At diagnosis peripheral blood (PB) or bone marrow from patients with juvenile chronic myelogenous leukemia (JCML) have shown two reproducible abnormalities when studied in cell culture: impaired growth of normal hematopoietic progenitors and excessive proliferation of monocyte-macrophage elements. We used these findings to assess quality of treatment response by serially studying PB specimens from four JCML patients (patients 5, 7, 8, and 9) in complete chemotherapy-induced remission. PB readily yielded high numbers of monocyte-macrophage colonies in CFU-C and CFU-GEMM assays when cultured in early remission, and the colonies were cytogenetically proven to have arisen from a malignant clone in patient 9. When studied later in remission, the abnormal cell proliferation persisted in three of the four patients, but in patient 8 PB colony growth resembled controls. Similarly, when PB from patient 8 was studied in liquid culture without using added growth factor, cell proliferation declined identical to controls, whereas PB from the other three patients showed exuberant growth of monocyte-macrophage elements. Patient 8 successfully completed therapy and has been in a long-term, disease-free remission. The other three had recurrent, ultimately fatal disease. The cell cultures have allowed detection of residual abnormal cells that circulate in PB of JCML patients in remission. Although patient numbers were small because of the rarity of JCML, the data suggested that persistence of leukemia cells in these patients had a bearing on clinical outcome.

**Materials and Methods**

Subjects. All four patients fit the classical clinical and laboratory description of JCML.\(^2\)\(^3\) Their data at diagnosis were reported previously in Blood\(^4\) and correspond to patients 5, 7, 8, and 9 in that series. They presented with varying combinations of lymphadenopathy, skin manifestations, variable splenomegaly, pallor, and hemorrhagic signs. Most had anemia, leukocytosis, monocytosis, thrombocytopenia, and nucleated RBCs in PB. BM aspirates were cellular with granulocytic hyperplasia, monocytosis, active erythropoiesis, and reduced-to-absent megakaryocytes. All patients were treated with combined chemotherapy used for acute nonlymphocytic leukemia\(^5\) and were followed for 12 months to 3 years. Their chemotherapy was given as described\(^7\) and consisted of varying combinations of daunomycin, cytosine arabinoside, 5-azacytidine, VP-16-213, 6-thioguanine, and 6-mercaptopurine. These studies were performed with informed consent and were approved by the Human Experimentation Committee of our institution.

**CFU-C and CFU-GEMM assays and liquid cultures.** Heparinized PB cells from the four patients and PB from five healthy volunteers used as controls were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 x g, 20 min) to separate the mononuclear cells. The PB cells were washed and resuspended in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2.0 U/mL of erythropoietin (Connaught Laboratories, Willowdale, Ontario), and 5 x 10\(^{-5}\) mol/L of 2-mercaptoethanol. All cultures were evaluated after 14 days using an inverted microscope. A CFU-C colony was defined as a cluster of 20 or more cells consisting of granulocytes, monocyte-macrophages, or both. Microscopically pure granulocytic colonies could be easily distinguished from pure macrophage colonies because of smaller cells and tighter clustering in the former. For the liquid cultures, nonadherent PB mononuclear cells (5 x 10\(^{6}\) cells/mL) were incubated in a-medium containing 10% fetal calf serum (FCS) for 10 days as previously described.\(^9\)

The CFU-GEMM assay was performed in methylcellulose as previously described\(^10\) using 20% colony stimulating activity (CSA) prepared from peripheral blood leukocytes of a normal volunteer.\(^10\) Colonies were counted after 14 days using an inverted microscope. A CFU-GEMM colony was defined as a cluster of 20 or more cells consisting of granulocytes, monocyte-macrophages, or both. Microscopically pure granulocytic colonies could be easily distinguished from pure macrophage colonies because of smaller cells and tighter clustering in the former.

For the liquid cultures, nonadherent PB mononuclear cells (5 x 10\(^{6}\) cells/mL) were incubated in a-medium containing 10% fetal calf serum (FCS) for 10 days as previously described.\(^9\)

The CFU-GEMM assay was performed in methylcellulose\(^10\) using 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA-LCM),\(^11\) human erythropoietin 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver) or sheep erythropoietin 2.0 U/mL (Connaught Laboratories, Willowdale, Ontario), and 5 x 10\(^{-5}\) mol/L of 2-mercaptoethanol. All cultures were evaluated after 14 days for the number of BFU-E colonies, defined as an aggregate of greater than 500 hemoglobinized cells or three or more erythroid subcolonies, CFU-C colonies of granulocytic or macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a
micropipette and were analyzed for cellular composition and karyotype.

**Cell characterization and cytogenetic studies.** Individual colonies were punched from cultures with a micropipette, spread on glass slides, and the cellular composition was determined microscopically using Wright's stain and nonspecific (alpha naphthyl butyrate) esterase staining with and without treatment with fluoride.24 The cell surface antigens of individual CFU-C colonies were assessed using the following monoclonal antibodies (MoAbs) with intestinal alkaline phosphatase as previously described15-27: OK1a (Ortho Diagnostic Systems Inc, Raritan, NJ) that detects HLA-D framework antigen; Leu-M3, OKM1 (Becton-Dickinson, Mountain View, CA) that identifies monocytes and macrophages; Leu-1 and Leu-4 and B1 and B4 (Becton-Dickinson) that detect T and B cells respectively. The slides were counterstained in hematoxylin, and the presence of surface antigen was indicated as bright red granular cellular inclusions seen by light microscopy. Morphological details were well preserved, and a very high degree of correlation between morphology and surface antigen expression was observed. The intensity of staining was defined as strong (+ + +), moderate (+ +), and weak (+).

BM and PHA-stimulated PB mononuclear cells were cultured for 24 and 72 hours respectively and were then harvested for cytogenetic studies by routine procedures.29 Individual JCML CFU-C colonies were punched for cytogenetic analysis according to published methods.30

**Statistical analysis.** The probability of significant differences between colony numbers was determined by Student's t test.

**RESULTS**

The four patients attained complete clinical, hematologic, and BM remission following one to four chemotherapy courses.13 During remission all signs and symptoms of JCML disappeared, and blood counts and BM aspirates were normal.

Patient 5 was given 21 courses of chemotherapy; the remission lasted 21 months but was followed by acute myeloblastic leukemia. Patient 7 received 16 courses of chemotherapy; the remission lasted 21 months but was followed by acute myelomonocytic leukemia. Patient 9 received 9 courses of chemotherapy; JCML recurred after 11 months. Patients 5, 7, and 9 have subsequently died of progressive disease. Patient 8 received 16 courses of chemotherapy over an 18-month period, after which treatment was stopped; at the time of writing patient 8 has been in complete remission for 15 months.

PB mononuclear cells from the four patients were studied at diagnosis and serially during remission using the CFU-C assay (Table 1). At diagnosis there was excessive proliferation of CFU-C colonies compared to the controls (P < .005). Characterization studies of individual colonies by morphology and cytochemistry confirmed that they were comprised of monocyte-macrophage elements; 85% of the cells stained intensely for nonspecific esterase and the stain was inhibited by fluoride. Immunocytochemically, 90% of cells in JCML colonies were positive for OK1a, greater than 90% were positive for Leu-M3, and greater than 80% expressed OKM1. All cells were negative for Leu-1, Leu-4, B1 and B4.

Cultures were performed several times during remission in the four patients when WBC counts and differential counts were normal. Representative results are shown in Table 1 at two time periods, in early clinical remission, and later while on long-term chemotherapy. Initially in early remission colony numbers were increased compared to control values, except for patient 7, and the cellular composition of the CFU-C colonies from all four was exclusively of monocyte-macrophage lineage. The cells had the identical morphological, cytochemical, and cell surface phenotype as the cells harvested from colonies at diagnosis. In contrast, control CFU-C colonies were about 50% granulocytic, 40% monocyte-macrophage, and 10% mixed lineage.

Later in remission, while receiving chemotherapy, colony numbers were decreased or comparable to controls in patients 7 and 8 respectively but still increased in patients 5 and 9. The cellular composition of the colonies in patients 5, 7, and 9 still showed exclusive proliferation of monocyte-macrophage colonies. Results from patient 8 were different. At the conclusion of 16 months of chemotherapy, the CFU-C colonies had changed in composition and consisted of about 50% granulocytic lineage, identical to controls. Chemotherapy was stopped for patient 8 because the treatment protocol was completed, and the patient was retested seven months later while still in remission (Table 1). Colonies were somewhat reduced in number but still consisted of a normal pattern of about 50% granulocytic, 40% monocyte-macrophage, and 10% mixed lineage.

When studied at diagnosis in liquid culture without added CSA, PB cells from the four patients showed a high cellular proliferative rate compared to control PB cells. After ten days in culture, patients' cells increased numerically by approximately 70% to 150%, whereas control cells decreased by about 10%. A differential count of nonadherent JCML cells obtained on day 10 consisted of 13% monoblasts, 32% early monocytes, 54% mature monocytes, and 1% premature and fully differentiated macrophages. In the control cultures the nonadherent cells were comprised primarily of granulocytic and lymphocytic-appearing elements and a small population (less than 5%) of mature monocytes and macrophages. The majority of monocyte-macrophage cells in the control cultures were found in the adherent layer.

In remission, PB cells from patients 5, 7, and 9 still showed the abnormal, excessive proliferation of monocyte-macrophage elements in liquid culture even though routine peripheral blood cell numbers and differential counts were unre-
Results from patient 8 were different. After ten days of culture cell numbers decreased, and most cells became adherent to the plastic culture flasks, similar to controls.

In the CFU-GEMM assay, PB cells from the four patients at diagnosis yielded large numbers of monocyte-macrophage CFU-C colonies but absent BFU-E and mixed colony formation (Table 2). During early remission CFU-C colonies declined in number compared to the initial studies, and BFU-E and mixed colonies were detected. However, CFU-C colonies in all four patients consisted of more than 90% monocyte-macrophage cells with an obvious background of individual monocytes and macrophages in the culture dishes. Cytogenetic analysis (17 metaphases) of CFU-C colonies from patient 9 showed the identical, abnormal karyotype (45,X,–Y) that was demonstrated in the diagnostic marrow, whereas concurrent analysis of PB lymphocytes showed a normal male karyotype (46,XY). Patient 5 was studied several months later while still in remission and on chemotherapy, and the CFU-C colony growth was increased and exclusively of monocyte-macrophage lineage (Table 2). Patient 8 was studied in remission 7 months after stopping chemotherapy. CFU-C numbers were decreased, and the cellular composition was approximately 50% granulocytic, similar to controls.

DISCUSSION

The unusual growth behavior of BM and PB JCML cells in vitro has facilitated characterization studies of this syndrome. In our recent study, the major finding in cell cultures of JCML BM and PB was an excessively high number of CFU-C colonies in CFU-C and CFU-GEMM culture assay systems. These CFU-C colonies were comprised exclusively of monocyte-macrophage elements, as confirmed by morphology, cytochemistry, and surface marker studies, and were proven to arise from a malignant clone by cytogenetic analysis of individual colonies. The growth requirements for JCML CFU-C were also atypical because added CSA and the presence of adherent cells were not essential for JCML CFU-C to proliferate into colonies. In liquid culture JCML cells showed similar, abnormal growth characteristics and proliferated rapidly and excessively in the absence of an added humoral growth factor. The clonal expansion of the malignant cells in liquid culture allowed us to perform detailed analysis of cellular lineage, functional properties, and potential for cellular differentiation.

Thus assays for quantitation of JCML cells have become established and validated. The cell cultures have been of central importance in our laboratory for making a definitive diagnosis of this disease in new cases presenting as difficult diagnostic problems. In the present study the objective was to determine whether the assays were sensitive enough to detect small numbers of residual JCML cells from patients in chemotherapy-induced complete remissions, similar to our findings in acute lymphoblastic leukemia.

The four JCML patients who were studied were all clinically and hematologically normal and hence in “complete remission.” Careful inspection of PB white cell morphology by detailed differential counting did not disclose abnormal cells. Yet in all four patients studied in early remission and in three patients studied later in the treatment course, the in vitro findings were clearly abnormal in the clonogenic assays and in liquid cultures and confirmed persistent, disordered hematopoiesis. Even in studies in which colony numbers were in control range, the cellular composition of the colonies was strikingly aberrant, being exclusively of monocyte-macrophage lineage. The findings in patient 8 were different because the initially abnormal growth pattern became identical to controls with therapy. This change in the in vitro findings was clear-cut and was readily identified by several observers in our laboratory when the cultures were examined “blindly.”

When tissue cultures are used to study leukemia cell proliferation, it is ideal to confirm the leukemic origin of cultured cells by demonstrating clonality. This may be accomplished by using cytogenetic markers, glucose 6-phosphate dehydrogenase isoenzymes in appropriate females, and by molecular approaches. Fortunately, JCML CFU-C from one of our patients showed a chromosomal abnormality that was previously described in this disorder.

Thus we were able to prove that the patient’s PB clonogenic CFU-C that were detected during remission were of leukemic origin. In the other cases our data indicate that the unusual growth behavior of JCML cells in vitro can be successfully utilized to detect residual disease. This is especially important in JCML patients who do not show a marrow cell cytogenetic abnormality at diagnosis.

Thus these assays allowed identification of abnormal cells that were circulating in PB of our JCML patients in remission. An obvious question that arises is whether the finding of persistent JCML cells had prognostic significance. The answer is difficult because JCML is rare, and large numbers of patients are not available for study. However, of our four cases, the three that showed evidence of persistent, residual JCML had recurrent, fatal disease. The other case, in whom

| Table 2. Peripheral Blood CFU-GEMM Assay at Diagnosis and During Remission |
|-----------------------------|-----------------------------|-----------------------------|
| Patient ID No. | At Diagnosis | During Clinical Remission |
| CFU-C | Mixed | BFU-E | CFU-C | Mixed | BFU-E |
| 5 | >1000 | 0 | 0 | 45 | 4 | 80 |
| 7 | >1000 | 0 | 0 | 85 | 5 | 50 |
| 8 | 189 | 0 | 0 | 58 | 3 | 82 |
| 9 controls | 137 | 0 | 0 | 26 | 0 | 12 |
| 9 | 90 ± 10 | 8 ± 3 | 130 ± 12 |

Data shown are mean colony numbers from duplicate cultures.

*See reference 15.
†Patient no. 5 was studied twice after 12 and 21 months of chemotherapy (upper and lower values, respectively). Patients no. 7 and 9 were studied after 10 and 13 months of chemotherapy, respectively. Patient no. 8 was studied after 16 months of chemotherapy and after being off therapy for 7 months (upper and lower values, respectively).
JCML colony-forming cells were no longer detected, completed the therapy protocol and at the time of writing has been in long-term complete remission, off treatment. The data indicate that persistence of leukemia cells in our patients had a bearing on clinical outcome. Cell cultures should be useful, therefore, in assessing the degree of efficacy of treatment programs that are designed to eradicate the disease.

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Residual juvenile chronic myelogenous leukemia cells detected in peripheral blood during clinical remission

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