Predicting Response or Progression of Human Leukemia by Premature Chromosome Condensation of Bone Marrow Cells

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ABSTRACT

The object of this study was to apply the phenomenon of premature chromosome condensation to the prediction of the future course of the disease in leukemic patients during chemotherapy and to the forecasting of relapse if they are in complete remission.

The proliferative potential index (PPI) of bone marrows from leukemic patients compared with that of solid tumor patients with no bone marrow involvement. This technique involves the fusion of bone marrow cells with mitotic Chinese hamster ovary cells with the use of UV-inactivated Sendai virus. The morphology of the prematurely condensed chromosomes (PCC) of the bone marrow cells reflects their position in the cell cycle, i.e., G0, S, or G2. In addition, the G2 PCC exhibited a wide range of variability in their degree of condensation and were arbitrarily ranked on a scale of 1 to 6. The value of 6 represents the most decondensed and extended G2 PCC. The PPI of a bone marrow was defined as a percentage of highly extended G2 PCC (belonging to Classes 4 to 6) among the total number of G2 PCC scored (Classes 1 to 6). The average PPI values are: 11.7% for solid tumor patients; 27.5% for untreated leukemic patients; 22.0 to 24.5% for leukemic patients responding to chemotherapy; and 39.0% for those not responding to therapy. Among the 13 leukemic patients in complete remission at the time of the bone marrow sample, 4 patients had PPI values greater than 35%, of which 3 relapsed within 6 months. Of the remaining nine patients who exhibited low PPI values, only one relapsed within the same time interval. These data suggest a correlation between the PPI values of the bone marrow cells and the prognosis of the disease. A lower PPI value is indicative of a good prognosis, i.e., impending or continued remission with therapy. A high PPI value during active phase of the disease or during remission, on the other hand, is indicative of continued progression or impending relapse.

INTRODUCTION

Although human cancers are most successfully treated when the tumor burden is relatively small, early detection of active disease is generally difficult to achieve. In the case of leukemia patients in remission, for example, there is always the risk of a relapse and yet, unless a reemergence of a karyotypically abnormal clone of cells can be detected (13), there is no way of predicting when the cancer is about to resume an active phase. The prediction requires the ability to detect a cell population stimulated to divide within a predominantly resting or non-dividing population even before the proliferation becomes clinically evident in the bone marrow. Most prognostic procedures, such as labeling index and pulse cytophotometry, indicate the kinetic status of the bone marrow at a given time but are not capable of detecting a subpopulation of cells, G0 or G1, in this case, that is stimulated but is not yet synthesizing its DNA. For prediction of leukemic relapse, a technique is needed that can indicate the future growth course of the bone marrow. This paper describes a new approach to this problem by the application of the phenomenon of PCC.

Normally, chromosomes in eukaryotic cells become visible as distinct structures only during mitosis or meiosis. However, when an interphase cell is fused with a mitotic cell with the aid of Sendai virus, the interphase chromatin condenses into discrete units that are known as PCC. The morphology of the PCC of the interphase cell is characteristic of the cell's phase in the cell cycle (G0, S, or G2) at the time of fusion (10, 12). For example, PCC with single chromatids are characteristic of G0 cells, whereas PCC with double chromatids are characteristic of G2 cells. PCC from S-phase cells exhibit a "pulverized" appearance. Moreover, the PCC morphology also indicates the relative position of a cell within a particular phase of the cell cycle, i.e., early, mid, and late. As a cell passes through G0 phase, for example, the chromatin becomes more decondensed (11), and consequently the PCC become more elongated (14). We recently reported that the G2 PCC of PHA-stimulated human leukocytes are decondensed and greatly extended as compared to those of unstimulated cells, even though at the time of sampling neither of the populations have entered S phase (8). Thus, the PCC technique appears to have the potential to distinguish a G0 or G1 population that is stimulated to enter (but has not yet entered) S phase from the one that is not stimulated.

In the light of these observations, we decided to use the PCC technique to evaluate the proliferative potential of bone marrow in a variety of leukemic patients before, during, and after therapy and to correlate these data with the clinical course of the disease. The results of this study suggest that the condensation pattern of the G2 PCC of the bone marrow cells from solid tumor patients differed from...
those of leukemic patients. Among the leukemic patients those who are responding to chemotherapy showed significantly different patterns from those who are not. This technique may therefore have predictive value in forecasting the course of leukemic disease after therapy and during remission. An abstract of the study has appeared elsewhere (9).

MATERIALS AND METHODS

Cells. Bone marrow cells were drawn by aspiration from the iliac crest of either leukemic or solid tumor patients with their informed consent. The buffy coat obtained was washed twice with Hank's BSS and then incubated at 37° with McCoy's medium (Grand Island Biological Co., Grand Island, N. Y.) with 16% fetal calf serum for 20 to 30 min in the presence of [3H]thymidine, about 2 to 5 μCi/ml (Schwarz/Mann, Orangeburg, N. Y.), specific activity 6.7 Ci/μm. The mononuclear marrow cells were then concentrated by a Ficoll-Hypaque gradient centrifugation (2) and washed twice in Hank's BSS without serum and glucose. CHO cells were routinely grown as monolayer cultures on plastic Petri dishes (Falcon Plastics Co., Oxnard, Calif.) at 37° in a humidified incubator in modified McCoy's medium supplemented with 16% heat-inactivated fetal calf serum (Grand Island Biological Co.) and 1% antibiotic mixture (penicillin, 10,000 units/ml, and streptomycin, 10,000 μg/ml). Mitotic CHO cells were obtained by 3- to 4-hr colcemid treatment followed by selective detachment of the mitotic cells. The mitotic index of such a population is routinely greater than 95%. These cells were then washed in Hank's BSS without serum.

Cell Fusion. The fusion procedure to obtain PCC and the preparation of slides have been described in earlier publications (6, 7). Briefly, this involved mixing the bone marrow and mitotic CHO cells, centrifuging the mixture, and resuspending the pellet in 0.5 ml of Hank's BSS without glucose, containing about 2000 hemagglutinating units of UV-inactivated Sendai virus. The mixture was placed at 4° for 15 min and then transferred to a 37° water bath for a 45-min incubation. By this time cell fusion and the induction of PCC was completed.

Slide Preparation. At the end of fusion, a small volume (0.2 ml) of fetal calf serum was added and the fusion mixture was resuspended in 12 to 15 ml of hypotonic 0.075 M KCl for 10 min. The cells were then centrifuged, fixed in methanol:glacial acetic acid (3:1), and dropped on wet slides. The air-dried slides usually were stained with Giemsa. In some cases the slides were first prepared for autoradiography by (6) before staining with Giemsa. For autoradiography dried slides were dipped in Kodak nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.), diluted 1:3. They were allowed to dry and were stored in light-tight boxes. After 2 days of exposure, the slides were developed in D19 developer for 2 min and in Kodak fixer for 2 min, washed, and then stained with Giemsa (Fisher Scientific Co., Fair Lawn, N. J.).

Scoring of Slides. With the use of the light microscope, 70 to 100 PCC spreads were first located under low power and then examined under high power. The PCC were scored for their positions in the cell cycle (G1, S, or G2). The G1 PCC exhibited various degrees of decondensation, which were arbitrarily graded on a scale of 1 to 6. The value of 6 represents the most decondensed and extended G1 PCC. To avoid any bias bone marrows were scored without knowledge of the case histories.

In a previous study (8) stimulation of lymphocytes with PHA resulted in an increase in the frequency of cells with extended G1 PCC belonging to Classes 4 to 6. Since these extended G1 PCC represent cells that were stimulated to proliferate, the proportion of extended G1 PCC within the G1 population could be used as a quantitative measure of growth potential. We therefore introduce the term PPI, which is defined as the ratio of the number of highly extended G1 PCC (of Classes 4, 5, and 6) to the total number of G1 PCC scored.

Patient Groupings. For this study we compared the PCC characteristics of 82 bone marrow samples taken from 65 leukemic and solid tumor patients at various stages of their disease. For analysis the patients were grouped into the following clinically defined stages at the time of the bone marrow aspiration: (a) untreated solid tumor patients with no bone marrow involvement to serve as a control; (b) leukemic patients before therapy; (c) leukemic patients either stable or in partial remission during chemotherapy; (d) leukemic patients in complete remission while on maintenance therapy; and (e) leukemic patients in relapse or progressing on therapy at the time of sampling.

RESULTS

All 3 types of PCC (i.e., G1, S, and G2) were observed in bone marrow populations from both solid tumor and leukemic patients after fusion with mitotic CHO cells (Fig. 1). The G1 PCC from different cells showed varying degrees of condensation. The 6 classes (arbitrarily based on the degree of condensation) of G1 PCC of bone marrow cells are shown in Fig. 2. Pulse labeling of the bone marrow cells for 20 min with [3H]thymidine before fusion and subsequent autoradiography showed label only on the S PCC. For practical purposes the percentage of S PCC is equivalent to the labeling index of the total bone marrow population. Usually, the frequency of G1 PCC was less than 10%.

Table 1 compares the PPI values and the percentage of S PCC of bone marrow of untreated leukemic patients with those of solid tumor patients having no evidence of bone marrow involvement. In general, the bone marrow samples from leukemic patients exhibited significantly higher PPI values than did those of solid tumor patients. Untreated

Table 1

<table>
<thead>
<tr>
<th>Disease category</th>
<th>No. samples studied</th>
<th>PPI values</th>
<th>% S PCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td>31</td>
<td>27.5</td>
<td>6.0-76.0</td>
</tr>
<tr>
<td>Solid tumor</td>
<td>5</td>
<td>11.7</td>
<td>4.0-18.2</td>
</tr>
</tbody>
</table>

Predicting Response in Human Leukemia

SMART READER SERVICES
April 24, 1978

417

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leukemic patients had an average PPI value of 27.5%, as compared to an average of 11.7% for the solid tumor patients. Among the untreated leukemic population, there was no correlation between the PPI and the percentage of leukemic blasts in the bone marrow. The percentages of S PCC observed in the 2 patient populations were remarkably similar (Table 1). Thus, there was no direct correlation between the PPI value and percentage of cells in S phase. Nevertheless, among the untreated leukemic patients, the patients with the highest percentage of S PCC generally had PPI values greater than 30%. However, the converse was not true.

Chart 1 is a scatter diagram showing the PPI values for each bone marrow sample of treated leukemic patients. Most of the patients represented in this scatter diagram were receiving chemotherapy on either an induction or maintenance regimen. Bone marrow samples were usually taken at the end of a recovery phase and just before the initiation of a new course of chemotherapy. The PPI values were grouped according to the clinically defined stage of the disease at the time of the sampling. The 48 sample points represent 28 different patients. The horizontal dashed line for each class of patients indicates the average PPI for that class of patients. In general, the patients clinically in relapse or evidently progressing despite therapy showed higher PPI values (39.0%) than did those patients responding to treatment (22.0 to 24.5%).

The PPI values varied widely within each group of patients represented in Chart 1. In the progressing group of leukemic patients, 6 of 8 exhibited markedly high PPI values (greater than 35%). Of the 2 patients with low PPI values (25.0% and 23.5%), 1 was relapsing slowly during treatment with Bacillus Calmette-Guérin alone, while the other was progressing on hydroxyurea treatment alone. Thus, progression was generally marked by high PPI values.

The patients clinically in complete remission showed the converse pattern (Chart 1); i.e., most of the patients were clustered around lower PPI values, but 4 patients (representing 5 values) showed values greater than 35% even though their bone marrows were morphologically within the normal limits at the time of sampling. Of these latter 4 patients with high PPI values (25.0% and 23.5%), 1 was relapsing slowly during treatment with Bacillus Calmette-Guérin alone, while the other was progressing on hydroxyurea treatment alone. Thus, progression was generally marked by high PPI values.

The patients clinically in complete remission showed the converse pattern (Chart 1); i.e., most of the patients were clustered around lower PPI values, but 4 patients (representing 5 values) showed values greater than 35% even though their bone marrows were morphologically within the normal limits at the time of sampling. Of these latter 4 patients with high PPI values, 3 relapsed clinically within 6 months despite maintenance therapy, whereas the fourth received late intensification treatment and remained in complete remission. In contrast, only 1 of the 9 patients in complete remission with PPI values less than 35% relapsed within a period of 6 months. For these patients, clinically in complete remission at the time of PCC analysis, the PPI values of the patients relapsing within 6 months differed significantly from those of patients continuing in remission for more than 6 months (p < 0.01 by x² analysis). Further, if one groups the patients in complete remission with PPI values less than 35% relapsed within a period of 6 months. For these patients, clinically in complete remission at the time of PCC analysis, the PPI values of the patients relapsing within 6 months differed significantly from those of patients continuing in remission for more than 6 months (p < 0.01 by x² analysis). Further, if one groups the patients in complete remission who progress within 6 months with those patients clinically progressing and compares them to the patients who remain in complete remission, the PPI values are also significantly different (p < 0.01). Thus, the presence of lower PPI values was prognostic of continued complete remission.

Fig. 1. PCC of human bone marrow cells. A, G, PCC consist of single chromatids and appear spiralized. B, S PCC, distinguishable by their "pul-
The chronic myelogenous leukemia patient with the lowest duration of complete remission was less than 2 months. than 3 months after the time of this sampling, yet the duration of complete remission was less than 2 months. The chronic myelogenous leukemia patient with the lowest PPI in this class (1.8) still remains in stable condition after more than 1 year. One patient with acute myeloblastic leukemia in this group showed PPI values of 23.5, 27.7, and 26.7% over a 4-month period during this study. He remained in a "smoldering" condition without further therapy for at least 6 months after the last sample point.

The average frequency of S PCC in leukemic bone marrows before therapy was 12.2% (Table 1). After treatment, the percentage of S PCC rose significantly in the bone marrows from leukemic patients. Nevertheless, those patients relapsing, progressing, or about to relapse despite therapy had S PCC values comparable to those of patients in partial or complete remission (27.7% compared to 27.3 and 30.3%). Thus, differences between responding and nonresponding patients are difficult to discern on the basis of the frequency of cells in S PCC after therapy. The potential utility of the PCC technique in the prognosis of leukemia is illustrated in the following case histories. Chart 2 represents the case of a patient with smoldering leukemia before therapy, showing the percentage of blasts in the bone marrow and the peripheral WBC as a function of sample date. At the time that the bone marrow was obtained for PCC evaluation, the blast and WBC levels were similar to previous values, yet the PCC method indicated a high PPI value of 44.1%. Subsequently, the percentage of blasts in the bone marrow started to increase and the WBC showed a sharp increase starting nearly 2 months after the PCC technique had indicated a high proliferative potential for this bone marrow.

The second example (Chart 3) represents a patient with acute lymphocytic leukemia clinically in complete remission at the time of the first bone marrow sample studies by the PCC technique. This patient was on a maintenance therapy with vincristine, 1-ß-D-arabinofuranosycytosine, and prednisone. When studied by the PCC technique, the patient's bone marrow showed a PPI value of 46%, which suggested an impending relapse. At this time, the percentage of S PCC was 50.5%. When the next bone marrow sample was taken 6 weeks later, the blast level in the marrow had risen to nearly 70% from a very low value of 2%. The patient was then placed on a new protocol (6-mercaptopurine, vincristine, methotrexate, and prednisone). At this point, the patient's bone marrow showed an unusually low PPI value of 11.5% even though the bone marrow showed greater than 70% blast cells and a significant portion of the marrow cells were in S phase (35.7%). The patient subsequently went into remission. In this case the PCC technique predicted not only the impending relapse about 6 weeks before the manifestation of any clinical evidence but also the later onset of remission.

The third example (Chart 4) was a patient with chronic myelogenous leukemia in blast crisis. At the time of the first sample studied by the PCC technique, the patient's bone marrow contained 60% blasts, but it showed a PPI value of only 8% with 27.8% S PCC. The patient subsequently responded to piperazinedione, 1-ß-D-arabinofuranosyladenine, and 5-azacytidine. At the time of the next bone marrow sample to be studied by the PCC technique, the patient was clinically in complete remission but had a PPI value of 38.3%. In the sample taken 1 week later, the PPI rose to 50% despite no detectable change in the percentage of blasts. Subsequently, the blast count increased. In this case, progression of disease was predicted nearly 3 weeks before it was clinically observable.

The fourth example (Chart 5) was a patient with acute lymphocytic leukemia. At the time of the first bone marrow sample for PCC analysis, this patient showed a high blast count as well as a high PPI (54.8%) value. This patient responded only marginally to several courses of therapy. However, the sample taken about 4 months later for PCC examination revealed a lower PPI value (28%). The patient subsequently went into remission.

DISCUSSION

Previous results in our laboratory indicated that a population of PHA-stimulated lymphocytes could be distinguished from unstimulated cells by the PCC technique even before these cells initiated DNA synthesis (8). This finding was not unexpected, since upon stimulation the chromatins of the cell decondenses as it changes from a resting state to a proliferating state (1). Similarly, Waldren and Johnson (14) had shown that the PCC of HeLa G, cells became longer and more extended as the cells passed through G, toward S phase. Thus, the PCC technique appeared to have the ability to detect the proliferative potential of a cell population, such as human bone marrow. The results of our retrospective study involving the examination of bone marrows from a number of leukemic and solid tumor patients by the PCC technique indicate that it is possible to predict the future course of disease. These findings may be summarized as follows: (a) the average PPI values for the leukemic patients before therapy are higher than those of solid tumor patients with no obvious bone marrow involvement; (b) in the case of leukemic patients responding to therapy, the average PPI value showed a decrease as compared to the pretreatment value; (c) leukemic patients not responding to treatment exhibited an average PPI value nearly twice that of responding patients; and (d) those leukemic patients who were clinically in remission (showing normal bone marrow morphology) but who showed high PPI values soon relapsed.

It is important to note the lack of correlation between the PPI values and the percentage of S PCC. Untreated leukemic patients exhibited nearly twice the PPI values of solid tumor patients, yet both showed the same percentage of S PCC (12.4 and 10.0%, respectively). Subsequent to therapy the percentage of S PCC increased in both responding and nonresponding patients (27.3 and 30.3%, compared to 27.7%, respectively). However, the PPI's for responders and nonresponders were significantly different (24 versus 39%). From these data it is obvious that the parameters measured by labeling index and PPI are different. The basic differences between the PPI and percentage of S PCC is as follows. The percentage of S PCC

FEBRUARY 1978
Chart 1. Scatter diagram showing the PPI's of bone marrow samples from leukemic patients. The patients were grouped into 3 categories according to the clinical status of their disease at the time of sampling, i.e., stable or in partial remission (PR) on therapy; complete remission (CR) on therapy; or progressing despite therapy. Each symbol, 1 bone marrow sample evaluated by the PCC method. Dashed line, average PPI value for each category. AML, acute myeloblastic leukemia; AUL, acute undifferentiated leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia.

Chart 2. Case history of a patient with smoldering leukemia. Top panel, percentage of blasts in the bone marrow as a function of time; lower panel, WBC in the peripheral blood at corresponding times. A high PPI value (44.1%) was seen about 1 month prior to an increase in blast count in the bone marrow and 2 months prior to a similar increase in WBC. or labeling index gives the proportion of cells in S phase and hence the rate of proliferation at the time of sampling. The PPI, on the other hand, which indicates the percentage of highly extended G1 PCC among the G1 fraction, is a reflection of the proliferative activity that will take place at a later time. The PPI values are not based on the frequency of G1 cells in a given cell population. In view of these results, it is suggestive that, in comparison with normal marrow, a significantly high proportion of cells in leukemic bone marrow is held at a more advanced state in G1 phase, as characterized by their highly extended PCC (Fig. 2). The biological basis for the changes observed in the PPI levels for leukemic bone marrow populations is not well understood. In the case of peripheral blood lymphocytes stimulated with PHA, a rise in the PPI was indicative of an impending rise in the fraction of cells in S phase. A similar trend may not exist for leukemic bone marrow, since no correlation was found to exist between the PPI and percentage of cells in S phase. In this paper we have described a situation in which, for patients clinically in remission with

Chart 3. Case history of a patient with acute lymphocytic leukemia in complete remission on maintenance therapy. As in Chart 2, a high PPI value (46%) was soon followed by a rise in the blast count in the bone marrow, which was subsequently followed by an increase in WBC in the peripheral blood. On the other hand, the low PPI value (11.5%) observed when the blast count was at its maximum predicted a subsequent clinical response. △therapy, change in therapeutic regimen.

Chart 4. Case history of a patient with chronic myelogenous leukemia in blast crisis. As in Chart 3, a low PPI value (8%) during active disease predicted impending remission while a subsequent increase in the PPI value (to 38% and then to 50%) during complete remission predicted an impending relapse. △therapy, change in therapeutic regimen.
Predicting Response in Human Leukemia

Table 2
Correlation between PPI values and the future course of disease in leukemic patients

<table>
<thead>
<tr>
<th>State of disease</th>
<th>PPI</th>
<th>Therapy</th>
<th>Future course of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable</td>
<td>High</td>
<td>–</td>
<td>Progression</td>
</tr>
<tr>
<td>(smoldering)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>High</td>
<td>+</td>
<td>Refractory</td>
</tr>
<tr>
<td>Active</td>
<td>Low</td>
<td>+</td>
<td>Remission</td>
</tr>
<tr>
<td>In remission</td>
<td>Low</td>
<td>+</td>
<td>Continued remission</td>
</tr>
<tr>
<td>In remission</td>
<td>High</td>
<td>+</td>
<td>Relapse</td>
</tr>
</tbody>
</table>

Normal bone marrow morphology (and low blast count), a high PPI value preceded clinical evidence of relapse, sometimes by months. Two possible explanations might account for these observations: (a) the cells arrested with a late G\textsubscript{i} PCC morphology represent normal bone marrow cells arrested in late G\textsubscript{i} phase due to the influence of residual growing leukemic cells or (b) they represent the building up of leukemic cells in late G\textsubscript{i} prior to a burst of proliferation. In support of the first possibility, in vitro studies have suggested that the presence of leukemic cells in the bone marrow may prohibit normal cells from proliferating (3, 5). If this were the case, the normal bone marrow cells might be stimulated out of a resting phase, yet might be blocked in late G\textsubscript{i} phase prior to initiation of DNA synthesis by leukemic factors. In support of the second possibility, Frenster et al. (4) have shown that leukemic cells can sometimes differentiate morphologically and still show loose chromatin patterns. If this were true the leukemic cells could be hidden within a normal bone marrow morphology. Experiments are underway to distinguish these possibilities.

One of the limitations of this study is the subjective method of scoring the various classes of G PCC for the determination of PPI. Nevertheless, the results obtained are encouraging, particularly in view of the low incidence of false positives or negatives. Further prospective studies involving a greater number of patients and sequential observations are being performed to determine the ultimate usefulness of the technique in the clinical setting and to explore the relationship between the PPI values, the particular type of leukemia involved, and prediction of relapse by other techniques (bone marrow colony formation and cytogenetic and cytokinetic analysis). From the technical point of view, the PCC method has an added advantage in that results can be obtained within hours of the bone marrow aspiration. In this laboratory 12 bone marrow aspirations are routinely analyzed by the PCC technique each day.

On the basis of these data and the 4 individual cases presented in greater detail, a correlation seems to emerge between the PPI values of the bone marrow cells and the future course of the disease (Table 2). A lower PPI value is indicative of a good prognosis, i.e., impending or continued remission with therapy. A higher PPI value during active phase of the disease or during remission, on the other hand, is indicative of continued progression or impending relapse. This advance information could be useful for the patient in remission. When the PPI in these patients begins to rise, which is indicative of impending relapse, the clinician could be alerted to place the patient on a more effective therapeutic protocol before the tumor burden becomes too great.

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REFERENCES

Fig. 2. G, PCC of human bone marrow cells showing varying degrees of chromosome condensation. The G, PCC were classified on the basis of condensation and arbitrarily rated from 1 to 6. They are as follows: A, 1; B, 2; C, 3; D, 4; E, 5; F, 6. The darkly stained chromosomes are from mitotic CHO cells. × 1280 to 1740.
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