the pellet and the following morning colony cells were collected from the culture plates with a pasteur pipet, pooled in a sterile plastic tube, and spun down; fresh medium containing either TdR (10−8M), TdR (10−7M) was added to the pellet and the culture was continued for 5 to 6 hr at 37°C. Further processing was similar to bone marrow or blood cultures. Banding techniques were used to identify the chromosomes; QFA (Q−banding by fluorescence using acridine orange), GTG (G-banding by trypsin-Giemsa technique) and CBG (C−banding by barium hydroxide using Giemsa) according to the Paris Conference (1971) supplement 1975.19

**Case History**

In November 1979 a 17 yr old man was admitted to the hospital with high fever (39°C) and a painful throat. The previous history was unremarkable. For several days he felt fatigue and was feverish. Gums hypertrophy and bilateral cervical lymphadenopathy were present. Liver and spleen reached 3 cm below the costal margins. A night before harvesting, medium containing methotrexate was added to the dishes. The following morning colony cells were collected from the culture plates with a pasteur pipet, pooled in a sterile plastic tube, and spun down; fresh medium containing either TdR (10−7M) or BrdU (10−7M) was added to the pellet and the culture was continued for 5 to 6 hr at 37°C. Further processing was similar to bone marrow or blood cultures. Banding techniques were used to identify the chromosomes; QFA (Q−banding by fluorescence using acridine orange), GTG (G−banding by trypsin−Giemsa technique) and CBG (C−banding by barium hydroxide using Giemsa) according to the Paris Conference (1971) supplement 1975.19

**RESULTS**

Chromosome analysis at diagnosis revealed three abnormal karyotypes, respectively designated as clones A, B, C (Table 1). Clone A, (Fig. 1A) showed an apparently balanced translocation (6;11) (q27;q23). Clone B, (Fig. 1B) the major clone at diagnosis stemmed from A, and showed 51 or 52 chromosomes with different trisomies in addition to the t(6;11) : 51, XY, t(6;11), +der(6), +3,+19,+19,+21 and in the majority of the cells one pair of double minutes (dm) which stained only with Q− and G−banding, not with R−banding and showed no positive C−bands. Clone C observed in blood culture at diagnosis, showed a divergent evolution from the presumed parental clone A: 53,XY,t(6;11), +der(6), +4, +10, +13, +18, +19, +21. A PHA stimulated blood culture yielded lymphocytic metaphases with a normal constitutional karyotype: 46,XY.

Following treatment, during partial remission (Feb. 6, 1980), the bone marrow yielded mostly cells with the normal karyotype, a few cells of clone A but none of clone B and C. Remarkably, at the same time colonies

<table>
<thead>
<tr>
<th>Table 1. A Selection of Hematological and Cytogenetic Data of Patient With Acute Monoblastic Leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Presentation</strong> (Nov., 1979)</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
</tr>
<tr>
<td>WBC (x 10⁹/l)</td>
</tr>
<tr>
<td>blast cells (%)</td>
</tr>
<tr>
<td><strong>Marrow</strong></td>
</tr>
<tr>
<td>M/E ratio</td>
</tr>
<tr>
<td>blast cells (%)</td>
</tr>
<tr>
<td><strong>Cytogenetics</strong></td>
</tr>
<tr>
<td>No. of cells analysed</td>
</tr>
<tr>
<td>with normal karyotype</td>
</tr>
<tr>
<td>with karyotype A</td>
</tr>
<tr>
<td>with karyotype B</td>
</tr>
<tr>
<td>with karyotype C</td>
</tr>
</tbody>
</table>

*Bone marrow taken before treatment
†includes (mono)blasts and all immature and abnormally looking cells with monocytoid appearance.
‡normal karyotype: 46,XY.
§Clone A: 46,XY,t(6;11) (q27;q23).
||Clone B: 51 or 52,XY,t(6;11), +der(6) +3, +19, +19, +21 ± dm.
||Clone C: 53,XY,t(6;11), +der(6) +4, +10, +13, +18, +19, +21.
One cell showed an additional translocation : 46,XY,t(6;11),t(3;5) (p12;q35). Blood culture with PHA was done on December 18, after one course of chemotherapy and revealed a normal lymphocyte karyotype in 16 cells.
DISTINCT SUBPOPULATIONS IN ACUTE LEUKEMIA

grown in large numbers from fractions 1.056 and 1.059 g/ml. They exclusively showed type A metaphases ($n = 32$), indicating their formation from the minority clone only.

Morphological studies of the cell fractions before culture did not reveal apparent differences between the cells from fractions 1.056 through 1.065 g/ml. The colony cells in the leucocyte feeder assay were mostly monocytic cells and macrophages. In the PHA–l.f. assays undifferentiated blast cells were grown and cytochemistry was inconclusive.

Similar cell fractionation was performed on bone marrow at the time of relapse (Table 3) and an even lighter density fraction 1.053 g/ml was isolated. Colony growth in both techniques was numerous. Chromosome analysis showed a monotonous karyotype of clone A in all metaphases before and after culture in both assays.

DISCUSSION

Fig. 1. Karyotypes observed in bone marrow cells of the patient at diagnosis. Upper section: 46,XY,t(6;11) (q27;q23) representative of clone A. Lower section: 51,XY,t(6:11), + der (6), +3, +18, +19, +21 representative of clone B. In this metaphase one of the der (6) is slightly contracted. R-bands with acridine orange.

grown from unfractionated bone marrow (leucocyte fed culture) showed only clone A metaphases ($n = 17$). On April 23, 1980, during florid relapse, three normal and 47 clone A karyotypes were identified.

Cell Fractionation Studies

Fractionation studies by the combined techniques of E–rosette forming cell depletion and discontinuous albumin gradient centrifugation require a large number of cells. They were applied to blood at diagnosis and to bone marrow at the time of relapse. Results of colony growth and cytogenetic analysis of the different blood cell fractions, before and after culture are given in Table 2. At diagnosis, numerically, clone B appeared the major one in fractions 1.056 and 1.059 g/ml. Grossly the same repartition between clones A and B were observed in colony cells in the PHA–l.f. assay. At the same time, leucocyte feeder colonies were

This study of one patient with acute monoblastic leukemia demonstrates that combined cytogenetic analysis and colony cultures may disclose within the neoplasm subclones with different karyotypes, growth requirements and therapeutic responses. The PHA–l.f. assay has been designed for acute leukemic blast colony growth and supports leukemic cell growth in about 80% of cases. The standard leucocyte feeder assay is suitable for normal myeloid stem cell proliferation rather than leukemia. In patients with AML usually no colony or minimal colony formation in the presence of large numbers of clusters is observed. Only rarely will large numbers of colonies arise from AML bone marrow.

In the case reported here, colonies were grown in both assays. The PHA–l.f. assay did not show selectivity of growth for different leukemic subpopulations and clones A and B were represented in colony cells approximately in the same proportions as in the cell suspensions before culture. In the conventional leucocyte feeder method on the other hand, only cells of clone A with the minimal chromosome rearrangement formed colonies. The selectivity was striking because colony cells showed merely clone A metaphases whereas cytogenetic analysis had revealed that 85% of blood and bone marrow metaphases belonged to clone B at diagnosis (Table 1 and 2) or to normal stem cells at partial remission (Table 1).

Morphological examination of these colonies suggested that the cells from clone A were still capable of some maturation along the monocytic cell lineage. The findings that cells of the ancestral clone with the minimal and apparently balanced chromosomal rearrangement (karyotype A) were still able to grow in the
Table 2. Cytogenetic Analysis on Blood Cell Fractions Prior to and After Culture in Conventional Leucocyte Feeder and PHA-Leucocyte Feeder Colony Assays (Nov. 21, 1979—at diagnosis)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1.056 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.059 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.062 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.065 g/ml Before Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of colonies†</td>
<td>—</td>
<td>229</td>
<td>10</td>
<td>—</td>
<td>165</td>
<td>185</td>
<td>—</td>
<td>79</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>number of cells with karyotype‖</td>
<td>clone A</td>
<td>2/8</td>
<td>16/16</td>
<td>1/5</td>
<td>0/12</td>
<td>16/16</td>
<td>2/6</td>
<td>1/3</td>
<td>3/4</td>
<td>6/8</td>
</tr>
<tr>
<td>clone B</td>
<td>6/8</td>
<td>0/16</td>
<td>4/5</td>
<td>12/12</td>
<td>0/16</td>
<td>4/6</td>
<td>2/3</td>
<td>1/4</td>
<td>2/8</td>
<td></td>
</tr>
</tbody>
</table>

*fractions obtained with concurrent E-rosette cell depletion and discontinuous albumin density gradient centrifugation.
†number of colonies per 10^5 cells plated.
§65/10^6 colonies were scored in the leucocyte feeder assay; these were not karyotyped.
§26/10^6 leucocyte feeder and 80/10^6 PHA-l.f. type colonies were counted, but not karyotyped.
‖number of metaphases with the clone A or B karyotype as a ratio of the total number of cells karyotyped.

leucocyte feeder assay and that the derived clones B and C were not, fit in with the concept of progressive dedifferentiation of subclones during malignant evolution.

While cells of clone B did not grow in the leucocyte feeder system, they produced large numbers of blast cell colonies when tested in the PHA-l.f. assay. It indicates that the cells of clone B were capable of expanding in vitro, when given specific growth stimulating factors.

Cells from clone C were not recognized in the cell fractions, neither before nor after culture. This is not too surprising. From their karyotypic changes one could expect these cells to behave similarly as the cells from clone B. The number of cells analyzed from each fraction was probably too small to recover metaphases from this minor subclone. In any case, one can safely conclude that the fractions studied were not enriched in cells from clone C and that the culture techniques used were not selectively growing these cells.

It is noteworthy that clones A and B, characterised by specific karyotypes, and typical requirements of colony growth in vitro had seemingly different fates and roles in the course of the disease. Before diagnosis clones B and C had overgrown the parental clone A and, in this sense, demonstrated faster growth. Clones B and C were successfully eradicated following cytotoxic treatment and were not identified again, which is consistent with a complete remission of these components of the leukemia. Clone A cells persisted and were responsible for therapy failure, i.e., only partial remission. During partial remission, a cytogenetic survey demonstrated 50 normal karyotypes and eight clone A cells among 58 metaphases, and at the same time the leucocyte feeder colonies (from unfractionated marrow) were exclusively of clone A karyotype. Meanwhile, in spite of further therapy, clone A grew out and progressively replaced normal marrow elements, which had transiently regenerated following elimination of B and C cells.

It has been proposed that some myeloid leukemias can be induced to revert from malignant to a nonmalignant phenotype by induction of differentiation. In this case we have an example of a leukemic population (clone A), still capable of some maturation in vitro (and possibly in vivo) that proved less amenable to treatment than the undifferentiated clone B. Differences in growth rates of both clones may possibly

Table 3. Cytogenetic Analysis on Bone Marrow Cell Fractions Prior to and After Culture in Conventional Leucocyte Feeder and PHA-Leucocyte Feeder Colony Assays. (April 23, 1980—at relapse)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1.053 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.056 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.059 g/ml Before Culture</th>
<th>Leucocyte Feeder Colonies</th>
<th>PHA-l.f. Colonies</th>
<th>1.062 g/ml Before Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of colonies†</td>
<td>—</td>
<td>1606</td>
<td>350</td>
<td>—</td>
<td>1596</td>
<td>585</td>
<td>—</td>
<td>936</td>
<td>577</td>
<td>—</td>
</tr>
<tr>
<td>No. of cells with karyotype A‡</td>
<td>n.t.</td>
<td>16/16</td>
<td>16/16</td>
<td>16/16</td>
<td>8/8</td>
<td>16/16</td>
<td>8/8</td>
<td>16/16</td>
<td>8/8</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

*fractions obtained by concurrent E-rosette cell depletion and discontinuous albumin density gradient centrifugation.
†no. of colonies per 10^5 cells plated.
‡number of metaphases with the clone A karyotype as a ratio of the total number of cells karyotyped
n.t. = not tested.
explain these different therapeutic susceptibilities: investigations along this line have not been made.

One important point which emerges from the findings in this patient is that in vitro assays can recognize subsets with qualitatively different proliferation/maturation capacities in human leukemia. We realize that we could make these observations on karyotypes related to growth abilities due to the unique circumstances in this patient. This limits the possibility to generalize from these findings at present. Nevertheless, if confirmed in situations of frequent karyotypic evolution (e.g. in CML), colony assays with varying selectivity may prove useful to characterize disparate cell populations among neoplasms for clinical monitoring. The principle of using assays, in which distinct cell clones exhibit preferential growth, could also potentially be applied to increase the sensitivity of cytogenetic analysis for minimal cell clones. The demonstration of pure clone A colonies from the minor clone A, as shown in our patient, is essentially in line with this assumption. One might suppose, that this approach could e.g., permit earlier detection of the emergence of new clones in hematological diseases (CML) which are prone to transform into acute leukemias.

REFERENCES

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