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27. We thank W. H. McClain and O. C. Uhlenbeck for communication of results prior to publication, and B. Bachmann for the overproducing strain of Arg-tRNA synthetase. Deoxyoligonucleotides were synthesized by a Core facility of Albert Einstein College of Medicine supported in part by National Science Foundation grant PCM-8400114. Supported by American Cancer Society grant NP19R.

13 September 1989; accepted 27 October 1989

A Model of Human Acute Lymphoblastic Leukemia in Immune-Deficient SCID Mice

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A human acute lymphoblastic leukemia (ALL) cell line that was transplanted into immune-deficient SCID mice proliferated in the hematopoietic tissues, invaded various organs, and led to the death of the mice. The distribution of leukemic cells in SCID mice was similar to the course of the disease in children. A-1 cells marked with a retrovirus vector showed clonal evolution after the transplant. SCID mice that were injected with bone marrow from three patients with non-T ALL had leukemic cells in their bone marrow and spleen. This in vivo model of human leukemia is an approach to understanding leukemic growth and progression and is a novel system for testing new treatment strategies.

MUCH PROGRESS HAS BEEN MADE recently in identifying regulatory genes involved in cell development and maturation by studying the genetic changes of transformed cells in both in vitro and in vivo model systems. Such models are not available for human leukemic cells, especially childhood acute leukemias. The difficulties in culturing primary human leukemic cells suggest that selective processes may alter the properties of such cells over time (*1*). In vivo models would aid in the development of treatment strategies and further our understanding of leukemic transformation and progression. Subcutaneous transplantation of lymphoid and myeloid cell lines (*2–9*), lymphomas (*8*), or primary patient material (*2*) into nude mice produces myelosarcomas or localized solid tumors (*10*) uncharacteristic of the primary leukemia. The growth of one human leukemia cell line in the hematopoietic tissues of nude mice has been described (*11*). This cell line was an aneuploid T ALL originally established and maintained as an ascites tumor in nude mice; the animals died within 2 to 4 weeks. However, growth as an ascites does not reflect the normal course of the disease in children. We, therefore, developed an experimental system in which the growth of human leukemic cells in murine hematopoietic tissues was more analogous to growth in patients with leukemia.

Non-T ALL is the most prevalent childhood leukemia and is characterized by a pre-B cell phenotype (*12*). A cell line (A-1) was established from the peripheral blood of a patient undergoing a terminal relapse of non-T ALL (*13*). The A-1 cells were HLA-DR$^+$, CD19$^+$, CALLA$^-$ (CD10), and CD20$^-$. No cytoplasmic or surface immunoglobulin was detected; it has one rearranged μ chain gene and unrearranged light chain genes, characteristic of a pre-B cell line (*13*). The A-1 line was EBV free, had a normal karyotype, and grew autonomously producing an unidentified factor (not interleukin-1, -2, -3, -4, -5, -6, granulocyte-, or granulocyte/macrophage–colony stimulating factor) that augmented its growth in semisolid and suspension cultures.

Normal human hematopoietic cells (*14–16*) and human tumors (*17*) can be engrafted into immune-deficient SCID (*18*) or *bg/nu/xid* (*19*) mice; thus, they may be better recipients than nude mice for the transplantation of human leukemic cells. Immune-deficient SCID and *bg/nu/xid* mice differ in their ability to be engrafted with normal human bone marrow; *bg/nu/xid* mice con-

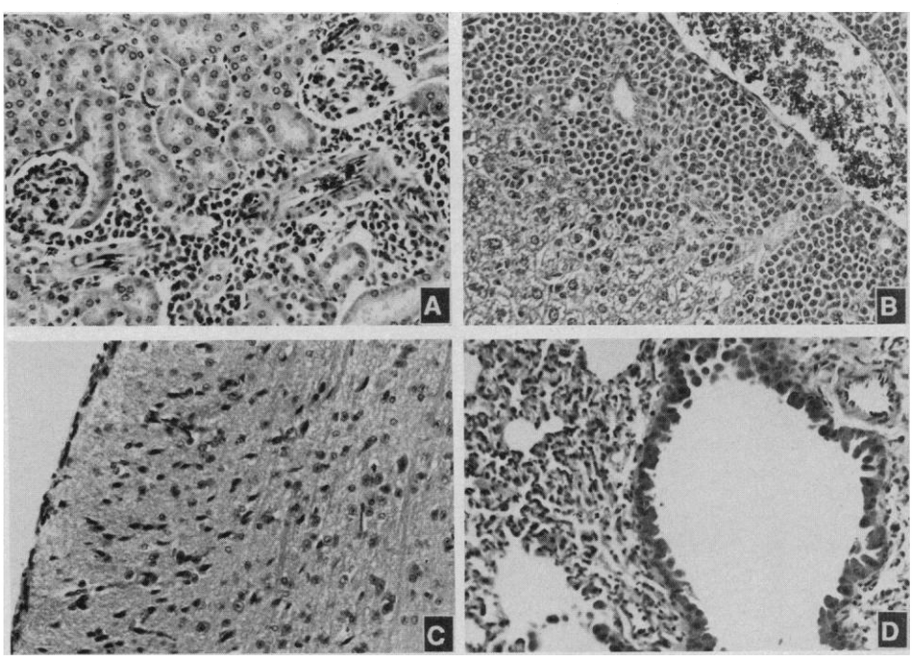

Fig. 1. Histological analysis of SCID mice 8 weeks after transplantation with the A-1 leukemic cells. Tissue sections were obtained from the kidney (**A**), liver (**B**), brain (**C**), and lung (**D**) of SCID mice transplanted with A-1 cells. The tissues were fixed in 10% formalin, paraffin embedded, and 4 μm sections were cut and stained with hematoxylin and eosin. Microscopic evaluation indicated that there was a light leukemic infiltration in the interstitial region between the tubules in the kidney (A) and in the periportal regions of the liver (B). Note the normal hepatocytes in the lower left hand corner of (B). No leukemic cells were observed in the cerebral cortex or meninges of the brain (C) or in the peribronchial region of the lung (D). Magnification is ×40.

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tained at least 500 times the myeloid progenitors than SCID mice after engraftment with primary human bone marrow (*16*). However, SCID mice can be engrafted with human lymphoid cells by intraperitoneal injection of peripheral blood leucocytes (*14*) or by implantation of fetal liver, thymus, and lymph nodes (*15*).

SCID mice were irradiated (*20*) before intravenous or intraperitoneal injection of 10^7 A-1 cells. The bone marrow and spleen were periodically analyzed by flow cytometry for the presence of $CD44^+$ cells. CD44 is a human cell surface glycoprotein implicated in lymphocyte-endothelial cell interactions (*21*) and expressed on A-1 cells. Preliminary experiments indicated that SCID mice injected intraperitoneally contained no detectable $CD44^+$ cells in the bone marrow or spleen after 4 weeks. These data are in contrast to results obtained by Mosier *et al.* (*14*), in which normal human lymphocytes that were injected intraperitoneally engrafted SCID mice. SCID mice injected intravenously showed a dissemination of leukemia reminiscent of that observed in many children with ALL. Four weeks after the transplant, the bone marrow contained 34% $CD44^+$ cells; no positive cells were detected in the spleen. After 8 weeks, the proportion of A-1 cells rose to 90% in the bone marrow and 61% in the spleen; this percentage of human cells was maintained in the animals at 10 and 12 weeks after engraftment. At 8 weeks after the transplant, the interstitial regions between the kidney tubules and the periportal regions of the liver contained small infiltrates of A-1 cells; no leukemic cells were observed in brain, lung, intestine, heart, or pancreas (Fig. 1). At 10 weeks A-1 cells were in the blood, and leukemic infiltrates filled most sinusoids of the liver and interstitial regions and perirenal fat of the kidney. Infiltrates were also observed in the peribronchial regions of the lung, in the stomach mucosa, and in the choroid plexus of the brain. After 12 weeks, infiltrates were present in the kidney, liver, brain, lung, intestine, and pancreas (Fig. 2). The animals began to die 12 weeks after the transplant.

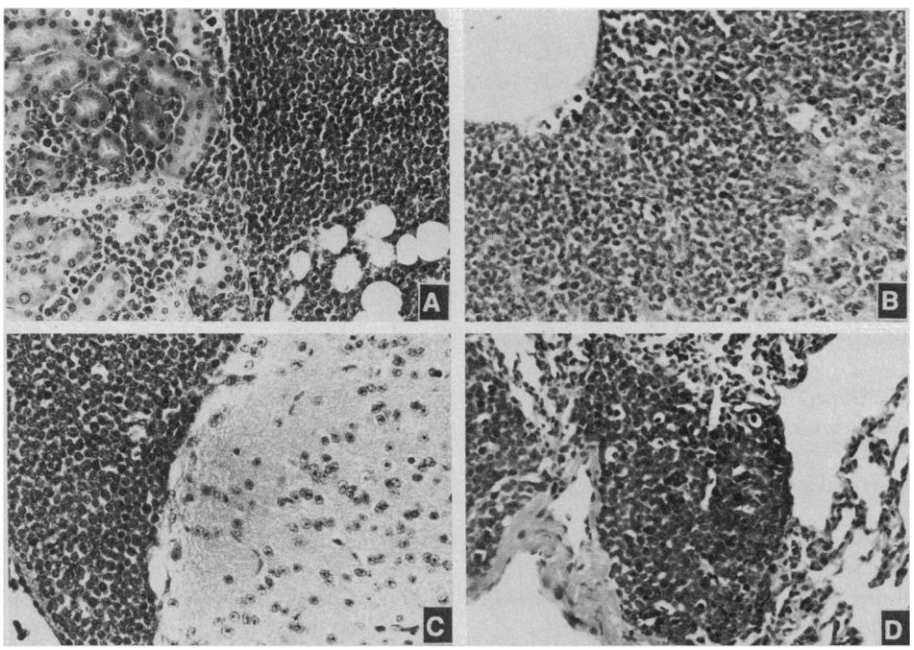

Fig. 2. Histological analysis of SCID mice tissues 12 weeks after transplantation with the A-1 leukemic cells. Tissue sections from the kidney, liver, brain, and lung from transplanted SCID mice were fixed and stained as in Fig. 1. (**A**) Transverse section of the kidney with leukemic infiltration in the interstitium (left-hand side) and perirenal fat (right-hand side). (**B**) Section through the liver demonstrating a much heavier portal infiltrate than at 8 weeks. Few normal hepatocytes are seen (lower right), and blasts can be seen throughout the liver tissue. (**C**) Section through the cerebral cortex demonstrating a dense infiltration of the meninges by leukemic cells. (**D**) An example of a nodular aggregate of leukemic cells, adjacent to pulmonary vasculature, in the peribronchial region of the lung.

Table 1. Flow cytometry analysis and in vitro colony formation of SCID mice transplanted with the A-1 leukemic cell line. The SCID mice bred in our defined-flora colony (Ontario Cancer Institute) were irradiated (400 cGy) from a cesium source and immediately given 1×10^7 A-1 cells by tail vein injection. At the times indicated, the bone marrow, spleen, and peripheral blood were analyzed by flow cytometry for $CD44^+$ cells (*34*). Bone marrow and spleen cells were also plated in methylcellulose cultures to quantitate the number of colony-forming cells. Briefly, 2×10^5 cells were plated in methylcellulose, 10% fetal calf serum, and 10% A-1 conditioned medium; colonies were scored after 14 days.

Weeks after transplant (number of mice)	% $CD44^+$ cells			A-1 colonies per 1×10^5 cells plated		
	BM	SPL	PBL	BM	SPL	PBL
Control ($n = 6$)	0	0	nd	0	0	0
4 ($n = 5$)	34*	0	nd	400†	0	0
8 ($n = 4$)	90‡	61§	nd	2000†,nd	370,tn	0
10 ($n = 2$)	86,93	58,68	nd	tn,tn	tn,tn	tn,tn
12 ($n = 2$)	72,35	97,99	nd	nd	nd	nd
13‖ ($n = 2$)	99,99	75,79	93	nd	nd	nd

*Median of 17, 17, 34, 47, and 86%. †Counts from the pooled BM of two animals. ‡Median of 76, 87, 93, and 95%. §Median of 53, 92, 63, and 58%. ‖These animals received 5×10^7 cells intravenously. nd, Not done; tn, too numerous to count (>3000 colonies).

For independent evidence of engraftment, DNA was prepared from bone marrow and spleen and the proportion of human DNA was estimated on Southern blots by hybridization to a human-specific chromosome 17 α-satellite probe (*22*) (Fig. 3). The bone marrow and spleen from animals sacrificed at 8, 10, and 12 weeks contained a 2.7-kb band characteristic of the human chromosome 17 α-satellite sequence. Comparison with control lanes that contained known mixtures of mouse and human DNA indicated that these tissues contained between 20 and 100% of human cells, consistent with the flow cytometry analysis. DNA was also analyzed from the bone marrow, spleen, and lung of animals sacrificed 3 hours after intravenous injection of A-1 cells. Even with long exposure times, human DNA could only be detected in the lung, which indicated that few of the injected cells seeded to the hematopoietic tissues; but were trapped initially in the lungs and liver (*23*).

Cell suspensions of bone marrow, spleen, and peripheral blood harvested at each time point were placed in liquid cultures and

assayed for colony formation of A-1 cells as a sensitive method of detection. The bone marrow had many colony-forming A-1 cells at all time points, whereas colonies, or liquid culture growth, was not detected in the spleen earlier than 8 weeks after engraftment (Table 1). The peripheral blood had colony-forming A-1 cells at 10 and 12 weeks; blood smears also had detectable leukemic blasts at these time points. Animals injected with 5×10^7 cells were examined 13 weeks after transplantation; A-1 cells constituted 99% of their bone marrow and spleen and 93% of the nucleated cells in peripheral blood (Table 1). Our results suggest that blasts expand first in the bone marrow, migrate to the spleen, and appear in the peripheral blood at the onset of infiltration into other tissues, a pattern reminiscent of the terminal stages of the disease in children.

To determine the effects of the growth of human leukemic cells on normal hematopoiesis, we measured the numbers of normal murine myeloid progenitors in the spleen and bone marrow. After 4 weeks, when A-1 constitutes only 30 to 40% of the bone marrow, the number of mouse granulocyte-macrophage progenitors in the bone marrow was reduced by 95%, whereas the spleen contained five times more progenitors than normal controls. The spleen contained no detectable A-1 cells at 4 weeks; perhaps A-1 has a local inhibitory effect on mouse hematopoiesis. The increased numbers of progenitors in the spleen may be related to the elevated splenic hematopoiesis of mice that have been irradiated. At 8 weeks after the transplant the number of progenitors in the spleen was reduced by 90% and none were detected in the bone marrow; the leukemic cells may have displaced or suppressed normal murine progenitors.

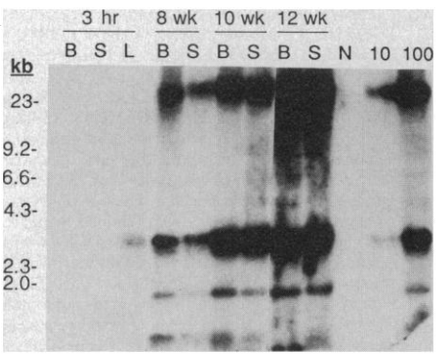

Fig. 3. DNA analysis of the hematopoietic tissues from SCID mice transplanted with A-1 cells. DNA was extracted from the bone marrow (B) and spleen (S) of SCID mice 8, 10, and 12 weeks after transplantation with A-1 cells. In addition, DNA was also extracted from the bone marrow, spleen, and lungs (L) of SCID mice 3 hours after injection of A-1 cells. DNA (2 μg) was digested with Eco RI, blotted according to standard procedures, and probed with p17H8, a human α-satellite probe specific for human chromosome 17 (*22*). Eco RI digestion of human DNA produces a characteristic 2.7-kb band; in addition, one-third of people have polymorphisms that give rise to a series of lower molecular weight minor bands (*16*). N is the negative control, 10 represents 10% human and 90% mouse DNA, and 100 represents 100% human DNA. The autoradiograph was exposed for 7 hours, except for the lanes with the 3-hour samples, which were exposed for 3 days. Molecular sizes are indicated in kilobases.

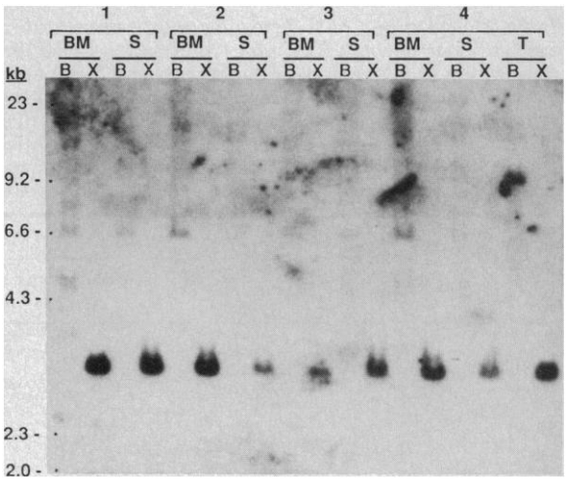

Fig. 4. DNA analysis of the bone marrow and spleen of SCID mice transplanted with A-1 cells infected with the PA317-N2 retrovirus vector (*25*). DNA was extracted 6 weeks (animals 1 and 2) and 7 weeks (animals 3 and 4) after transplant. DNA (5 μg) from the spleen (S), bone marrow (BM), and tumor (T) was digested with Xba I (X) or Bam HI (B), and analyzed by Southern blot using a *neo* specific probe. Xba I cuts once in each LTR to produce a 3.8-kb provirus band characteristic of N2 while Bam HI cuts once in the provirus outside the *neo* gene and in flanking cellular sequences to generate band sizes that are unique to the site of integration. Molecular sizes are indicated in kilobases.

An important problem in cancer biology is understanding the clonal evolution that occurs during the spread of neoplastic growth (*24*). A-1 cells were infected with a retrovirus vector that contained the dominant selectable *neo* gene (*26*) to uniquely mark each infected cell (*25*). DNA was prepared from the bone marrow and spleen of animals 6 and 7 weeks after transplantation with infected cells (Fig. 4). Digestion of DNA with Xba I, which produces a common 3.8-kb provirus band independent of the vector integration site, indicated that the bone marrow and spleen contained a substantial proportion of infected A-1 cells. Digestion of DNA with Bam HI produces a unique sized *neo*-containing band for each infected cell. All lanes had a light DNA smear indicative of multiple clones and a few more prominent bands with ≤10% of the intensity of the provirus band. One common clone was present in the bone marrow and spleen of different animals while other clones were unique to one tissue, such as the five clones seen in the bone marrow from animal 1. Animal 4 contained a small tumor growing near the kidney that arose from a clone different from those seen in the bone marrow and spleen. These data indicate that by six weeks some clones proliferate much faster than others; in the case of the clone common to different animals, this selection must have occurred in vitro; for other clones it occurred in vivo. It will be particularly interesting to use this approach to follow leukemic cell infiltration into the peripheral tissues.

The ability to engraft bone marrow directly from patients with leukemia into SCID mice could be a valuable tool for predicting the clinical course of the disease, detecting residual leukemias, and for developing individualized therapeutic strategies. Toward this objective, we injected bone marrow cells from three patients with non-T ALL into SCID mice. All three patients were non-T ALL group III according to immunologic classification (*27*), although patient 1 expressed only low levels of CD10 and may have a more immature phenotype. The proportion of human cells in the bone marrow and spleen was analyzed by flow cytometry, colony assay, and DNA analysis [Southern blot and polymerase chain reaction (PCR)] (Table 2). In all three cases, human cells were detected in the bone marrow and spleen of the engrafted animals. Animals transplanted with bone marrow from patient 2 still contained human cells at 10 weeks after transplant. No human cells could be detected after 16 weeks in the animals transplanted with bone marrow from patient 3. In addition to DNA analysis and flow cytometry, leukemic blast colonies were grown from the bone marrow and spleen of engrafted animals. Thus bone marrow obtained directly from patients with leukemia was engrafted into SCID mice. It is now important to determine whether different patterns of growth in SCID mice reflect biological parameters that correlate with clinical outcome.

We conducted parallel experiments with *bg/nu/xid* and nude mice. Nude mice transplanted 5 weeks previously contained very low levels of A-1 cells. The *bg/nu/xid* mice (Harlan Sprague-Dawley) had a subclinical infection of *Staphylococcus aureus*; none of the

Table 2. Engraftment of bone marrow from patients with non-T ALL into SCID mice. All three patients belong to non-T ALL group III defined as $CD10^+$, $CD19^+$, $CD20^-$, $HLA\text{-}DR^+$ (*27*). Bone marrow cells (1.0×10^7) were transplanted into SCID mice as described in Table 1. At the times indicated, the proportion of human cells in the bone marrow and spleen was quantitated by three methods: flow cytometry (FC) to determine the percentage of CD44, CD19, or HLA-DR positive cells (these markers were expressed on the original patient cells); DNA analysis (DNA) to detect human α-satellite sequences by Southern blot (Fig. 4) or PCR (*16*); and plating in semisolid cultures (colonies per 10^5 cells) to determine the number of leukemic blast colony-forming cells.

Patient	Time (weeks)	Bone marrow			Spleen		
		FC	DNA	Colonies per 10^5 cells	FC	DNA	Colonies per 10^5 cells
1	4	+	+	185	+	+	60
	7	+/−	+/−	82	+	+	94
2	4	+	++	30	−	+/−	nd
	10	nd	+	68	+/−	+	30
3	9	−	nd	10	+	+/−	10
	16	−	−	0	−	nd	0

Symbols represent the percentage of human cells as determined by FC, or the percentage of human DNA. For FC: ++, >10%; +, 6–10%; +/−, 1–5%; −, <1%. For DNA: ++, >10%; +, 1–10%; +/−, 0.1–1%; −, <0.1%.

animals had detectable A-1 engraftment in the bone marrow or spleen at 12 weeks after the transplants. When we later tested two defined-flora *bg/nu/xid* mice from our own colony, they contained 20% A-1 cells in the bone marrow 4 weeks after transplantation. While the mechanism of rejection in the infected mice is not known, it may be due to the stimulation of macrophages or NK cells. These preliminary data stress the importance of using pathogen-free mice for transplantation of human leukemic cells.

The growth of A-1 cells in culture, like that of most non-T ALL cells, requires an unidentified autocrine growth factor; SCID and *bg/nu/xid* mice may also provide the factors or microenvironment necessary for A-1 cell growth, or the transplanted cells can produce their own growth factors. We have successfully engrafted other leukemic cell lines into immune-deficient mice, including the myeloid lines K562 (*28*) and EM-2 (*29*), the megakaryocytic line MO7 (*30*), and the erythroid line OCI-M2 (*31*); thus these mice may support the growth of many types of human leukemic cell lines (*32*).

An in vivo model for the most common form of childhood leukemia provides a system to experimentally address a number of biological questions. For example, the identification of leukemic cells in bone marrow usually dictates the course of chemotherapy; however, histological methods for detecting low numbers of residual cells are neither sensitive nor precise. While recently reported in vitro assays for leukemic cells may improve both the sensitivity and precision of detecting residual leukemia (*33*), further refinement of the animal model described here could also offer a sensitive method to study residual cells from patients undergoing chemotherapy. New chemotherapeutic and immunotherapeutic protocols, combinations of biological response modifiers, or new unconventional therapies that are difficult to develop and evaluate by human experimentation can be tested in this in vivo model which mimics the progression of human leukemia. With high efficiency gene transfer technology (*25, 26*), individual leukemic cells have been marked to follow the growth and development of clones during the multistage progression of the disease. Such gene transfer in conjunction with our model system should allow the introduction of key growth regulatory genes, such as oncogenes or tumor suppressor genes, to determine how their aberrant expression affects normal hematopoiesis and leukemic transformation and progression.

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34. For flow cytometry, single cell suspensions were prepared from bone marrow and spleen; bone marrow cells were washed two times with PBS while spleen cells were fractionated through a Percoll gradient. Cells (5×10^5) were stained with the 50B4 monoclonal antibody to human CD44 antigen and that does not cross-react with murine cells. Washed cells were then incubated with FITC-$F(ab')_2$ goat antibodies to mouse immunoglobulin G, washed, and analyzed by flow cytometry using the Epics Profile Analyzer (Coulter Electronics).
35. The animal experiments were approved by the Animal Care Committee of our institution. Patient material was obtained after informed consent and used according to procedures approved by our Human Experimentation Committee.

Supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (J.E.D., M.L., R.A.P.), a Medical Research Council postdoctoral fellowship (S.K.-R.), a Research Scientist award (J.E.D.), and a Terry Fox Research Scientist award (M.L.) of the National Cancer Institute of Canada. We thank B. Murdoch, S. Chance, and W. Vanek for excellent technical assistance, P. Thorner for help in identifying and assessing the extent of leukemic infiltration, D. Pestche and A. Greaves for doing the flow cytometry, and L. Siminovitch, N. Iscove, and A. Bernstein for critically reading the manuscript.

18 September 1989; accepted 17 November 1989