# Detection of Leukemic Clone Maturation In Vivo by Premature Chromosome Condensation

By Walter N. Hittelman, Phylisha Agbor, Iskra Petkovic, Borje Andersson, Hagop Kantarjian, Ronald Walters, Charles Koller, and Miloslav Beran

**The purpose of this study was to determine the feasibility of using the technique of premature chromosome condensation to detect the in vivo maturation of abnormal elements in patients with chronic myelogenous leukemia (CML), myelodysplastic syndrome, and acute leukemia. Patients were chosen for study if there were a clinical suggestion of in vivo maturation and a leukemic clone exhibiting a distinguishable karyotypic abnormality. Mature peripheral blood granulocytes were enriched by two-step Ficoll-Hypaque gradient sedimentation and fused with mitotic Chinese hamster ovary cells to induce the formation of prematurely condensed chromosomes (PCC). These PCC were then analyzed for chromosome number per cell (in the case of patients with a numerical abnormality) or by G-banding (in the case of specific translocations). Of 13 patients chosen for study, 12 showed karyotypic evidence for maturation of the abnormal elements in vivo. Maturation was observed in a number of clinical situations including before treatment in benign CML and myelodysplasia, after low-dose and high-dose chemotherapy in myelodysplasia and acute myelogenous leukemia (AML), and in remission. These results suggest that the technique of premature chromosome condensation can be a powerful tool in better understanding the biology of disease and mode of response to therapy in vivo in patients with leukemia and preleukemic syndromes, especially during treatment with agents thought to induce maturation of the leukemic elements.**



THE DEVELOPMENT of malignancy in general and leukemia in particular is thought to be a multistep process involving both the regulation of proliferation and the control of maturation.[1-3] In some hematopoietic diseases, eg, the benign stage of chronic myelogenous leukemia (CML), the major problem appears to concern growth regulation while maturation continues.[4] In other hematopoietic diseases such as myelodysplastic syndromes (MDSs), there is abnormal regulation of maturation.[5] Finally, in acute leukemia or the blastic phase of CML, proliferation is unregulated, and maturation is impaired.[6,7] In such situations, therapeutic intervention has focused on cytotoxic elemination of the leukemic elements to allow repopulation of the bone marrow with normal hematopoietic elements.

In recent years, evidence has been accumulating that leukemic cell maturation can take place in vitro and in vivo. For example, experiments with human and mouse myeloid leukemic cells grown in culture have shown that some clones of leukemic cells can be induced to mature by a variety of chemotherapeutic and growth and differentiation regulatory molecules.[8-10] Similarly, many of these same substances have been found in mouse model systems to retard or prevent the growth of leukemic cells in vivo.[11] As a result of such experiments, therapeutic trials have been initiated with the intent of inducing the maturation of abnormal elements in both acute leukemia and in myelodysplasia.[12,13] Such trials involve the use of low doses of chemotherapeutic agents (eg, low-dose cytosine arabinoside [Ara-C] or mithramycin) or bioregulatory agents (eg, *cis*-retinoic acid or recombinant colony-stimulating factors).

The biologic basis of response in such trials has been difficult to assess since it is not known whether the mature elements arising after treatment are of normal or malignant origin.[14-16] The occurrence of a hypoplastic phase before response has led some investigators to suggest that the efficacy of low-dose Ara-C, for example, is due to its cytotoxic effect or to its inhibitory effect on the self-renewal properties of the leukemic clone.[17] On the other hand, the lack of a hypoplastic phase before response has led other investigators to suggest that the clinical effect is due to the induced maturation of the leukemic clone.[18,19]

To determine whether maturation of leukemic elements occurs in these varied clinical situations, one must determine whether the mature elements, eg, peripheral blood polymorphonuclear cells or monocytes, are of normal or malignant origin. Because such mature cells are incapable of proliferation, conventional cytogenetic techniques cannot be used to detect their origin. Several approaches to this question have involved the detection of clonality in the mature populations by using probes that detect either glucose-6-phosphate dehydrogenase (G6PD) isoenzyme patterns[20] or DNA restriction fragment–length polymorphisms[21,22] in heterozygous individuals. While these approaches are powerful, they are limited to subsets of patients where informative probes are available. Another approach has been to label the myeloblasts in vivo with bromodeoxyuridine (BUdR) and determine whether the granulocytes derived after treatment contain BUdR.[23] However, this finding simply means that the granulocyte progenitor cell was cycling at the time of BUdR administration but does not positively identify the origin of the mature granulocyte.

The purpose of this study was to determine whether the technique of premature chromosome condensation could be

*From the Departments of Medical Oncology and Hematology, The University of Texas M.D. Anderson Cancer Center at Houston.*

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*Address reprint requests to Walter N. Hittelman, PhD, Department of Medical Oncology, Box 19, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030.*

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**Table 1. Clinical Characteristics of the Patients**

| Patient | Diagnosis | Treatment Before Study | Stage of Disease at Time of Study | Karyotype Marker |
|---|---|---|---|---|
| 1 | CML | Unknown | Benign | 45, X, −Y, Ph¹+ |
| 2 | CML | None | Benign | 46, XY, Ph¹+ |
| 3 | CML | Hydrea, IFN-α | Accelerated phase | 46, XY, Ph¹+, t(3;14) |
| 4 | RAEB | Surgery, x-rays, CCNU (3 year earlier for astrocytoma) | Untreated | Hypodiploid, minutes |
| 5 | MPD | None | Untreated | Ring chromosome |
| 6 | RAEB-T | Hexamethylmelamine, Adriamycin, CTX (4 yr earlier for ovarian carcinoma) | Untreated | 42-44 chromosomes |
| 7 | AML (M1) | AMSA-OAP × 2, 1 yr earlier | Stable, no treatment | 47, XY, +8 |
| 8 | AML (M2) | Dauno, Ara-C, 6TG, vincristine, low-dose Ara-C | Partial remission | 45, X, −Y, t(8;21) |
| 9 | AML (M4) | MOPP, XRT (2 yr earlier), low-dose Ara-C | CR | 48, XY, +8, INV(16), +21 |
| 10 | AML (M1) | AdOAP (CR) MTX, 6MP, DOAP (CR), High-dose Ara-C, mitoxantrone, mithramycin, Hydrea | PR | 46, XX, t(8;9), t(18;21) |
| 11 | AML (M1) | AMSA-OAP (no response) | PR | 47, XY, +8 |
| 12 | RAEB-T past AML | AMSA-OAP, AdOAP, CTX, BCNU, VP16, BM TX | CR | 47, XY, +8 |
| 13 | AML (M1) | AMSA-OAP, CTX, BCNU, VP16, Auto BM Tx | CR | 45, X, −Y |

Abbreviations: AdOAP, Adriamycin, Oncovin, Ara C, prednisone; AMSA-OAP, Amsacrine, Oncovin, Ara C, prednisone; Auto BM Tx, autologous bone marrow transplant; CR, complete remission; CTX, Cytoxan; Dauno, daunomycin; DOAP, daunomycin, Oncovin, Ara C, prednisone; IFN, interferon; MOPP, nitrogen mustard, Oncovin, procarbazine, prednisone; 6MP, 6-methyl purine; MTX, methotrexate; PR, partial remission; RAEB, refractory anemia with excess blasts; RAEB-T, RAEB in transformation; 6TG, 6-thioguanine; XRT, x-irradiation.

used to detect the presence of the abnormal cytogenetic clone in the mature elements. This technique involves the fusion of mitotic tissue culture cells with the mature cells in question so as to result in the immediate condensation of the chromatin of the interphase cells into discrete chromosomes, ie, prematurely condensed chromosomes (PCCs).[24,25] This procedure thus permits karyotypic evaluation of nondividing cell populations. For example, we recently reported that, in a patient with acute myelogenous leukemia (AML) who had been treated with low doses of Ara-C, the mature granulocytes were found to be derived from the karyotypically abnormal clone.[26] To further determine the feasibility of this approach in various clinical conditions, we have chosen to examine the mature cells of patients with distinct cytogenetic abnormalities in whom their clinical situation suggested that leukemic cell maturation might be occurring. The results of this study indicate that the premature chromosome condensation technique can be a powerful technique for detecting leukemic cell maturation in vivo as well as for determining the extent of multilineage involvement in various forms of leukemia.

## MATERIALS AND METHODS

*Patient selection.* All patients in this study presented to the University of Texas M.D. Anderson Cancer Center for treatment of CML, MDS, or AML. Patients were selected for study on the basis of their clinical parameters (ie, suspected maturation of leukemic elements) and the presence of a distinct cytogenetic marker in the leukemic population as demonstrated by conventional mitotic analysis of bone marrow populations. These studies were approved by the M.D. Anderson Hospital Surveillance Committee and are in accord with an assurance filed with and approved by DHHS. These studies included three patients with CML, three patients with MDS or myeloproliferative disorder, and seven patients with AML (Tables 1 and 2).

**Table 2. Hematologic Data of the Patients at the Time of PCC Analysis**

| | | | Differential Count of WBC (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Diagnosis | WBC ($10^3/\mu L$) | Blasts | Pros | Myel | Meta | Bands + Polys | Eos/Baso | Lymph | Mono |
| 1 | CML | 39.1 | 0 | 0 | 4 | 10 | 69 | 7 | 7 | 3 |
| 2 | CML | 141.0 | 4 | 16 | 15 | 12 | 72 | 1 | 9 | 2 |
| 3 | CML | 91.0 | 32 | 21 | 18 | 5 | 13 | 1 | 8 | 2 |
| 4 | RAEB | 9.9 | 1 | 1 | 5 | 7 | 50 | 7 | 17 | 12 |
| 5 | MPD | 7.2 | 0 | 0 | 0 | 0 | 55 | 6 | 26 | 13 |
| 6 | RAEB-T | 10.3 | 15 | 4 | 3 | 1 | 16 | 4 | 37 | 20 |
| 7 | AML | 20.0 | 9 | 3 | 18 | 5 | 48 | 0 | 14 | 3 |
| 8 | AML | 0.5 | 0 | 0 | 0 | 0 | 94 | 0 | 5 | 0 |
| 9 | AML | 1.5 | 0 | 0 | 0 | 0 | 20 | 0 | 80 | 0 |
| 10 | AML | 4.1 | 6 | 0 | 0 | 0 | 65 | 0 | 27 | 2 |
| 11 | AML | 2.5 | 0 | 0 | 0 | 0 | 92 | 0 | 6 | 2 |
| 12 | AML | 4.6 | 0 | 0 | 0 | 0 | 34 | 0 | 56 | 9 |
| 13 | AML | 4.8 | 0 | 0 | 0 | 0 | 32 | 0 | 60 | 8 |

Abbreviations: MPD, myeloproliferative disorder; Pros, promyelocytes; Myelo, myelocytes; Bands, band-form neutrophils; Polys, polymorphonuclear neutrophils; Eos/Baso, eosinophils and basophils; Lymph, lymphocytes; Mono, monocytes.

*Peripheral blood fractionation.* After informed consent of the patients, peripheral blood specimens obtained by venipuncture, and bone marrow aspirations were obtained from the iliac crest during diagnostic procedures. For enrichment of particular cell populations, a two-step Ficoll-Hypaque gradient system was used[27] that produced mononuclear cells at the top interface, polymorphonuclear cells at the intermediate interface, and erythrocytes in the pellet. In one case, the patient's monocyte fraction was enriched by adherence to plastic.[28]

Aliquots of the interface populations were placed on slides by using a Shandon cytocentrifuge, stained with Wright-Giemsa, and monitored for cell type. In some cases, cells on slides were monitored for peroxidase activity by incubation in $H_2O_2$ and diaminobenzimide followed by staining in Wright-Giemsa.

*Premature chromosome condensation.* The procedure for cell fusion and induction of PCCs has been previously described in detail.[29] Briefly, the fractionated peripheral blood or bone marrow cells were washed free of serum with Hanks' balanced salt solution and fused with mitotic Chinese hamster ovary (CHO) cells (obtained by selective detachment of Colcemid-arrested cells) by using Sendai virus. The duration of fusion ranged from 45 to 75 minutes to allow increased chromosome condensation.[30] At the completion of fusion and induction of PCC, the fusion mixture was treated with hypotonic 0.075 mol/L KCl for ten minutes, fixed in methanol–glacial acetic acid (10:1 and then 3:1 vol/vol), and dropped onto clean, wet microscope slides. These chromosome preparations were stained with Giemsa (Fisher, Houston). Cells fused in the $G_1$ phase yield a single chromatid per chromosome, whereas $G_2$ cells yield two chromatids per chromosome. The PCC of S phase yield a pulverized appearance.

In some cases, it was desirable to identify the morphologic origin of the cell involved in a fusion and PCC formation. To ensure that there is not a selective fusion of contaminating immature cells in a predominantly mature fraction, in preliminary experiments the fusion process was aborted early (after cell fusion but before nuclear envelope breakdown and PCC formation), and the morphology of the nucleus was examined. No preferential fusion of immature cells over polymorphonuclear cells was observed.

To further ensure the identity of the fused cells since nuclear morphology is lost during chromosome condensation, the fusion products were tagged with markers characteristic of cell origin. To accomplish this, we used a modification of the technique described by Teerenhovi et al.[31] The fusion population was treated with hypotonic KCl as before and an aliquot removed and placed directly on slides by using the cytocentrifuge (Shandon-Elliot Cytospin, Selwickley, PA). These slides could then be processed before acid fixation for cell type–specific identification (eg, peroxidase activity) and then treated with successive fixatives of absolute methanol, methanol–glacial acetic acid (10:1), and then methanol–glacial acetic acid (3:1) to improve chromosome morphology. After the slides were stained in Giemsa, a one-to-one correspondence could be made between cell type and karyotype. Peroxidase activity proved to be a good marker in many cases because the level of peroxidase activity in the mature fractions were several orders higher in the mature cells as compared with the immature cells.

We were able to adapt the G-banding procedure of Seabright[32] and the C-banding technique[33] to the premature chromosome condensation methodology.[30] Four cases reported here were analyzed by G-banding techniques. The abnormal clone karyotype was identified in the other cases by the presence of cells with distinctive markers (eg, minutes, ring chromosomes, or aneuploidy). After the PCC preparations were stained with Giemsa, they were examined under the light microscope for the presence or absence of the cytogenetic marker of the abnormal clone.

## RESULTS

This study represents a feasibility study rather than a prospective study of the incidence of leukemic cell maturation in patients with leukemia. Therefore, the results are presented case by case wherein each patient's clinical and laboratory characteristics are described followed by the PCC cytogenetic findings. The results are grouped according to disease status at the time of analysis by the premature chromosome condensation technique.

*Normal controls.* Fusion of peripheral blood mononuclear cells and mature polymorphonuclear cells from normal controls yielded $G_1$ PCCs (Fig 1A) that exhibited 46 chromosomes per $G_1$ PCC (Fig 2). If cytocentrifuge slide preparations of the fusion population were made and stained with an appropriate marker for cell type, the same $G_1$ PCCs could be simultaneously examined for karyotype and cell type. This is illustrated in Fig 1B, which shows a cell containing a $G_1$ PCC derived from a peripheral blood granulocyte that had been treated with diaminobenzidine and $H_2O_2$ to show peroxidase activity of the cell. It is important to note that the CHO mitotic cells had no demonstrable peroxidase activity.

*Chronic granulocytic leukemia.* To determine whether premature chromosome condensation could be used to detect maturation of the abnormal clone in benign-phase CML, we

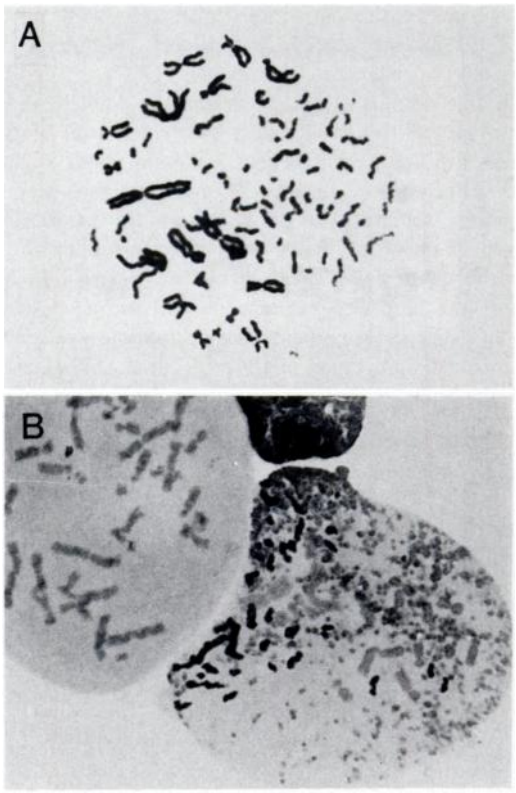


**Fig 1. (A) $G_1$ PCCs derived from peripheral blood granulocytes after fusion with a mitotic CHO cell. (B) A cytocentrifuge preparation of the fusion mixture reacted with $H_2O_2$ and diaminobenzidine and demonstrating residual peroxidase activity of the target granulocyte.**

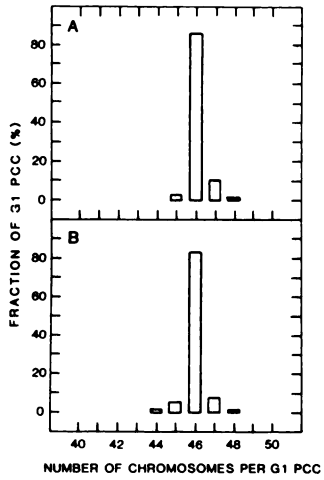


**Fig 2. Frequency distribution of chromosome number per cell in peripheral blood cells determined by premature chromosome condensation. (A) Mononuclear fraction. (B) Polymorphonuclear fraction.**

chose a patient who exhibited an aneuploid clone of 46 X, −Y, Ph[1] by conventional mitotic analysis (Table 1, patient 1). Fusion of this patient's peripheral blood granulocytes with mitotic CHO cells demonstrated a predominant clone with 45 chromosomes per cell (Table 3).

To more definitively identify the Philiadelphia chromosome in the peripheral blood granulocytes, $G_1$ PCCs of mature granulocytes from two additional patients with CML were G-banded (patients 2 and 3, Table 1). As shown in Fig 3A, B, the Philadelphia chromosome could be identified in the $G_1$ PCCs.

*MDS and myeloproliferative disorder.* MDSs represent a group of hematopoietic disorders characterized by bone marrow dysplasia and abnormal maturation of the granulocytic series. Since approximately 50% of these patients exhibit abnormal cytogenetic patterns,[34] they present a second clinical situation for probing whether the mature granulocytes are derived from the normal or abnormal clone.

A 32-year-old man (Table 1, patient 4) presented to the M.D. Anderson Hospital Leukemia Service with a smolder-

**Table 3. Enumeration of the Abnormal Clone in the Granulocyte-Rich Fractions of Patients with Numerical Aneuploidies**

| Patient | No. Cells Scored | No. Aneuploid Cells (%) |
|---|---|---|
| 1 | 44 | 32 (72.7) |
| 4 | 49 | 44 (89.8) |
| 6 | 46 | 36 (78.3) |
| 7 | 40 | 27 (67.5) |
| 8 | 64 | 40 (62.5) |
| 9 | 125 | 73 (58.4) |
| 11 | 127 | 57 (44.9) |
| 12 | 45 | 16 (35.6) |
| 13 | 77 | 42 (54.5) |

The granulocyte-rich fraction was obtained by two-step Ficoll-Hypaque sedimentation and contained ≥90% maturing granulocytes.

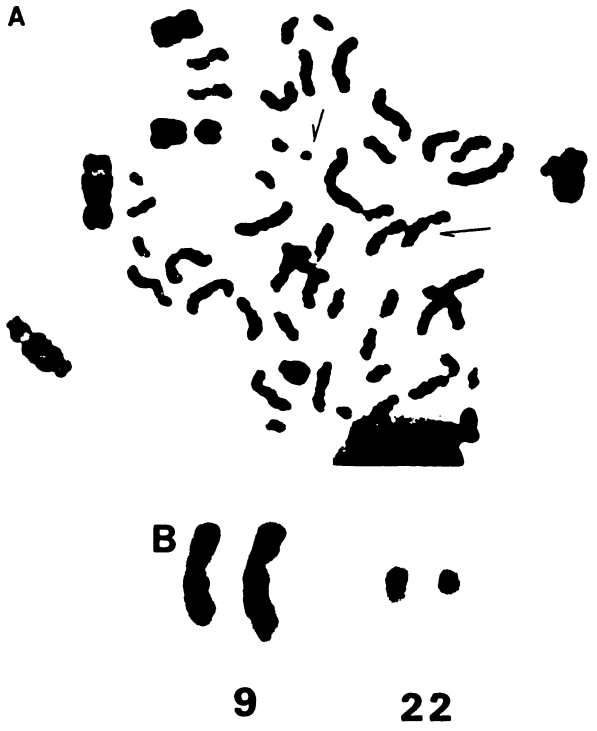


**Fig 3. G-banded $G_1$ PCCs derived from granulocytes obtained from patient 2 with CML demonstrate the presence of the Philadelphia chromosome, ie, t(9;22). (A) Banded $G_1$ PCC chromosome spread. (B) Partial karyotype.**

ing form of secondary leukemia (RAEB) 3 years after having been treated for an astrocytoma in the frontal brain. Examination of the bone marrow aspirate revealed a 60% cellular marrow, with 7.4% blasts, 31.2% progranulocytes, 19.3% metamyelocytes, and more mature elements. Conventional cytogenetic analysis of the bone marrow mitoses showed damaged chromosomes, a hyperdiploid karyotype, and double minute chromosomes. Since minute chromosomes are easily detected in PCC preparations,[35] blast-free peripheral blood from this patient was fractionated by density sedimentation and analyzed by premature chromosome condensation. Minute chromosomes could be found in PCCs derived from cells from both density fractions, which were predominantly single in $G_1$ PCCs and double in $G_2$ PCCs (Fig 4). Nearly 90% of the cells in the granulocyte-rich fraction contained at least one minute chromosome (Table 3) whereas 29% of the mononuclear fraction did not contain minute chromosomes. Bone marrow fibroblasts were also obtained by long-term bone marrow culture and were found to lack minute chromosomes. The results in this first patient with MDS therefore suggest that the abnormal cell clone was maturing along the granulocytic pathway before remission induction therapy.

The next patient, a 37-year-old man (Table 1, patient 5) with myeloproliferative disorder, presented with a 50% cellular bone marrow with 28.6% unclassified blasts and some evidence of maturing myeloid, erythroid, and lymphoid

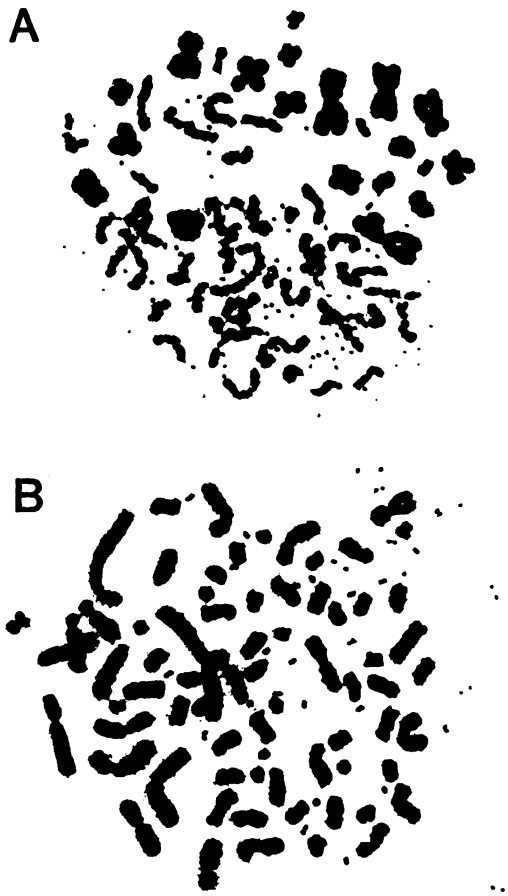


Fig 4. Evidence of minute chromosomes in (A) $G_1$ PCCs and (B) $G_2$ PCCs derived from peripheral blood cells of patient 4 with smoldering leukemia. Note that the minute chromosomes are single in $G_1$ PCCs and double in $G_2$ PCCs.

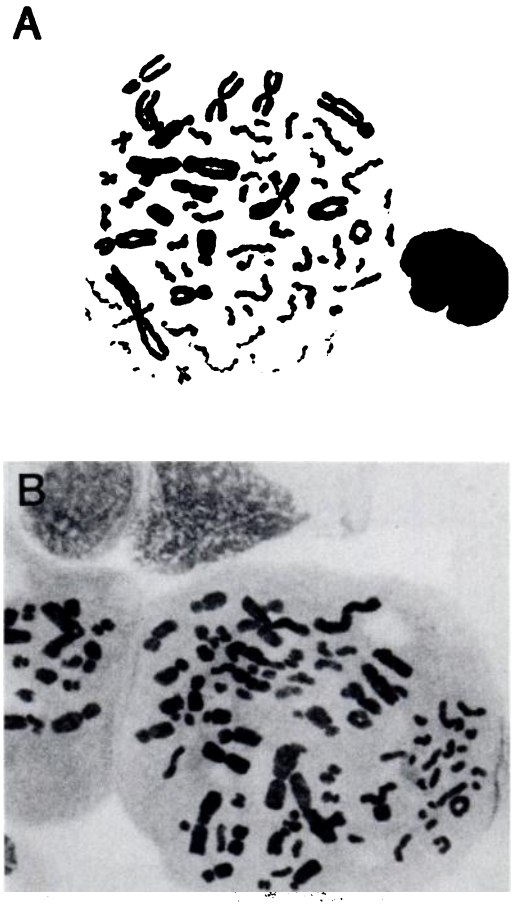


Fig 5. Demonstration of a ring chromosome in a $G_1$ PCC of a blast cell derived from patient 5 with myeloproliferative disorder. (A) Chromosome spread. (B) Cytocentrifuge preparation.

elements. At this time, no blasts were present in the peripheral blood (Table 2). Conventional cytogenetic analysis of the bone marrow revealed the presence of a ring chromosome in the leukemic clone. Similarly, PCC analysis of the light and dense fractions of bone marrow cells separated by two-step Ficoll-Hypaque sedimentation revealed that 19 of 55 and 2 of 50 $G_1$ PCCs, respectively, exhibited the ring chromosome (Fig 5 and Table 4). To determine whether the leukemic population containing the ring chromosome was maturing along any pathway, PCC analysis was also performed on a fractionated peripheral blood population. Only three of 100 $G_1$ PCCs examined in the mononuclear fraction exhibited the ring chromosome, whereas zero of 100 $G_1$ PCCs examined from the polymorphonuclear fraction exhibited the ring chromosome (Table 4). This result suggested that the leukemic clone was not maturing along any pathway in this patient. More likely, the 3% incidence of cells with ring chromosomes in the light fraction were leukemic cells that were not morphologically apparent on routine staining (eg, small blasts).

After the aforementioned analysis, morphologically identifiable blasts began to appear in the peripheral blood of this patient: this was accompanied by a rise in the monocyte fraction to 32%. It was therefore postulated that the monocytes might be involved in the leukemic process. However, PCC analysis of the light-density fraction of the peripheral blood at that time showed that cells with ring chromosomes occurred at the same frequency as the fraction of blasts in the population (Table 4), which suggests that blasts are not preferentially included in fusions. Further PCC analysis of cells selected by adherence to plastic (ie, enriched in monocytes) showed a lower frequency of $G_1$ PCCs with ring chromosomes. Thus, despite the coordinate occurrence of blasts and monocytes in the peripheral blood, there was no evidence for maturation of leukemic elements along any pathway in this patient. Nevertheless, the PCC technique was useful in detecting occult blasts in the peripheral blood.

The next patient (Table 1, patient 6), a 60-year-old woman, presented with RAEB-T 4 years after chemotherapy for an ovarian carcinoma. At the time of PCC analysis, a bone marrow aspiration demonstrated 40.2% blasts and 11.4% promyelocytes. Conventional cytogenetic analysis of

**Table 4. Frequency of $G_1$ PCCs With a Ring Chromosome in Bone Marrow and Peripheral Blood Fractions in a Patient With Smoldering Leukemia**

| Cell Fraction | Number of $G_1$ PCCs Scored | Number of $G_1$ PCCs With Ring Chromosomes (%) |
|---|---|---|
| November 2, 1984* | | |
| Bone marrow (top)† | 55 | 19 (35) |
| Bone marrow (bottom) | 50 | 2 (4) |
| Blood (top) | 100 | 0 (0) |
| Blood (bottom) | 100 | 0 (0) |
| January 14, 1985‡ | | |
| Bone marrow (top) | 100 | 34 (34) |
| Bone marrow (bottom) | 68 | 0 (0) |
| Blood (top) | 100 | 19 (19) |
| Blood (bottom) | 100 | 2 (2) |
| Blood (adherent fraction) | 100 | 8 (8) |

*At this time, the bone marrow contained 28.6% unclassified blasts, while no blasts were present in the peripheral blood.

†The top and bottom fractions refer to the two layers of a two-step Ficoll-Hypaque density gradient.

‡At this time, the peripheral blood contained 16% blasts and 32% monocytes.

the bone marrow population showed karyotypic instability, with chromosome numbers ranging from 43 to 46 chromosomes and some cells containing a ring chromosome. The peripheral blood at that time contained a mixture of myeloblasts, monocytes, lymphocytes, and maturing granulocytes (Table 2). Premature chromosome condensation analysis of the granulocyte-rich fraction (79.5% granulocytes) of the peripheral blood revealed karyotypic instability, with 83% of the cells exhibiting numerical aneuploidy. Interestingly, in the granulocyte-rich fraction, the clone with 44 chromosomes predominated (Fig 6). In contrast, in the blast-rich fraction of the bone marrow, equal frequencies of cells with 43, 44, and 45 chromosomes were observed. These results suggest that while several of the abnormal clones were still capable of maturation in this patient at the time of analysis, some clones were more proficient than were others in differentiation capability. Thus not only were cells heterogeneous in karyotype, they were also heterogeneous in biologic properties. Patients with karyotypic heterogeneity might be interesting to examine in detail to search for chromosomes

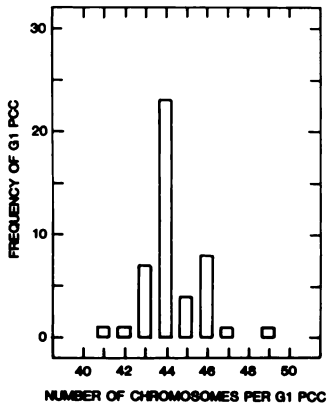


**Fig 6. Frequency distribution of chromosome numbers per cell in the granulocyte-rich fraction of the peripheral blood of patient 6 with RAEB-T. Note the presence of cells with 42 to 44 chromosomes.**

associated with biologic characteristics such as maturation capacity.

*AML.* While AML is generally accompanied by limited granulocytic maturation, some patients present with mature granulocytes in the peripheral blood, and in some patients, the absolute number of mature granulocytes increases during therapy without an interceding hypoplastic phase. To determine whether premature chromosome condensation might be useful in determining whether maturation of the leukemic elements can occur in vivo, we examined the granulocytes of seven patients with AML in whom leukemic cell maturation was suspected on clinical grounds.

The first patient, a 22-year-old man (Table 1, patient 7) presented with a smoldering acute leukemia (AML M1). Bone marrow examination showed a cellularity of 95% and 29.7% myeloblasts, and 100% of the dividing bone marrow cells exhibited 47 chromosomes with trisomy 8. Despite two courses of remission induction treatment, the patient continued to exhibit a subacute leukemic pattern, and therapy was discontinued until there were clinical signs of disease progression. One year later, the patient still continued to exhibit a smoldering leukemia pattern. Peripheral blood analysis at this time revealed 9% blasts and 48% mature granulocytes (Table 2). PCC analysis of the fraction containing 92.1% mature granulocytes showed a predominance of cells with 47 chromosomes (Table 3).

The next patient (Table 1, patient 8), a 42-year-old man with AML (M2), was studied after treatment with low-dose Ara-C (7.5 mg/m$^2$ daily) for 21 days for a smoldering relapse of a second complete remission. Examination of his bone marrow before treatment showed a cellularity of 35% with 40.5% Auer's rod–positive blasts and a cytogenetic clone of 45, X, −Y, t(8;21). With low-dose Ara-C treatment, the fraction of blasts in the bone marrow decreased and blasts disappeared from the peripheral blood. Although the fusion was limited technically, analysis of the concentrated granulocytes at this time showed a high fraction of cells with 45 chromosomes (Table 3).

Patient 9 (Table 1) is a 30-year-old man who was studied in complete remission after low-dose Ara-C treatment of a

low-infiltrate, secondary leukemia. Before treatment, conventional cytogenetic analysis of the bone marrow showed a karyotype of 48, XY, +8, INV(16), +21. Low-dose Ara-C treatment resulted in a clinical complete remission and a reversion of the bone marrow to a diploid cytogenetic pattern. Nevertheless, premature chromosome condensation analysis of his peripheral blood leukocytes during the first course of maintenance therapy showed aneuploidy in 58.4% of the cells scored (predominantly cells with 47 rather than 48 chromosomes) even though no blasts were present in the peripheral blood (Tables 2 and 3). This would suggest that an abnormal clone was still present; however, this clone was capable of maturation. Conventional cytogenetic analysis of bone marrow aspirates after the second course of low-dose Ara-C showed 40% of the mitotic cells with the original abnormal clone, and 75% abnormal metaphases were observed after the third course. The patient soon relapsed clinically.

Patient 10 (Table 1), a 46-year-old woman, was originally diagnosed in March 1980 with AML (M1). Conventional cytogenetic analysis demonstrated an abnormal clone with 46 XX, t(8;9) (p11;q34) in 100% of the metaphases analyzed. The patient achieved complete remission and then received 2 1/2 years of maintenance therapy. However, the patient's disease evolved into a pattern resembling a variant form of CML. Conventional cytogenetic analysis at this time showed a major clone with t(8;9) and t(18;21), a minor clone of t(8;9), and some diploid cells. The patient then achieved two transient complete remissions with high-dose chemotherapy, but her disease soon progressed to resemble CML in blast crisis. The patient was then treated with mithramycin (plicamycin) and hydroxyurea since this combination had previously been reported to be useful in CML.[36]

At the initiation of therapy, the bone marrow exhibited 73.5% blasts and 7% polymorphonuclear cells. With plicamycin treatment, the fraction of blasts in the peripheral blood decreased to 6%, and the granulocyte fraction increased to 65%. The granulocyte-rich fraction (34% polymorphonucleocytes and 66% bands) was then examined by premature chromosome condensation. Of 13 $G_1$ PCCs analyzed by G-banding procedures, 12 showed evidence of t(8;9) (ie, eight showed t(8;9), three showed 8p−, one showed

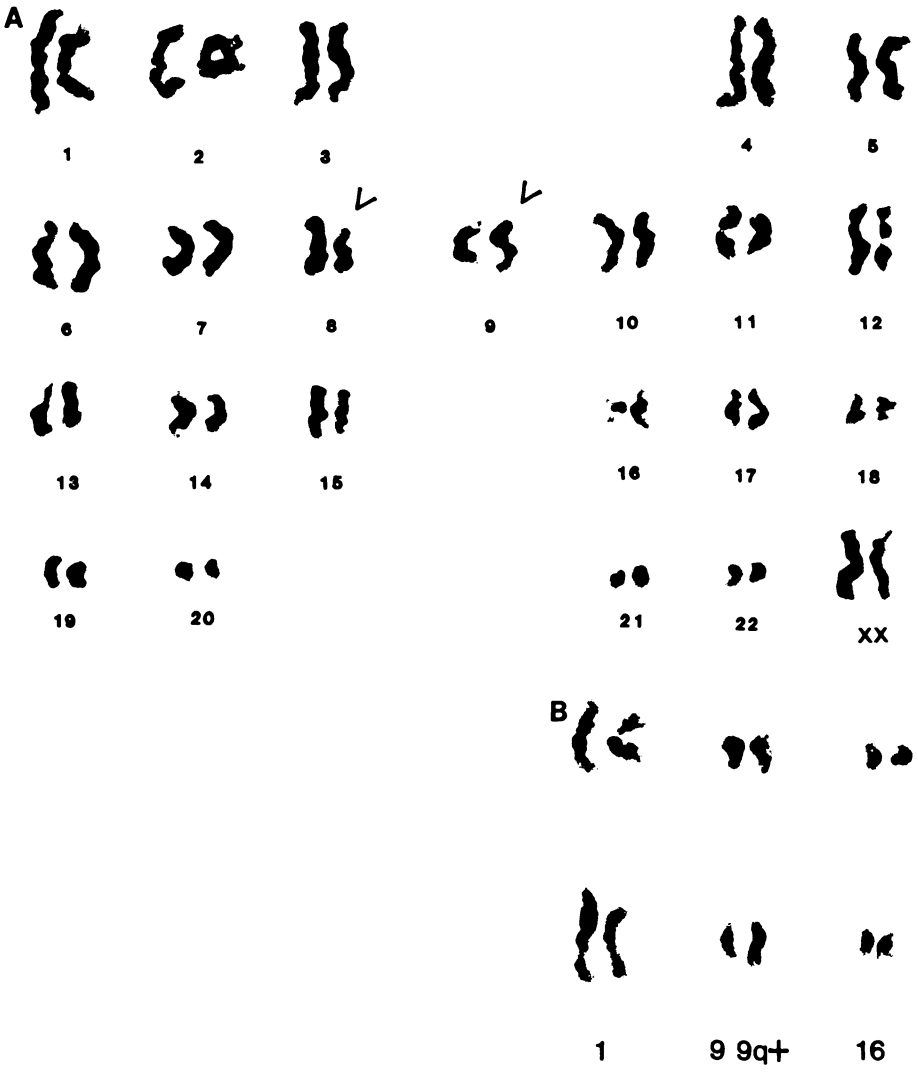


**Fig 7. (A) G-banded $G_1$ PCC karyogram of a mature granulocyte derived from patient 10 with AML who had been treated with low-dose plicamycin. Note the presence of the t(8:9), typical of the leukemic clone before treatment. (B) C-banded preparations demonstrating the presence of a chromosome 9q+ in the mature granulocytes.**

9q+). A $G_1$ PCC karyogram of a peripheral blood granulocyte is shown in Fig 7A. By C-banding procedures, seven of eight adequately banded $G_1$ PCCs showed evidence of a 9q+ chromosome (Fig 7B). Thus, of 21 informative $G_1$ PCCs of peripheral blood granulocytes, 19 of 21 showed evidence of t(8;9). It therefore appears that the clone containing t(8;9) was capable of maturation in vivo after plicamycin treatment.

The previous cases suggested that granulocytes derived from the abnormal clone could be found after low-dose treatment with Ara-C or plicamycin. The question then arises whether the abnormal clone might be capable of granulocytic maturation after high-dose therapy. This appeared to be the case for a 66-year-old man with AML (M1) (patient 11, Table 1) who was treated with fluoro-Ara–adenosine monophosphate (F-Ara-AMP) as salvage therapy. Before F-Ara-AMP treatment, conventional cytogenetic analysis of the leukemic blasts showed a karyotype of 47, XY, +8. After two courses of F-Ara-AMP, since there was some evidence of recovery of normal bone marrow, PCC analysis was performed when the peripheral white blood count was $2.5 \times 10^3/\mu L$ with 92% polymorphonuclear cells. PCC analysis of the concentrated polymorphonuclear cells showed that nearly 45% of the cells exhibited aneuploidy (Table 3).

The next patient (Table 1, patient 12) first presented with RAEB-T. Conventional cytogenetic analysis of the bone marrow aspirate showed an abnormal clone of 47, XY, +8. The patient received remission induction therapy (resulting in complete remission), maintenance therapy, intensification therapy, an autologous bone marrow transplant, and continued maintenance therapy, and then therapy was discontinued. Three months later (March 1986) during complete remission, peripheral blood was obtained, and the granulocyte-rich fraction was analyzed by the PCC technique. As shown in Table 3, approximately 35% of the $G_1$ PCCs showed 47 chromosomes. The presence of trisomy 8 in some of the cells was confirmed by G-banding of the PCCs (Fig 8). Soon after this sample was obtained (May 1986), the patient began to show clinical evidence of relapse. This result suggests that for some patients in remission a clone with the karyotype of the leukemic population might still be present; however, it might not be detected due to its ability to undergo maturation.

**Fig 8. G-banded $G_1$ PCCs derived from granulocytes of patient 12. Note the presence of three chromosomes 8s (arrows).**

A similar occurrence was found in the next patient; however, the event did not precede relapse. This 24-year-old man (Table 1, patient 13) first presented with AML (M1) in June 1984. Conventional cytogenetic analysis of the bone marrow showed 100% hypodiploidy (45, X, −Y). This clone was not observed in phytohemagglutinin-stimulated blood cells. The patient achieved complete remission, which was continued with maintenance and intensification therapy. Nevertheless, peripheral blood granulocytes obtained during complete remission still exhibited 45 chromosomes when examined by premature chromosome condensation (Table 3). Thus the detection of an abnormal clone in the granulocytes does not necessarily predict clinical relapse.

## DISCUSSION

The purpose of this study was to determine whether premature chromosome condensation might be useful in the study of maturation of abnormal elements before, during, and after therapy. By visualizing the chromosomes of mature, nondividing, peripheral blood cells by fusion with mitotic tissue culture cells, we have shown in several clinical situations that the mature cells were derived from the karyotypically abnormal clone. It is important to note that the patients presented in this feasibility study were selected for study on the basis of clinical evidence that leukemic cell maturation might be occurring. Future studies will determine the frequencies of such occurrences and the relationship of abnormal cell maturation to the biology and response of the patient's disease.

The individual situations in which maturation of abnormal elements were demonstrated are deserving of some discussion because of their implications for the biology of these diseases. The patients with CML in the benign phase were chosen as likely positive controls since several lines of evidence have already suggested that the mature granulocytes in these patients are derived from the abnormal clone. First, karyotypic analysis of the bone marrow populations of these patients generally demonstrates that 100% of the cells exhibit the Philadelphia chromosome.[37] Second, isoenzyme analysis of the mature granulocytes of black female patients with constitutive polymorphism of the G6PD locus demonstrates the clonal origin of these cells.[38] The clonality of the mature granulocytes has also been demonstrated in patients with constitutive DNA polymorphisms at other loci, including the c-*abl* locus involved in the 9:22 translocation associated with the Philadelphia chromosome.[39] In fact, the abnormal clone has been observed to mature along a variety of hematopoietic pathways including the B-lymphocyte pathway,[40] which suggests stem cell involvement in this disease.

In the natural history of CML, progression of disease to

the transitional and blast phases is often accompanied by karyotypic evolution and an apparent loss of the differentiative capacity of the leukemic clones.[41] It is therefore possible that the newly developed clones are less capable of maturation into peripheral blood granulocytes. For techniques that involve probes for polymorphism, since both the original Philadelphia-positive clone and the evolved clones are of the same clonal origin (and will exhibit identical isoenzyme and DNA polymorphism patterns), new DNA probes will have to be developed that can distinguish the evolved clone from the original clone. On the other hand, if cytogenetic evolution has been demonstrated in the dividing cells, then karyotypic analysis of the mature cells by premature chromosome condensation can be useful in determining the relative maturation efficiency of the evolving clones. This information will be important both before and after treatment for understanding the biology of disease evolution.

MDSs and smoldering leukemia represent other classes of diseases whose in vivo biology could be better understood by using premature chromosome condensation to determine the clonal origin of the maturing elements. These syndromes often occur with limited myelodysplastic maturation, and the origin of the residual granulocytic elements have not been well characterized. In this paper, we have demonstrated that the karyotypically abnormal clone can be found in the maturing elements of some of these patients before therapy. For patients with MDS and AML exhibiting monosomy 7, a similar conclusion was recently reported by Kere et al[22] who used restriction fragment–length polymorphism techniques. Thus it appears that despite an accumulation of immature forms in many of these patients there is still a capacity for maturation of the abnormal elements. It should be recognized, however, that morphologic maturation of abnormal clones does not imply full functionality of the mature cells. In fact, the mature granulocytes found in patients with active disease are often found to be deficient in one or more functional activities,[42-44] and this might explain the continuing increased risk of infections in these patients, even when adequate numbers of granulocytes are present.

It has been generally thought that acute leukemic disease is associated with an accumulation of immature forms with limited capacity for maturation. Thus, response to remission induction therapy has often been interpreted as cytotoxic elimination of the abnormal clone, thereby allowing regrowth of normal residual hematopoietic elements. This conclusion has also been reached in cases where patients with acute leukemia have been treated with therapies postulated to induce maturation of leukemic elements.[17] On the other hand, it has been proposed that low-dose Ara-C, for example, can induce maturation of leukemic elements and result in both the loss of renewal capacity of the abnormal clone and regrowth of normal elements.[45] The results presented in this paper show that, at least in these selected patients treated with low-dose Ara-C, plicamycin, and even F-Ara-AMP, the abnormal clone could mature into mature granulocytes during therapy.

It is not definitely known, however, whether these results represented therapy-induced maturation of leukemic cells or a return to a preexisting clonal hemopathy with maturation capacity. Since many of these patients with AML had secondary leukemias or had evolved from MDS, the latter possibility must be considered. However, several lines of evidence support the notion of induced maturation. First, in some patients an increase in the granulocyte count early in treatment accompanies a drop in the blast count, which leads to an increase in the absolute number of granulocytes before a period of regeneration. Second, the granulocytes must have been derived from blasts at some time in their development. Third, in vitro studies have shown that blast cells from some patients can be induced to mature under the influence of chemotherapeutic agents. Fourth, the detection of the abnormal clone in the granulocyte fraction preceded clinical evidence of relapse in some of these patients studied. The definitive answer to this question will have to depend on future studies where the degree of clonal maturation is monitored carefully before and during treatment by the PCC technique.

The results for clonality reported here, along with those previously published for other probes, therefore suggest that remission induction therapy for myelodysplastic disease, AML, and CML in blast crisis involves maturation induction as well as cytotoxic mechanisms. The role of each mechanism probably depends on both the therapy being used and the biology of each patient's disease. If mouse myeloid leukemia model systems are indicative of human disease, it is likely that some patients have leukemic cells capable of maturation induction while others do not. It has previously been suggested that patient leukemic cells should be prescreened with various differentiating compounds in vitro to predict whether maturation therapy might be a useful therapeutic modality.[46] In a similar vein, residual peripheral blood granulocytes at disease presentation could be analyzed by using the PCC technique to determine whether the abnormal clone has maturation potential in vivo before therapeutic intervention.

While DNA recombinant probes have proved to be an elegant approach to these questions, the PCC technique still has some advantages. First, the PCC technique requires comparatively small numbers of cells for analysis (ie, fewer than $10^6$ cells). Second, this technique can be applied to all those patients with cytogenetic abnormalities (ie, greater than 50% of the patients with MDS or acute leukemia). Third, where clonogenic evolution has occurred and can be detected at the cytogenetic level, the PCC technique can be used to better understand the biology of clonal evolution. Finally, a one-to-one correspondence can be made between karyotype and cell type where functional markers (eg, peroxidase activity) or immunologic markers (eg, antibodies against cell-specific antigens on the surface or within the cell) exist. This will prove to be crucial when heterogeneous populations are being probed for maturation down different hematopoietic pathways. Premature chromosome condensation should therefore prove to be effective in achieving a better understanding of the multistep pathway of leukemogenesis in individual patients before, during, and after therapy as well as during remission. Further, similar approaches could be used effectively to study the evolution of other malignant diseases that exhibit premalignant states as well as metastatic behavior.

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## REFERENCES

1. Knudson AG: Hereditary cancer, oncogenes and antioncogenes. Cancer Res 45:1437, 1985

2. Land H, Parada LF, Weinberg RA: Cellular oncogenes and multistep carcinogenesis. Science 222:771, 1983

3. Fialkow PJ, Martin PJ, Najfeld V, Renfold GK, Jacobson RJ, Hansen JA: Evidence for a multistep pathogenesis of chronic myelogenous leukemia. Blood 58:158, 1981

4. Champlin RE, Golde DW: Chronic myelogenous leukemia: Recent advances. Blood 65:1039, 1985

5. Hamblin TJ, Oscier DG: The myelodysplastic syndrome—A practical guide. Hematol Oncol 5:19, 1987

6. Champlin R, Gale RP: Acute myelogenous leukemia. Recent advances in therapy. Blood 69:1151, 1987

7. Hoelzer D, Gale RP: Acute lymphocytic leukemia in adults: Recent progress, future directions. Semin Hematol 24:27, 1987

8. Sachs L: Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. Nature 274:535, 1978

9. Hozumi M: Fundamentals of chemotherapy of myeloid leukemia by induction of leukemia cell differentiation. Adv Cancer Res 38:121, 1983

10. Sachs L: Cell differentiation and bypassing of genetic defects in the suppression of malignancy. Cancer Res 47:1981, 1987

11. Honma Y, Kasukabe T, Okabe J, Hozumi M: Prolongation of survival time of mice innoculated with myeloid leukemia cells by inducers of normal differentiation. Cancer Res 39:3167, 1979

12. Bloch A: Induced cell differentiation in cancer therapy. Cancer Treat Rep 68:199, 1984

13. Spriggs DR, Stone RM, Kufe DW: The treatment of myelodysplastic syndromes. Clin Haematol 15:1081, 1986

14. Koeffler, HP: Induction of differentiation of human acute myelogenous leukemia cells: Therapeutic implications. Blood 62:709, 1983

15. Desforges JF: Cytarabine: Low-dose, high-dose, no dose? N Engl J Med 309:1637, 1983

16. Ross DW, Capizzi RL: Differentiation versus cytoreduction during remission induction in acute nonlymphoblastic leukemia treated with sequential high-dose Ara C and asparaginase. Cancer 53:1651, 1984

17. Tagawa M, Shibata J, Tomonaga M, Amenomori T, Yoshida Y, Kuriyama K, Matsuo T, Sadamori N, Ichimaru M: Low-dose cytosine arabinoside regimen induced a complete remission with normal karyotypes in a case with hypoplastic acute myeloid leukemia with No. 8-trisomy: In vitro and in vivo evidence for normal haematopoietic recovery. Br J Haematol 60:449, 1985

18. Housset M, Daniel MT, Degos L: Small doses of Ara C in the treatment of acute myeloid leukemia: Differentiation of myeloid leukemia cells? Br J Haematol 51:125, 1982

19. Castaigne S, Daniel MT, Tilly H, Herait P, Degos L: Does treatment with Ara C in low dosage cause differentiation of leukemic cells? Blood 62:85, 1983

20. Fialkow PJ, Jacobsen RJ, Papayannopoulou T: Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet, and monocyte/macrophage. Am J Med 63:125, 1977

21. Fearon ET, Burke PJ, Schiffer CA, Zehnbauer BA, Vogelstein B: Differentiation of leukemia cells to polymorphonuclear leukocytes in patients with acute nonlymphocytic leukemia. N Engl J Med 315:15, 1986

22. Kere J, Ruutu T, dela Chapelle A: Monosomy 7 in granulocytes and monocytes in myelodysplastic syndrome. N Engl J Med 316:499, 1987

23. Raza A, Preisler H: Evidence of in vivo differentiation in myeloblasts labeled with bromodeoxyuridine. Cancer J 1:15, 1986

24. Johnson RT, Rao PN: Mammalian cell fusion: Induction of premature chromosome condensation in interphase nuclei. Nature 226:717, 1970

25. Hittelman WN, Rao PN, McCredie KB: Premature chromosome condensation studies in human leukemia, in Arrighi FE, Rao PN, Stubblefield E (eds): Genes, Chromosomes and Neoplasia. New York, Raven, 1981, p 379

26. Beran M, Hittelman WN, Andersson BS, McCredie KB: Induction of differentiation in human myeloid leukemia cells with cytosine arabinoside. Leuk Res 10:1033, 1986

27. English D, Anderson B: Single-step separation of red cells, granulocytes, and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 5:249, 1974

28. Koller CA, King GW, Hurturise PE, Sagone AL, LoBuglio AF: Characterization of glass adherent human mononuclear cells. J Immunol 111:1610, 1973

29. Hittelman WN: Premature chromosome condensation for the detection of mutagenic activity, in Hsu TC (ed): Cytogenetic Assays of Environmental Mutagens. Totowa, NJ, Allanheld, Osmun & Co, 1982, p 353

30. Hittelman WN, Petkovic I, Agbor P: Improvements in the premature chromosome technique for cytogenetic analysis. Cancer Genet Cytogenet 30:301, 1988

31. Teerenhovi L, Knuutila S, Ekblom M, Rossi L, Borgstrom GH, Tallman JK, Andersson L, de la Chappelle A: A method for stimultaneous study of the karyotype, morphology, and immunologic phenotypic of mitotic cells in hematologic malignancies. Blood 64:1116, 1984

32. Seabright M: A rapid banding technique for human chromosomes. Lancet 2:971, 1971

33. Scheres JMJC: CT banding of human chromosomes. Hum Genet 31:293, 1976

34. Yunis JJ, Brunning RD: Prognostic significance of chromosomal abnormalities in acute leukemias and myelodysplastic syndromes. Clin Haematol 15:597, 1986

35. Barker PE, Drwinga HL, Hittelman WN, Maddox A: Double minutes replicate once during S-phase of the cell cycle. Exp Cell Res 130:353, 1980

36. Koller CA, Miller DM: Preliminary observations on the therapy of the myeloid blast phase of chronic granulocytic leukemia with plicamycin and hydroxyurea. N Engl J Med 315:1433, 1986

37. Whang-Peng J, Canellos GP, Carbonne PP, Tsio JH: Clinical implications of cytogenetic variants in chronic myelocytic leukemia (CML). Blood 32:755, 1968

38. Fialkow PJ, Jacobson RJ, Papayannopoulou T: Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet, and monocyte/macrophage. Am J Med 63:125, 1977

39. Collins SJ, Groudine MT: Chronic myelogenous leukemia: Amplification of a rearranged c-*abl* oncogene in both chronic phase and blast crisis. Blood 69:893, 1987

40. Fialkow PJ, Denman AM, Jacobsen RJ, Lowenthal MN: Chronic myelogenous leukemia: Origin of some lymphocytes from leukemic stem cells. J Clin Invest 62:815, 1978

41. Spiers ASD, Baikie AG: Cytogenetic evolution and clonal proliferation in acute transformation of chronic granulocytic leukemia. Br J Cancer 22:192, 1968

42. Ruutu P, Ruutu T, Vvopio P, Kosunen TU, de la Chapelle A: Function of neutrophils in preleukemia. Scand J Haematol 18:317, 1977

43. Rausch PG, Pryzwansky KB, Spitznagel JK, Herion JC: Blood Cells 4:369, 1978

44. Suda T, Onai T, Maekawa T: Studies on abnormal polymorphonuclear neutrophils in acute myelogenous leukemia: Clinical significance and changes after chemotherapy. Am J Hematol 15:45, 1983

45. Steinberg HN, Tsiftsoglou AS, Robinson SH: Loss of suppression of normal bone marrow colony formation by leukemic cell lines after differentiation is induced by chemical agents. Blood 65:100, 1985

46. Lotem J, Sachs L: Potential pre-screening for therapeutic agents that induce differentiation in human myeloid leukemia cells. Int J Cancer 25:561, 1980