Premature Chromosome Condensation Studies in Human Leukemia: 4. Characterization of Albumin Density Fractionations of Bone Marrow at Presentation, Remission, and Relapse

By Walter N. Hittelman, Lijda Vellekoop, Axel R. Zander, and Karel A. Dicke

Previous studies have suggested that the technique of premature chromosome condensation (PCC) is useful in the study of human leukemia, both as a predictive tool for course of disease and as a probe to better understand the biology of the disease process. The purpose of this study was to determine how the various subcomponents of bone marrow populations contribute to the overall PCC pattern. Thirty bone marrow aspirations from persons at various stages of disease (2 normal, 11 untreated, 12 in remission, and 5 in relapse) were fractionated according to density in albumin gradients, and the various fractions were characterized by determining the proliferative potential index (PPI, or the fraction of G1 cells in late G1) using the PCC technique. In general, normal bone marrow and marrow from patients in remission showed lower PPI values throughout the gradients, with the most dense and most mature cells yielding the lowest PPI values in the gradient. In contrast, bone marrow from newly presenting patients and from patients in relapse showed higher PPI values, with the highest PPI values found in the most dense fractions containing the most mature cells. Thus, residual mature cells from patients with active disease exhibit the malignant characteristic of accumulating in late G1 phase. It is postulated that these morphologically mature cells from patients with active disease either represent matured cells of malignant origin or represent residual normal cells pushed beyond the restriction point in early G1 phase by a putative tumor growth factor synthesized by the leukemic cell population.

IN AN EFFORT to better understand the cell biology of human leukemia, the technique of premature chromosome condensation (PCC) has been used to study the cytokinetic characteristics of bone marrow populations patients with leukemia. Consistent with in vitro observations that normal cells tend to come to rest in early G1, while transformed cells accumulate in late G1 phase, it has previously been reported that bone marrow populations from patients with acute leukemia exhibit three to four times, on the average, as many cells in late G1 phase than do bone marrow populations from normal persons. Interestingly, however, there was no correlation between the fraction of G1 cells in late G1 phase (defined as the proliferative potential index, PPI) and the fraction of blast cells in the specimen. The PPI values were observed to fall to intermediate levels accompanying complete remission. However, in the months just prior to clinical evidence of relapse, the PPI values returned to high values. This latter finding was puzzling, since it suggested that morphologically normal cells were yielding chromatin patterns typical of malignant cells.

While the PCC technique is useful for monitoring the course of disease and for predicting relapse, little is known about which cell types within the bone marrow are responsible for the observed PCC patterns. In order to approach this question, we fractionated 30 bone marrow aspirations on albumin density gradients and analyzed the resulting subpopulations using the PCC technique. We report here that the PCC patterns observed correlated with the stage of disease. Interestingly, the most mature cells of marrows from nonleukemic persons and from patients in good complete remissions of their disease showed the lowest PPI values of the gradient, while the most mature cells from newly diagnosed and relapsing patients yielded the highest PPI values of the gradient. Thus, the PCC technique detects a chromatin characteristic of bone marrow cells that is more indicative of the state of the disease than the actual morphologies of the cells.

MATERIALS AND METHODS

Patient Selection

Bone marrow populations from 30 persons were examined by the PCC technique, and these are included in the following four categories: (A) 2 from normal, healthy volunteers; (B) 11 newly presenting, untreated patients with leukemia (4 with acute myeloblastic leukemia (AML), 3 with acute monomyeloblastic leukemia (AMML), 2 with acute lymphocytic leukemia (ALL), 1 with chronic myelogenous leukemia (CML), and 1 with chronic monomyelogenous leukemia (CMML)); (C) 12 patients clinically in complete remission (6 AML, 1 AMML, 4 ALL, and 1 CML); and (D) 5 patients in clinical relapse (3 AML, 2 ALL). Bone marrow aspirations from patients in remission were obtained at the same time bone marrow was removed for frozen storage. In the event of relapse, this autologous remission marrow would be given to the patient after high-dose cytoreductive treatment.

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therapy. More specific patient characteristics are given in Tables 1 and 2 and in the Results section.

**Bone Marrow Fractionation**

Bone marrow aspirations were fractionated on discontinuous albumin density gradients. This procedure has been described previously in detail. Briefly, erythrocyte-poor marrow cell populations were suspended in a 17% albumin solution (bovine serum albumin-BSA, fraction V, Sigma) in phosphate-buffered saline with Tris buffer, and pipetted onto the top of a multistep albumin density gradient consisting of 19%, 21%, 23%, and 25% albumin solutions. The osmolarity of the stock solution (35% albumin) was 350 mosmole, and the density of the layers varied from 1.0525 (17% layer) to 1.0730 (25% layer).

The cell gradients were then centrifuged for 30 min at 10°C in an International centrifuge at 2000 rpm, corresponding to 1000 g at the bottom of the centrifuge tube. After centrifugation, distinct layers of cells were visible in the gradient near the interfaces between the different albumin density layers. The cell fraction between the 17% and 19% BSA layers has been labeled fraction 1, between 19% and 21% BSA as fraction 2, between 21% and 23% as fraction 3, between 23% and 25% as fraction 4, and the bottom of the tube as fraction 5. Each fraction was then collected, washed twice with Hanks' balanced salt solution (GIBCO, Grand Island, N.Y.), cell number determined, resuspended in medium containing serum, and stored at 4°C until PCC analysis. In several cases, cytocentrifuge preparations were made of the prefractionated marrow and its density fractions and stained with Wright-Giemsa for morphological determinations.

When normal bone marrow populations were fractionated in this manner, fractions 1 and 2 consisted mostly of early blastic precursors; fraction 3 predominantly contained early myelocytic cell types (promyelocytes, myelocytes, metamyelocytes), lymphocytes, monocytes, and erythroblastic precursors; fraction 4 contained maturing myelocytic cells, lymphocytes, monocytes, and normoblasts; and fraction 5 contained mostly mature granulocytes and nucleated red cells. In marrow from normal persons and patients in remission, the least dense fractions 1 and 2 contained few cells and often were combined to provide enough cells for analysis. In patients with active leukemia, the cell population shifted toward lighter densities. While fewer cells were found in the more dense fractions during active disease, the dense fractions were still enhanced with the more mature elements. Details of the cell distributions and colony-forming activity of gradients observed in patients with active disease and in remission will be reported elsewhere.

### Table 1. Clinical Characteristics of Untreated Patients

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<th>Patient</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Percentage of Blasts</th>
<th>WBC (x 10³/µl)</th>
<th>Percentage Blasts</th>
<th>Hb (g/dl)</th>
<th>Platelets (x 10⁵/µl)</th>
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<th>Response</th>
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<td>75</td>
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<td>LFU*</td>
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<td>156</td>
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*LFU: Lost to follow-up.*

### Table 2. Clinical Characteristics of Patients Studied in Remission

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<th>Therapy at Time of Study</th>
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<td>OAP</td>
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<tr>
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<td>ALL</td>
<td>6.0</td>
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<tr>
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<td>6.3</td>
<td>CBV</td>
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<tr>
<td>11</td>
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<td>3.0</td>
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</tr>
<tr>
<td>12</td>
<td>CML</td>
<td>25.0</td>
<td>BuGsulf</td>
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</tbody>
</table>

*ED: Early death during CR.*

**Premature Chromosome Condensation**

The procedure for cell fusion and induction of PCC has been previously described in detail. Briefly, the fractionated 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) using Sendai virus. At the completion of cell fusion and the induction of PCC, the fusion mixture was treated with hypotonic 0.075 M KC1 for 10 min, fixed in methanol: glacial acetic acid (3:1 v/v), and dropped onto wet microscope slides. These chromosome preparations were then stained with Giemsa (Fisher, Houston, Tex.).

### Scoring of Slides

For each fusion between fractionated bone marrow cells and mitotic CHO cells, 100 PCC were located at low power and then scored at high power with the light microscope. Each PCC was scored for its position in the cell cycle (G1, S, or G2) according to PCC morphology, and each G1 PCC was graded for its degree of condensation on an arbitrarily defined scale of 1-6, with a value of 6 representing the most extended G1 PCC (late G1). The proliferative potential index (PPI) is defined as the ratio of the number of highly extended G1 PCC to the total number of G1 PCC observed. The PPI, therefore, represents the fraction of G1 cells in late G1 phase. While the PPI determination is rather subjective, the...
variation in scoring is small. For example, during the training of a research assistant, the PPI values of 29 consecutive bone marrow PCC preparations were compared. The mean difference in PPI values determined by two observers was 4.4 with a standard error of the mean of 0.77.

RESULTS

Bone Marrow From Hematologically Normal Persons

Two bone marrow aspirations were obtained from normal healthy volunteers, and the fractions obtained after density sedimentation were analyzed by the PCC technique. Both fractionations yielded similar patterns, one of which is illustrated in Fig. 1. Two characteristics of these normal gradients are of interest here. First, the PPI values throughout the gradient are generally low, in this case all under 20% (there were too few cells in fraction 1 for PCC analysis). Second, the more dense, and thus more mature, cells yield the lowest PPI values of the gradient.

Bone Marrow From Untreated Patients With Leukemia

Marrow from 11 patients with previously untreated leukemia was analyzed by density fractionation followed by PCC analysis. These patients' marrow and blood characteristics at the time of these studies as well as their subsequent responses to therapy, are listed in Table 1. Nine of the patients presented with acute leukemia, with a median bone marrow blast frequency of 69.6% (range 44%–96%). All but one of the patients with acute leukemia were previously untreated. Patient 5 had received melphalan previously for breast cancer but had been untreated for 2 yr prior to this admission. Five of the 9 patients with acute leukemia received remission induction therapy consisting of rubidazone, vincristine, cytosine arabinoside, and prednisone (ROAP), and one patient received OAP therapy. Two patients received cyclophosphamide in place of cytosine arabinoside (CROP), and one of these also received methotrexate and L-asparaginase. Patient 3 died prior to the initiation of therapy. Four of these patients achieved complete responses with remission induction therapy, one obtained a partial remission, two patients had no response, and one patient died on day 12 of the first course of therapy.

The buffy coats of the bone marrows of 7 of the 9 patients with acute leukemia were evaluated by the PCC technique prior to marrow fractionation. The median PPI value for this group was 40.0 (range 15.7–62.0). This is similar to our previously reported results in untreated patients with acute disease.1

All of the fractionated bone marrows from this group of patients presenting with acute disease showed one of two general patterns, neither of which resemble the normal patterns illustrated in Fig. 1. Figure 2 illustrates the pattern observed in 7 of the 9 patients presenting with acute leukemia. The PPI was observed to rise to very high values in the more dense fractions. For the gradient illustrated in Fig. 2, the first 3 fractions accounted for the bulk of the cells in the gradient (88%) and most of the morphologically identifiable leukemic cells. Fraction 5 contained only 4% of the total cells in the gradient, of which 34.5% were maturing granulocytes, 27.5% lymphocytes, and 30.5% maturing red cells, yet it showed the highest PPI value of the gradient (62.2%).

The remaining two bone marrow fractionations from untreated patients with acute leukemia showed high PPI values throughout the
gradient, even in fractions 4 and 5, which were enriched in maturing granulocytes and red cells.

Patient 2 (Table 1) had the lowest overall PPI value for this group of patients, yet showed a similar pattern (Fig. 3). Interestingly, fractions 1, 2, and 3 contained 88% of the cells on the gradient, consisting of 91%, 91.5%, and 73% morphologically identifiable leukemic cells, respectively, yet they had the lowest PPI values on the gradient. In contrast, fraction 5 contained 71% maturing granulocytes (only 14.5% leukemic cells), and it had the highest PPI value of the gradient (46.5%).

Bone marrow aspirations from two patients with untreated chronic myelogenous leukemia were also fractionated and analyzed by the PCC technique. Patient 10 presented with 7% blasts with adequate evidence of myelocytic maturation, was treated with busulfan, and returned home. While most of the cells in the density gradient were in fractions 3, 4, and 5, the PPI pattern resembled that of Fig. 2 and 3, where the most dense fractions containing the most mature cells had the highest PPI values. Patient 11 presented with a smoldering leukemia pattern, diagnosed as CML, with 23.2% unclassified cells.

In summary, all the fractionated bone marrows from untreated patients studied showed PPI patterns characterized either by elevated PPI values throughout the gradients or increasing PPI values in the more dense fractions consisting of morphologically maturing cells.

Thus, while these more dense cell populations appeared morphologically normal, they exhibited the malignant characteristic of accumulating in late G1 phase. This was in sharp contrast to normal bone marrow fractionations, where the most mature cells yielded the lowest PPI values in the gradient.

Bone Marrow from Patients in Remission

Bone marrow aspirations from 12 patients clinically in complete remission of their disease were also fractionated and studied by the PCC technique. The durations of complete remission and therapies at the time of aspiration for this group of patients are given in Table 2. Most of these aspirations were part of a large bone marrow pull for storage and subsequent use in the event of relapse or for bone marrow transplantation in remission. Included in this group were five patients who initially presented with AML, one with AMoL, one with AMML, four with ALL, and one with CML in benign phase. Excluding patient 12 (chronic CML), who had been maintained on busulfan for 2 yr, the medium duration of complete remission until the time of bone marrow aspiration was 6 mo (range, 3–30 mo) and the median time to relapse after bone marrow aspiration was 5 mo (range 2 to > 67 mo).

Of the 11 patients with acute leukemia who were studied in remission, 8 exhibited patterns similar to that observed in normal bone marrow, i.e., either low PPI values throughout the gradient or PPI percentages decreasing to low values in the more dense and more mature populations. Figure 4 illustrates such a remission marrow fractionation. In this case, 80% of the bone marrow cells in the gradient were located in fractions 4 and 5, which contained mostly mature granulocytes, maturing red cell elements, and some lymphocytes.

Patient 7 showed a distribution similar to that shown in Fig. 4, with the most mature fractions showing the lowest PPI values; however, the PPI values throughout the gradient were elevated and the PPI value for the unfractionated cells was also high (55.2%). Patient 12, with CML under control with busulfan treatment, had PPI values under 30% throughout the gradient, except in fraction 5, which yielded a PPI value of 64.9%. This value is subject to question.
since fraction 5 contained many of the cells of the gradient yet the
PPI value for the unfractionated gradient was 28.6%. This patient’s
disease is still under control 18 mo after bone marrow aspiration.

Two patients, clinically in remission at the time of study, showed
patterns typical of those observed at disease presentation. Patient 10,
whose fractionation is illustrated in Fig. 5, had been in complete
remission for 6.3 mo and was in a program of repeated high-dose
chemotherapy with autologous bone marrow transplants during
remission. This patient relapsed clinically 5 mo after this marrow
fractionation. Patient 11 also showed a fractionation pattern similar
to that shown in Fig. 2. This patient showed clinical signs of relapse 2
mo after PCC determination. In contrast, the median time to relapse
in the eight patients in remission who showed normal fractionation
distributions was 9 mo (range 3 to > 67 mo).

The last two patients described, both of whom initially presented
with ALL, showed an interesting phenomenon. Patients 10 and 11
had 65% and 69%, respectively, of the cells in the fractionations
located in fraction 5, consisting of 92.5% and 81.7% mature granulo-
cytes, respectively. Yet the PPI values for these fractions were high
(44.4% and 60.3%, respectively). Thus, while both patients clinically
had lymphocytic leukemia, their mature granulocytes showed the
malignant characteristic of accumulating in late G1 phase.

Bone Marrow From Patients in Relapse

The fractionated marrows of five patients were studied while they
were in a period of clinically evident relapse. Two patients were in
full relapse (64.6% and 88.2% bone marrow blasts), while the other
three patients were in earlier stages of relapse. One patient was
relapsing with patchy disease and one patient showed a smoldering
relapse. In four of the five patients in whom PCC was induced in
prefractionated cells, the PPI values were 40.0%, 37.9%, 31.0%, and
26.1%. Despite differing marrow blast frequencies, all five gradients from
relapsing patients showed patterns typical of those observed in newly
diagnosed patients, i.e., the most dense fractions containing the
highest fraction of mature cells yielded the highest PPI values. For
example, the patient relapsing with patchy disease (ALL) had 57%
of the cells in fraction 5, with 91.5% maturing granulocytes, yet the
PPI value for this fraction was 46.7%. Conversely, fractions 1 and 2,
containing 6% of the cells on the gradient (83% leukemic cells), had
a PPI value of only 16.2%.

A comparison of the PPI values and the cell morphologies of
fraction 5 samples are given in Table 3 for patients in whom cell
differentials were determined. While some fraction 5 samples are
contaminated with leukemic cells, most contain a predominance of
maturing granulocytes and erythocytic precursors. Nevertheless, the
PPI values of fraction 5 samples from untreated and relapsed patients
yield high PPI values, while those from normal and remis-
sion patients yield low PPI values. The two patients with ALL
showing high fraction 5 PPI values despite apparent CR status soon
relapsed. Interestingly, this finding of high PPI values in the most
dense fraction of cells from patients with active disease was observed
irrespective of whether the patient had myelocytic or lymphocytic
disease.

Correlation of Fractionated Components to
Unfractionated Marrow

One of the reasons for this study was to determine which cells in
the bone marrow give rise to the overall PPI value obtained routinely
for bone marrow populations. For example, in patients with high PPI
values just prior to relapse, it might have been possible that the
residual leukemic cells were much more sensitive to PCC induction
and would predominate in the PPI determination. If this were true,
one would expect the unfractionated marrow PPI to correspond to
the PPI obtained in the fractions high in leukemic cells. This result
was not observed. In fact, if one compares the weighted average PPI
for 20 fully evaluable fractionated marrows (obtained by summing
the multiplicands of the percentage of cells and PPI values in each
fraction) with the PPI value for the unfractionated cell population, a
good correlation exists (Fig. 6). The correlation coefficient for these
results is 0.903. This result also suggests that the PPI determination
is a reliable measurement.

DISCUSSION

The early studies using the PCC technique as a
probe of human bone marrow produced a number of
puzzling observations. While the fraction of G1 cells in
late G1 phase (defined as the proliferative potential
index, or PPI) in untreated patients with acute leukae-
mia was, on the average, three to four times that of
normal bone marrow, there was a great deal of vari-
bility from patient to patient. Interestingly, there
was no correlation between the PPI value and the
fraction of blasts or the blast infiltrate in the bone
marrow. Thus, some patients with few blasts had high
PPI values, while some patients with high blast counts
had low PPI values.

A second puzzling situation occurred just prior to
leukemic relapse. In the course of monitoring patients
clinically in complete remission, we observed that a
rise in the PPI to values above 35% was predictive of an

![Fig. 5. PCC analysis of fractionated bone marrow from a
patient with ALL in remission. In this case, the PPI values were
high throughout the gradient, typical of that found in patients with
active disease. This patient relapsed 5 mo after this aspiration was
obtained.](image)
# Table 3. Comparison of PPI Value and Cell Differential in Fraction 5 Samples

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<th>Diagnosis</th>
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impending relapse. Since these soon-to-relapse patients were in complete remission at the time of study, the bone marrow populations were morphologically normal. Conversely, their bone marrow populations showed the malignant characteristic of a high proportion of cells in late G1 phase. Thus, morphologically normal cells exhibited a malignant chromatin pattern just prior to relapse.

To better understand the cellular basis for these puzzling observations, we decided to determine how the various cell types within bone marrow populations contribute to the patterns observed while using the PCC technique. In this study, bone marrow aspirations from patients in various stages of their leukemia were fractionated according to cellular density and analyzed by the PCC technique. The general finding of the studies described here is that bone marrow fractionations from patients with active disease (at presentation of acute leukemia and at relapse of disease) show entirely different PCC patterns than those from normal persons and from patients in remission. Patients with active disease either yielded high PPI values (>35%) throughout the gradient or rising PPI values in the fractions containing cells of increasing density and maturation. In contrast, normal bone marrow and those from patients in remission showed generally low PPI values, with the most dense and mature cells exhibiting the lowest PPI values.

These results are interesting for a number of reasons. First, in newly presenting and relapsing patients, the PPI values in the least-dense fractions (containing the preponderance of leukemic cells) were found to vary from patient to patient. Interestingly, in preliminary studies we have found that, in general, those patients who exhibit high PPI values at presentation of disease also show high PPI values at relapse (Hittelman, unpublished results). Thus, these results suggest that the PCC technique measures some biologic characteristic of leukemic cells that distinguishes one person's disease from another's.

The meaning of the PPI values in those fractions rich in leukemic cells is not yet understood. By definition, cell populations with high PPI values have increased numbers of cells in late G1 phase. Yet this does not necessarily mean that these populations are rich in actively cycling cells. In fact, in a previous study, there appeared to be a slight negative correlation between PPI values and fraction of cells in S phase.
suggest that while not all leukemic cells are situated in late G1 phase, they tend to accumulate in late G1 phase when restrictions are placed on their growth.

The second interesting observation in these experiments is that the most mature cells in bone marrow populations from patients with active leukemia accumulate in late G1 (high PPI value). This is in sharp contrast to the situation found in normal and remission bone marrow where the mature elements accumulate in early G1 (low PPI value) (Table 3).

Figure 7 shows the cells from fractions 5 and 4 from patients 10 and 5 (Table 2), both of whom were in remission at the time of study. While the cells from both patients look morphologically similar, the PPI value for the cells in Fig. 7A was 44.4%, while that for Fig. 7B was 19.4%. Patient 10 relapsed 5 mo later, while patient 5 relapsed after 18 mo.

The biologic explanation for these results is not well understood. Two possibilities have been proposed.5,8 The morphologically mature cells in the patient with acute leukemia might represent leukemic cells that were capable of maturation.9 This notion gains support from the fact that leukemic cell lines have been derived that can be induced to differentiate in culture3 and from situations where leukemic cells obtained from patients can be induced to mature upon the addition of agents like phorbol esters and retinoids.14,15 This possibility would be especially interesting in light of our observation that, even in patients with lymphocytic leukemia, the mature granulocytes were arrested in late G1 phase. Thus, it would mean that either (A) the granulocytes were derived from the lymphocytic leukemic clone or (B) the malignant transformation event took place in an earlier stem cell than that anticipated in patients with ALL. We have previously shown that malignant cells grown in culture are distributed throughout G1 phase during exponential growth and accumulate in late G1 phase when growth restrictions are placed on the cells. In contrast, normal cell populations accumulate in early G1 phase during growth restriction.11 If one can apply these findings to the gradient fractions rich in leukemic cells, it would
by the morphology of the leukemic cells at presentation. If proposition (A) were true, it would suggest that during the leukemic process, normal differentiative processes are altered and cells of lymphoid origin might inappropriately mature into granulocytes. If proposition (B) were true, it would suggest that the leukemic event occurred at an early stem level and the maturation arrest occurs in only one of the cell lineages, yet both cell lineages might be involved in the leukemic event.

An alternative second possibility to explain these findings is that the leukemic cells are expressing or causing the expression of a factor that causes residual normal cells to go beyond the restriction point in early G1 and arrest in late G1 phase. We have previously shown that normal cells come to rest in early G1 phase, and these cells can be stimulated into late G1 by mitogenic stimulation. For example, PHA treatment of whole blood induces lymphocytes to progress from early G1 phase into late G1 phase and then into S phase. But recent studies have shown that this transition is multifactorial and requires both a lectin and the expression of a lectin-stimulated T-cell growth factor. In an analogous fashion, the putative leukemia growth factor might resemble a lectin's action by allowing maturing cells to pass beyond the restriction point yet not enter into S phase. This might resemble other tumor growth factors reported in other tumor situations.

On the other hand, if these morphologically normal cells accumulated in late G1 phase are truly derived from nonmalignant precursors, they might represent cells blocked by some leukemic factor from entering into S phase. The production of leukemic inhibitory activity by leukemia cells has been reported by Broxmeyer et al. This activity is thought to reside in an acid isoferritin molecule, which acts on colony-forming units in S phase as measured in liquid agar studies. Since it is not known how such activity would be recognized by the PCC technique, studies are being initiated in our laboratory to determine the effect at the PCC level of leukemic inhibitory activity on normal maturing cells in liquid culture and in methylcellulose.

At present we cannot determine whether the maturing cells arrested in late G1 in patients with active disease are derived from normal or leukemic elements. Experiments are continuing to distinguish between these two possibilities, or whether both processes might be occurring in the same patient. Nevertheless, the fact that this phenomenon is observed both in patients with active disease and in patients about to relapse suggests that it might be useful to monitor the mature elements in the blood to predict for relapse of disease in human leukemia.

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Premature chromosome condensation studies in human leukemia: 4. Characterization of albumin density fractionations of bone marrow at presentation, remission, and relapse

WN Hittelmann, L Vellekoop, AR Zander and KA Dicke

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