Abnormal In Vitro Differentiation of Peripheral Blood Clonogenic B Cells in Common Acute Lymphoblastic Leukemia During Complete Remission

By R. Consolini, J. Béraud, A. Bourinbaier, A. Goutner, V. Georgoulas, C. Canon, E. Brugerie, and G. Mathé

An in vitro B cell colony assay system was used to evaluate B cell growth from peripheral blood precursors in common acute lymphoblastic leukemia (CALL) patients in remission during maintenance therapy and in normal controls. Major differences between the two groups were found in the phenotypic and morphologic features of pooled colony cells. In both cases, the cells were E-.

Controls' cells were surface immunoglobulin (slg)-positive, and some (mean, 25%) expressed la determinants. By Wright-Giemsa staining, they appeared as plasmacytid cells. In contrast, patients' cells had predominantly a lymphoblastoid appearance, fewer cells had developed slg, and a large fraction (mean, 43%) were la-positive. Moreover, the CALL antigen (CALLA) was expressed by a mean of 18% (range, 2% to 72%) of the patients' colony cells, whereas CALLA was never found in control colonies. Thus, cells with immature features persist in the colonies of CALL patients. Secondary colonies could be generated from the patients' cultured cells, indicating their self-renewal capacity. CALLA+ cells were also present in the secondary colonies. Finally, cytogenetic studies showed that a fraction of the patients' colony cells had karyotypic abnormalities similar to that of the original lymphoblasts. It is believed that in CALL patients this B cell assay permits the clonal expansion of residual circulating cells linked to malignant clones that are not detectable by classic hematologic and cytologic methods.

Numerous studies of the cellular distribution of CALLA have, however, shown that CALLA+ cells are never detected in the peripheral blood (PB) of healthy subjects.

We found that a small percentage of circulating CALLA+ cells were present in some CALL patients in remission receiving maintenance chemotherapy. A similar finding has been reported by Greaves et al in an early study on the specificity of an anti-CALLA heteroantiserum. Such a phenotype seemed compatible with either the presence of residual leukemic cells or that of normal immature cells released inappropriately in the blood. The very low representation of these cells within the PB, however, makes the determination of their nature technically difficult. We therefore induced in vitro the clonal expansion of circulating B cells in these patients. We used a recently described technique that permits the generation of B cell colonies from both normal and malignant B lymphocyte progenitors. The specificity of the technique resides in the exhaustive depletion of T lymphocytes from the starting population and in the use of irradiated T cell feeders together with T-conditioned medium. This assay allows for the phenotypic and morphologic evaluation of the colony cells at the end of the culture. We have thus compared the differentiation of clonogenic B cells in CALL patients in complete remission (CR) to that of normal controls as well as T-ALL patients subjected to a similar maintenance chemotherapy regimen.

MATERIALS AND METHODS

Patients. A total of 20 CALL, five T-ALL, and two acute myeloid leukemia (AML) patients (aged 4 to 22 years) entered the study; 16 of 18 CALL patients were in first remission, and four were in second remission. CR was checked in all cases by standard clinical and hematologic (less than 5% lymphoblasts in BM aspirates of normal cellularity) criteria. Patients were also monitored for the expression of CALLA+ cells in BM and PB. CALL and T-ALL patients were on the same maintenance chemotherapy protocol (6-mercaptopurine daily, methotrexate weekly, and vincristine twice a month). A total of seven normal donors (aged 28 to 40 years) from the laboratory staff were also studied.

Cell separation. Peripheral blood lymphoid cells (PBL) were separated on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). E- cells were obtained by rosette formation with two to five aminoethylisothio-uronium bromide hydrobromide.
DIFFERENTIATION OF B CELLS IN CALL

(AET) (Sigma Chemical Co, St Louis)–treated sheep red blood cells at 4 °C for two hours followed by two successive Ficoll gradients. E– cells were depleted of CALLA+ cells by complement-mediated lysis with the J5 monoclonal antibody kindly provided by Dr J. Ritz. Optimal lysis was obtained after a 30-minute incubation of 150 μL of J5 per 106 cells (dilution, 1/800, vol/vol in minimum essential medium [MEM, GIBCO, Grand Island, NY]) followed by a 45-minute incubation with 50 μL/106 cells of pretreated guinea pig complement (dilution, 1/5 vol/vol, in MEM) (Institut Pasteur Production, Marne la Coquette, France). Complete removal of CALLA+ cells by this procedure was checked by indirect immunofluorescence in all cases. Treated cells were incubated with J5 antibody (1/800) and, after three washes, with fluorochrome (fluorescein isothiocyanate)-labeled goat F(ab')2, antimouse Ig (Cappel Laboratories, Cochranville, Pa). The cells were then examined with a Zeiss microscope equipped with epillumination.

In some experiments, E-J5– cells were further depleted of la+ cells; 106 cells were incubated with 150 μL of an anti-la monoclonal antibody (anti-112) (Coulter Electronics, Hialeah, Fla) for 45 minutes at 4 °C with a subsequent incubation with guinea pig complement as described. In some cases, the presence of residual la+ cells, as detected by indirect immunofluorescence, led to a second complement-mediated lysis.

**B cell colony assay.** The method described by Izaguirre et al has been used. In brief, 2 × 105 cells from both cell fractions (E– and E-J5+) were seeded in 0.8% methyl cellulose in MEM supplemented with 10% fetal calf serum (GIBCO), 20% conditioned medium, and 3 × 105 normal irradiated T lymphocytes obtained from two normal donors. Conditioned medium was prepared by incubating normal T cells with 1% phytohemagglutinin (PHA, HA-16) (Wellcome) in growth medium (MEM, 10% FCS) at 37 °C in a 5% CO2 atmosphere for two days. Supernatants were collected, filtered, and stored at 4 °C. Such preparations are termed PHA–T cell–conditioned medium (PHA-TCM). Normal irradiated E+ cells were prepared from normal PBL by rosette formation with AET-SRBC, and centrifugation was done over a Ficoll-Hypaque gradient. E+ cells were recovered from pellet by hypotonic lysis of the SRBC, washed twice, and irradiated with 2,500 rad from a Co source. The same T lymphocyte donors and PHA-TCM batch were used in this study. Measured 0.1-mL aliquots of this preparation were distributed into the wells of a microwell plate with a flat bottom (Falcon Labware, Oxnard, Calif). The plates were incubated at 37 °C for five to seven days in a moist atmosphere with hypoxic conditions (7% O2).

**Colonies characterization.** Aggregates of more than 20 cells were counted as colonies under an inverted microscope. Individual colonies were then picked with a fine-capillary pipette containing carbonate-buffered saline and pooled and washed for further characterization. The cells were tested for expression of the E receptor (by SRBC rosette formation) and for the presence of surface immunoglobulins (slg) with a fluorescein-labeled goat F(ab')2, anti-human F(ab')2, fragment (polyvalent) (Cappel Laboratories). The cells were also phenotyped with various monoclonal antibodies for the presence of la (Anti-112) (Coulter Electronics), CALLA (iS) and J5 antibody kindly provided by Dr Nadler). 11, iS, and J5 were tested by indirect immunofluorescence in all cases. Treated cells were incubated with 4 #{176}C for two hours followed by two successive Ficoll gradient. E+ cells were recovered from pellet by hypotonic lysis of the SRBC, washed twice, and irradiated with 2,500 rad from a Co source. Measured 0.1-mL aliquots of this preparation were distributed into the wells of a microwell plate with a flat bottom (Falcon Labware, Oxnard, Calif). The plates were incubated at 37 #{176}C for five to seven days in a moist atmosphere with hypoxic conditions (7% O2).

**Chromosome analysis.** Chromosome analysis was performed in the colony cells of four patients. To obtain metaphases, individual colonies were picked and pooled in 0.2 mL PBS. Colcemid was added at a final dilution of 0.01 μg/mL, and cells were incubated at 37 °C for 60 to 90 minutes. Cells were exposed subsequently to a hypotonic solution with 0.075 mol/L KCl and fixed in methanol-acetic acid (3:1, vol/vol). Air-dried slide preparations were made, stained with Giemsa, and examined microscopically for well-spread metaphases.

**RESULTS**

**CALLA+ cells in BM and PB.** The percentages of CALLA+ cells in BM and PB of 20 CALL patients in remission are presented in Table 1. As can be seen, more than 5% J5+ cells were detected in the BM of four of these patients. BM cellularity was normal in these cases, with no evidence of lymphocytosis. Of note is patient 11 who presented with a regenerating BM with 25% lymphocytes but had no detectable J5+ cells. The proportion of CALLA+ cells in the PB of these patients was 0% to 3% and reached 15% in one case. Removal of T lymphocytes prior to testing resulted in a minor change (1% to 2% increase) in the remaining E– population; 200 cells were examined (Table 1). PBL from five T-ALL and two AML patients as well as seven healthy volunteers were negative for J5 except for one T-ALL patient who had 2% circulating CALLA+ cells. It has recently been reported that polymorphonuclear cells react with J5. However, no correlation could be made

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Abbreviations: N, normal; M, median; H, hypoplastic; ND, not determined.

*E– cells purified after rosette formation of PBL with AET-SRBC.

†Hypoplastic with lymphocytosis.
between the weak staining that we found on these cells and the bright fluorescence of J5+ lymphoid cells.

**Colony formation.** Normal PB E− cells could generate between 100 and 900 colonies/10⁵ plated cells. Cells from the colonies were pooled and characterized for various surface markers. They had no receptor for SRBC, and 65% were slg+ and 25%, Ia+. In addition, in two subjects tested, 40% and 58% of the colony cells expressed the antigen B1. This phenotype (E−, slg+, B1+) is characteristic of mature B cells and demonstrates that these culture conditions allow the specific growth of B lineage cells. Morphologic studies confirmed these observations since most of the colony cells had a plasmacytoid appearance as judged after Wright-Giemsa staining. Of note is the fact that none of the cells present in these colonies bore the CALLA antigen, as shown by their lack of reactivity with the J5 antibody.

E− cells from CALL patients in remission were cultured under the same conditions, and the assay was technically satisfactory in 15 patients (cases, 1 to 15). From 20 to 2,400 colonies/10⁵ cells were generated. As was the case for normal subjects, very few E− cells were present in the colonies. A total of 57% of these cells were slg+, which is less than in controls, and 43% were Ia+, significantly more than in normals (P < .01). Moreover, in two cases out of two, more than 50% of these cells were stained by the B1 monoclonal antibody (68% and 50%). These results are thus suggestive of a B cell nature for most of these patients' colony cells. A major phenotypic difference, however, was found between the cells derived from CALL patients and those of normal subjects since a mean of 18% of the former expressed CALLA (range, 2% to 72%; Fig 1). The morphology of the patients' colony cells also showed that they were not similar to the controls' cells, since most of them had a lymphoblastoid appearance. Both surface markers and cytologic criteria therefore seemed to indicate that colony cells derived from B precursors are less differentiated in CALL patients in remission than in normal subjects.

To assess whether chemotherapy could affect the differentiation of B cells, five T-ALL and two AML patients in remission and under the same maintenance treatment were tested. From 150 to 500 colonies/10⁵ plated cells were generated in T-ALL. A total of 65% of these cells were slg+, and 28% Ia+, the phenotype being similar to that observed in normal colonies. In all cases, no J5+ cells were detected in the colonies, even in T-ALL patient with circulating J5+ cells (Fig 1).

**Characterization of B colony-forming cells (B-CFC).** An interesting observation was the lack of statistically significant correlation between the proportion of J5+ cells in the colonies of CALL patients and the level of circulating J5+ cells. Therefore, in these patients we studied the differentiation of B-CFC under the same culture conditions but after elimination by complement-mediated lysis of the J5+ cells present in the E− populations. The colonies could be analyzed in 11 patients (cases 1 to 11); lysis of J5+ cells prior to culture did not significantly alter either the plating efficiency (268 ± 145 v 354 ± 260 colonies/10⁵ cells in the controls, 858 ± 891 v 628 ± 526 colonies/10⁵ cells in the CALL patients) or the phenotype (E−, slg+, Ia+) in both controls and CALL patients. A major phenotypic difference, however, was found between the cells derived from CALL patients and those of normal subjects, since a mean of 17% of the former expressed CALLA (range, 2% to 65%). These observations seemed to indicate that in CALL patients, CALLA+ cells can be generated in vitro from CALLA− progenitors.

To further characterize the phenotype of B-CFC, E− cells were treated with anti-Ia antibody (1:600 vol/vol) in addition to J5 (1:800 vol/vol) in two CALL patients and three controls. In both groups this pretreatment led to an abrogation of colony growth (27 ± 6 v 104 ± 31 colonies/10⁵ cells in the controls, 50 ± 12 v 415 ± 49 colonies/10⁵ cells in CALL patients), suggesting that the B progenitor cells in normal subjects as well as in the patients bear the Ia antigen. The presence of Ia antigen on normal B-CFC has already been reported by Taetle et al. To assess that the effect of the anti-Ia antibody was due not to a depletion of an accessory cell population, which could be critical to colony growth, increasing numbers of autologous (10⁶ to 10⁷ cells/mL) adherent cells were added to the E−, J5−, Ia− cell population. The presence of these monocytes could not restore colony growth (unpublished data).

**Self-renewal.** To investigate the clonogenic capacity of B colony cells from CALL patients and controls, primary colonies were picked individually, pooled, and replated as primary colonies. A significant secondary plating efficiency (PE2) was observed in four of four patients studied. Although considerable variation was present among the patients, essentially no differences were evident between E− and E-J5− secondary plating efficiencies. The proportion of J5+ cells in the secondary colonies was comparable to that found in the primary cultures. These results seem to indicate that some colony cells present self-renewal capacity similar to that of stem cells. In contrast, PE2 in controls was very low (inferior to five colonies/10⁵ seeded cells) either in E− or in E-J5− fractions.

**Cytogenetic analysis.** The karyotype of cultured cells was studied in four patients for whom chromosomal abnormalities had been found in the original lymphoblasts (Table 2). During the acute phase, three patients had a mosaic composition of BM lymphoblasts, with 33% of the cells carrying a marker for the first patient, 55% for the second, and 25% for the third patient, the marker being different in each case. Cytogenetic examination of 22 mitoses in the colony cells of the first patient revealed that one cell (ie, 5%)
had the same marker as the lymphoblasts. In the second patient, out of 20 mitoses studied, one cell had the same marker (mar 1) as the original malignant clone, one cell displayed a new marker (mar 2) and one cell had both markers (mar 1 + mar 2). Thus a total of 15% of the cells had chromosomal aberrations. In the third patient, cytogenetic examination of colony cells revealed a reduction of the hyperploidy observed on 25% of the initial lymphoblasts (from 53 to 47 chromosomes) in 20 mitoses studied; two of them had the same marker as the original malignant clone. The fourth patient had Down’s syndrome, presenting a chromosomal aberration identical to that previously found in the initial lymphoblasts. It thus appeared that some colony cells were derived from the original malignant clone. The expansion of these circulating precursors during chemotherapy was achieved by classic cytogenetic methods. All patients are still in remission, two of them more than 18 months after being tested; sig is the standard antibody for the detection of surface immunoglobulin (slg).

**DISCUSSION**

To induce the clonal expansion of circulating B cells in CALL patients in remission, we used an in vitro assay that was found suitable for the generation of B cell colonies from malignant progenitors in CALL.**2** Chronic lymphocytic leukemia, B cell non-Hodgkin’s lymphoma, and from normal precursors in healthy subjects. B cell colonies derived from normal controls are composed of E−, slg+, Ia+, B1+ cells that have a plasmacytoid appearance. Patient’s colony cells are composed of E−, B1+ (in two cases tested); slg is expressed on fewer cells than in normals. The Ia determinant, which is lost during the final stages of B cell maturation, is retained by a significantly greater proportion of the patients’ colony cells. Moreover, a variable fraction of these cells express CALLA, which is associated with immaturity B cells. In addition, they predominantly have a lymphoblastoid appearance contrasting with the uniform plasmacytoid features of normal colony cells. Taken together, these results suggest that some circulating B cells from CALL patients in remission cannot differentiate in vitro under culture conditions that permit the maturation of normal B cells.

Since it has been shown that chemotherapeutic treatment can induce abnormalities of in vivo lymphopoiesis in leukemic patients, we have studied T-ALL and AML patients during maintenance therapy. In contrast to CALL, no circulating J5+ cells were detectable in any of these patients except one. In addition, the phenotype and appearance of their B colony cells were identical to those of normal controls. Thus, it seemed unlikely that the chemotherapeutic regimen induced the abnormal in vitro growth pattern of B cells in CALL patients.

A high number of secondary colonies was observed in all four patients for whom primary colony cells were replated, whereas very few secondary colonies could be generated in the controls. Thus, some of the patients’ colony cells were capable of self-renewal, a property usually associated with stem cells and malignant cells.

The results of cytogenetics studies in four patients clearly showed that some colony cells were derived from the original malignant clone. In all cases, a fraction of the cultured cells presented a chromosomal aberration identical to that previously found in the initial lymphoblasts. It thus appeared that despite complete hematologic remission these patients had in their PB residual cells belonging to the malignant clone. The expansion of these circulating precursors during the culture allowed the detection of their abnormal progeny by classic cytogenetic methods. All patients are still in remission, two of them more than 18 months after being studied. Since chromosome examination and study of surface markers cannot be done on the same cells, we could not determine the phenotype of the colony cells with chromoso-
nal aberrations. It should be noted that in all cases the percentage of J5+ cells and number of abnormal metaphases in the colonies were comparable. However, further work is needed to establish whether the clonal cells express the J5 determinant. Double staining for J5 and surface or cytoplasmic light chains could be helpful but is subjected to the concomitant expression of CALLA and Ig light chains by the same cell. Such a phenotype has not been described in normal cells and can be induced in vitro in CALL leukemic blasts of only some patients. In any case, fluoresceinated anti-κ and anti-λ chains will be used instead of polyvalent anti-F(ab′)2 in our subsequent studies to evaluate the distribution of these markers in the whole colony population and eventually in the J5+ fraction.

Since the elimination of CALLA+ cells from the starting population did not alter the phenotype of the cultured cells, it appears that the J5+ colony cells are not, or at least not exclusively, derived from the circulating J5+ cells detected in these patients. The J5+ antibody has been shown to induce the modulation of the CALLA molecule. However, under the technical conditions used in this study (ie, incubation with J5 at 4 °C and subsequent incubation at 37 °C after the addition of complement), lysis is likely to occur prior to any modulation. These results can be compared to the data of Izaguirre and Greaves who showed, by separating CALLA+ cells into J5+ and J5− fractions, that CALLA does not seem to be a marker for all potentially clonogenic CALL blasts.

Finally, it is worth mentioning that during the course of this study, three patients (cases 1, 2, 13) relapsed 1, 5, and 2 months, respectively, after being studied. The proportion of J5+ cells in the colonies was 50%, 72%, and 13% respectively. Two of these patients (1, 13) had virtually no BM or PB CALLA+ cells at the time of study. Moreover, no patient in the group of less than 5% CALLA+ colony cells has relapsed thus far. More extensive follow-up might indicate whether a correlation between the percentage of CALLA+ colony cells and subsequent clinical course can be established.

In conclusion, we have shown that some circulating B clonogenic cells of CALL patients in remission do not differentiate in vitro under experimental conditions that allow the maturation of normal B cells. The self-renewal capacity of some of those cells, the presence of an identical karyotypic abnormality in some cultured cells in the original lymphoblasts, and the subsequent relapse of three patients who had a relatively high proportion of J5+ colony cells seem to indicate that this culture system permits the clonal expansion of residual circulating cells linked to the malignant clone. This observation might be of biologic as well as clinical importance, since these residual cells cannot be detected by classic hematologic, cytologic, and even immunologic methods. It would be of interest to study with the same colony assay the differentiation of B cells in CALL patients who are in long-term remission and not receiving therapy.

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