

MATURE T-LINEAGE LEUKEMIA WITH GROWTH FACTOR-INDUCED MULTILINEAGE DIFFERENTIATION

BY FRANK GRIESINGER,* DIANE C. ARTHUR,*[†] RICHARD BRUNNING,*
JANET L. PARKIN,* AUGUSTO C. OCHOA,* WESLEY J. MILLER,[§]
CHRISTOPHER W. WILKOWSKI,* JAMES M. GREENBERG,*[‡]
CAROLE HURVITZ,^{||} AND JOHN H. KERSEY*[‡]

*From the *Departments of Laboratory Medicine and Pathology, [†]Pediatrics, and [§]Medicine,
University of Minnesota, Minneapolis, Minnesota 55455; and the ^{||}Department of
Pediatrics, Cedars-Sinai Medical Center, Los Angeles, California 90048*

Human multipotent stem cell lines are of great interest for studies of hematopoietic differentiation and maturation and of leukemogenesis. The advent of different stem cell assays (1-3) and the availability of recombinant or highly purified growth factors (4-7) made possible the identification of precursor cells and delineation of their growth factor dependency. Acute leukemias, which are believed to represent clonal expansions of normally occurring progenitor cells, have proved useful in defining different stages of normal differentiation (8). The use of such cells to study early events of stem cell differentiation and maturation and to elucidate interlineage relationships has been limited by the lack of differentiation capacity in most leukemic cells. Recently, a T-ALL and a T-cell lymphoblastic lymphoma with in vivo and/or in vitro multilineage differentiation potential have been described that might serve as models to study hematopoietic stem cells. The T-ALL displayed in vitro and in vivo spontaneous and growth factor induced multilineage differentiation potential (9, 10), while the lymphoblastic lymphoma showed conversion to a bilineage T-lymphoid and myeloid leukemia at relapse (11). Both lymphoblastic processes displayed T cell precursor phenotypes of early thymocytes (CD2⁺ CD3⁻ CD5⁺ CD7⁺) or prothymocytes (CD2⁻ CD3⁻ CD5⁻ CD7⁺ HLADR⁺ TdT⁺) (12-14). These studies led to the hypothesis that CD3⁻ CD4⁻ CD7⁺ CD8⁻ precursor T-ALLs may represent malignant counterparts of a multilineage stem cell (15).

In this manuscript we report a T-ALL with a more mature T lymphocyte phenotype (CD2⁺ CD3⁺ CD5⁺ CD7⁺ WT31⁺) than those previously reported, which also has the capability of multilineage differentiation and maturation in the presence of recombinant human growth factors. In the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF)¹ and IL-3, the leukemic cells gave rise to ter-

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¹ *Abbreviations used in this paper:* IMDM, Iscove's modified Dulbecco's medium; GM-CSF, granulocyte/macrophage colony-stimulating factor; MPO, myeloperoxidase; NBT, nitroblue tetrazolium, NSE, nonspecific esterase; PE, phycoerythrin; SB, Sudan Black; TdT, terminal deoxynucleotidyl transferase; WBC, white blood cell.

minally differentiated functionally active segmented neutrophils. In the presence of IL-2, leukemic cytotoxic T lymphocytes with MHC nonrestricted cytotoxicity were derived. In the absence of any growth factor, a cell line with an immature blast morphology and mixed lineage phenotype was established. The derivation of these different leukemic cultures from the same malignant clone was unambiguously demonstrated by unique monoclonal rearrangements of the constant region of the TCR- δ gene and of the joining regions of the TCR- β , - γ and - δ genes.

Materials and Methods

Clinical Course of the Patient. A 25-year-old Caucasian male (MT) presented with multiple petechiae, conjunctival hemorrhage, hepatosplenomegaly, and an anterior mediastinal mass in March 1987. The white blood cell count (WBC) was 759,000/ μ l, with 99% lymphoblasts. A bone marrow aspirate showed infiltration with atypical CD2⁺ and TdT⁺ and CD4⁻ and CD8⁻ lymphoblasts. After polychemotherapy, the patient achieved a complete remission lasting 5 mo. At first relapse, the bone marrow showed infiltration by lymphoblasts displaying the same immunophenotype as at diagnosis. With reinduction therapy, the patient achieved a short, unsustained remission. At three different time points during second relapse, leukemic cells were obtained for the present studies. The patient died one year after diagnosis.

Leukemic Cells. PBMC were isolated by centrifugation through a Ficoll-Hypaque density gradient. The fresh leukemic cells were either immediately processed for cell culture, immunophenotyping by flow cytometry, or frozen in RPMI 1640 tissue culture medium (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated FCS (Gibco Laboratories) and 10% DMSO (Sigma Chemical Co., St. Louis, MO) and stored at -196°C.

Cell Culture. Fresh leukemic cells were set up in 24-well tissue culture plates (Costar, Cambridge, MA) at a concentration of 10⁶ cells/ml in Iscove's modified Dulbecco's medium (IMDM) (Gibco Laboratories) supplemented with 20% FCS (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (Gibco Laboratories), 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco Laboratories), 25 mM HEPES buffer (Sigma Chemical Co.), 2 g/liter NaHCO₃ in a humidified 5% CO₂, 95% ambient air atmosphere at 37°C. Recombinant human growth factors were added to the cell cultures at the following concentrations: IL-2 (Amgen Biologicals, Thousand Oaks, CA), 100 U/ml; GM-CSF (Genetics Institute, Cambridge, MA), 50 ng/ml during the first 3 wk, 10 ng/ml subsequently; IL-3 (Genetics Institutes), 10 U/ml (1 U/ml is defined as the amount of IL-3 that induces a half-maximal [³H]thymidine incorporation in chronic myelogenous cells in vitro). Control cells were cultured without growth factor as a control. The cultures were fed twice a week with fresh medium and growth factors. IL-2-dependent and GM-CSF-dependent leukemic cells were established from these cultures. Cells from the GM-CSF leukemic cell cultures were subcloned by limiting dilution, as described before (16). Cells were plated in 96-well microtiter plates at concentrations of 0.4, 1, 3, 10 cells per well in the presence of 50 ng/ml GM-CSF. A control plate containing no GM-CSF yielded no clones. The leukemic cells grown in IL-3 were weaned from IL-3 after 10 wk in culture and a growth factor-independent leukemic cell culture was established.

Morphological and Cytochemical Characteristics. Differential counts were performed on Wright's Giemsa-stained smears of the fresh leukemic cells, from which the different cultures were established, and on cytopspins prepared from IL-3, GM-CSF, IL-2-dependent, and growth factor-independent leukemic cell cultures. Cytochemical stains included staining for myeloperoxidase (MPO), periodic acid-Schiff (PAS), α -naphthyl butyrate esterase (nonspecific esterase, NSE), Sudan Black (SB) and toluidine blue. Terminal deoxynucleotidyl transferase (TdT) activity was determined by immunofluorescence using a rabbit anti-TdT mAb and a fluorescein isothiocyanate conjugated goat anti-rabbit IgG (TdT iF Kit; Super Tech Inc., Bethesda, MD). 200 cells were evaluated under an immunofluorescence and phase contrast microscope (Carl Zeiss, Inc., Thornwood, NY).

Immunophenotype. The expression of surface membrane antigens on fresh and cultured leukemic cells was determined on an Ortho Spectrum III cytofluorograf (Ortho Diagnostic

TABLE I
List of Monoclonal Antibodies

Cluster designation	Monoclonal antibody	Conjugate	Supplier	Antigen/specificity
CD1	OKT 6	u*	Ortho Diagnostic	Cortical thymocyte
CD2	13B3	u	D. A. Valleria [‡]	Sheep erythrocyte receptor
CD3	OKT 3	u	Ortho Diagnostic	Mature T cell antigen
	Leu-4	PE	Becton Dickinson & Co.	associated with TCR
CD4	Leu-3	u, FITC	Becton Dickinson & Co.	T cell helper/inducer
CD5	T101	u	Hybritech, Inc., San Diego, CA	Pan-T lymphocyte
CD7	3A-1	u	B. Haynes [§]	Pan-T lymphocyte
	G3.7	u	Oncogene Science, Inc., Mineola, NY	
	Leu-9	PE	Becton Dickinson & Co.	
CD8	OKT 8	u	Ortho Pharmaceuticals	T cell suppressor/cytotoxic
	Leu-2	FITC	Becton Dickinson & Co.	
CD9	BA-2	u	T. W. LeBien [‡]	Lymphoid and myeloid precursor-associated antigen
CD10	BA-3	u	T. W. LeBien [‡]	cALLA
	J5	PE	Coulter Immunology	
CD13	MCS-2	u	J. Minowada	Pan-myelomonocytic antigen
CD14	MY 3	u	Coulter Immunology	Pan-monocytic antigen
CD16	Leu-11	FITC	Becton Dickinson & Co.	Fc γ -receptor
CD19	B43	u	F. Uckun [‡]	Pan-B
	Leu-12	PE, FITC	Becton Dickinson & Co.	
CD24	BA-1	u	T. W. LeBien [‡]	Pan-B
CD33	MY 9	u, PE	Coulter Immunology	Myeloid precursor antigen
CD34	MY 10	u	Coulter Immunology	Hematopoietic precursor antigen
nd	MY 8	u	Coulter Immunology	Myeloid lineage cells
nd	Leu-7	FITC	Becton Dickinson & Co.	NK cells
nd	Leu-19	PE	Becton Dickinson & Co.	NK cells
nd	Anti-TCR-1	FITC	Becton Dickinson & Co.	TCR α/β framework
nd	δ TCS-1	FITC	T Cell Sciences, Inc., Cambridge, MA	Subpopulation of TCR- γ/δ lymphocytes
nd	T γ A	u	T. Hercend [¶]	Subgroup of TCR- γ/δ lymphocytes expressing V γ 9-J γ 1.2

List of the mAbs used in this study according to the CD classification, where available. In other cases, the antigen detected by the mAb is given.

* u, Unconjugated.

[‡] University of Minnesota, Minneapolis, MN.

[§] Duke University Medical Center, Durham, NC.

^{||} Hayashibara Biochemical Laboratories, Inc., Okayama, Japan.

[¶] Institute Gustav-Roussy, Villejuif, France.

** nd, Not designated.

Systems Inc., Raritan, NJ) or a FACS IV (Becton Dickinson & Co., Mountain View, CA) using unconjugated (for indirect immunofluorescence) and FITC- or phycoerythrin (PE)-conjugated mAbs (Table I). For indirect immunofluorescence, 10^6 cells were suspended in 250 μ l fluorescence buffer (PBS, 4% newborn calf serum (Gibco Laboratories), 0.1% sodium azide),

and stained with a predetermined concentration of mAb. Control ascites protein served as a negative control. Cells were washed twice with fluorescence buffer and subsequently incubated with a mixture of FITC-conjugated goat anti-mouse IgG and IgM. After three additional washes in fluorescence buffer, cells were fixed in 1% paraformaldehyde and analyzed. Two-color fluorescence studies were carried out in 250 μ l incubation volume with directly PE- and FITC-conjugated mAbs following the manufacturers' instructions. Isotype-matched, directly conjugated antibodies served as controls. All staining and washing steps were carried out at 4°C.

Functional Studies. NK and LAK activity of IL-2- and GM-CSF-dependent, and the growth factor-independent, leukemic cell cultures were assessed as previously described (17). Peripheral blood lymphocytes obtained from normal donors were cultured for 14 d in IL-2 and served as controls. Briefly, NK-sensitive K562 and NK-resistant HL-60 were labeled with 250–750 μ Ci of Na⁵¹CrO₄ (5,000 μ Ci/ml; New England Nuclear, Boston, MA), for 60–90 min at 37°C. Cells were washed once in RPMI 1640 supplemented with 6% pooled human serum and resuspended in culture medium and incubated at room temperature for 60 min. Cells were then washed twice, resuspended in fresh medium, and 500 cells/well were added to effector cells in 96-well V-bottomed multititer plates (Costar). E/T ratios ranged from 30:1 to 0.1:1. Plates were centrifuged at 65 *g* for 5 min and incubated in 5% CO₂ at 37°C for 4 h, after which 150 μ l of the supernatant was harvested from each well. Radioactivity released in the supernatant was counted in a liquid scintillation counter (LKB 1216) and percent cytotoxicity was determined as follows: Percent cytotoxicity = 100 \times [(experimental mean cpm – spontaneous release mean cpm)/(maximal mean cpm – spontaneous mean cpm)].

The ability of cells to produce oxygen radicals in response to stimuli was assessed by the reduction of nitroblue tetrazolium (NBT; Sigma Chemical Co.) as previously described (18). Briefly, 4 \times 10⁵ cells were incubated with 10 μ g/ml NBT for 20 min at 37°C and cytospin Wright, Giemsa preparations were counted to determine the number of cells containing blue-black formazan deposits. NBT reduction was measured in unstimulated cells as well as in those stimulated by PMA (5 ng/ml; Sigma Chemical Co.) and the synthetic chemotactic peptide FMLP (Calbiochem-Behring Corp., La Jolla, CA) at a concentration of 10⁻⁷ M.

Ultrastructural Studies. Ultrastructural localization of MPO and platelet peroxidase was performed according to the methods of Graham and Karnovsky (19) and Breton-Gorius et al. (20). Cells were fixed for 30 min at 4°C and subsequently incubated at room temperature in a buffered diaminobenzidine/hydrogen peroxide solution for 60 min. Control cells were incubated in a solution lacking the hydrogen peroxide. Detection of lymphocyte parallel tubular arrays was performed as described before (21).

Cytogenetic Studies. Metaphase chromosome spreads were obtained from the GM-CSF, the IL-3, and the growth factor-independent leukemic cell cultures using standard methods. Briefly, 0.5 \times 10⁶ and 1.0 \times 10⁶ cultured cells were harvested directly using two techniques. In the first, the cells were exposed to a mixture of 0.075 M KCl (9 ml), 0.25% Trypsin-EDTA (1 ml), and colcemid (70 μ l; final concentration, 0.07 μ g/ml) at 37°C for 20 min. In the second, the cells were suspended in 10 ml RPMI 1640 medium supplemented with 20% FCS and L-glutamine (29 mg/ml), exposed to colcemid (0.07 μ g/ml) for 20 min at 37°C, and then resuspended in 0.075 M KCl for 12 min at 37°C. Cells from both preparations were fixed in 3:1 methanol/glacial acetic acid, and slides were made in the usual fashion. Chromosomes were G-banded using the Wright's technique of Sanchez et al. (22). A total of 10 metaphase cells from each of the original cultures were fully analyzed, and a minimum of two photographic karyotypes were constructed for each abnormal clone. Karyotypes were designated according to the ISCN 1985 (23).

DNA Extraction. High molecular weight DNA was extracted from fresh and cultured leukemic cells by proteinase K digestion in TE, NaCl 0.1 M, 1% SDS, followed by two phenol/chloroform/isoamyl alcohol (25:24:1) and two chloroform/isoamyl alcohol (24:1) extractions. DNA was precipitated with absolute ethanol, dried, and resuspended in TE pH 7.4.

Detection of Gene Rearrangement. High molecular weight DNA was digested to completion with Bam HI, Eco RI, or Xba I (Bethesda Research Laboratories, Gaithersburg, MD), size fractionated on a 0.8% agarose gel, and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, NH) in 10X SSPE. After washing and baking, the membrane was

prehybridized for 2–12 h and hybridized for 20 h at 42°C according to the manufacturer's instructions. Probes were labeled to high specific activity by the random primer method (24). After hybridization, membranes were washed two times for 15 min with 6 × SSPE, 0.1% SDS at room temperature, two times for 15 min with 1 × SSPE, 0.5% SDS at 37°C and finally with 0.1 × SSPE, 1% SDS at 60 to 65°C for 30 min. Autoradiography was carried out on Kodak XR film with two intensifying screens for 14–36 h at –70°C.

Probes. p δ 7 is a genomic 1.6-kb Eco RI–Eco RI DNA probe that detects the human TCR- δ constant region. The probe was derived from a human chromosome 14 library with the mouse TCR- δ constant region probe described by Chien et al. (25). Rearrangements of the TCR- δ gene were detected using the cDNA probe O/240–38 (26), kindly provided by M. S. Krangel (Dana-Farber Cancer Institute, Boston, MA). This probe contains the V δ region, used in the cell line IDP2, and the leukemic cell line PEER (26, 27), the J δ 1 region, and part of the TCR- δ constant region (C δ). Rearrangements of the TCR- β were detected with a 770-bp cDNA constant region probe kindly provided by T. W. Mak (University of Toronto, Toronto, Ontario, Canada). Rearrangements of the TCR- γ gene were detected with a cDNA probe pT γ 1 kindly provided by R. Holcombe (Harvard University, Boston, MA) (28) detecting the constant (C γ 1 and 2), joining regions (J γ 2.3 and 1.3), and a member of the V γ 1 family. Rearrangements of the IgH chain joining region were detected with a genomic 5.4-kb joining region probe (JH), kindly provided by S. Korsmeyer (Washington University, St. Louis, MO). To detect rearrangements of the breakpoint cluster region, the human *bcr* (Pr-1) (Oncogene Science, Inc., Mineola, NY) probe was used. This is a 1.2-kb Hind III–Bgl II fragment of genomic DNA that detects rearrangements of chromosome 22, resulting in formation of the Philadelphia chromosome in patients with chronic myelogenous leukemia.

Results

Establishment of Different Leukemic Cell Cultures with Various Growth Factors from a Patient with T-ALL. Fresh peripheral blood leukemic cells of the patient obtained at second relapse were cultured with no growth factor or in the presence of human IL-3, GM-CSF, or IL-2. In the presence of IL-3 or GM-CSF, after a period of 6 and 10 wk, respectively, proliferating cells began forming clumps and clusters and the cultures could be expanded. Leukemic cells initially cultured in the presence of IL-3 were weaned from this growth factor after 10 wk and have been in continuous culture without the addition of any growth factor for 5 mo. In the presence of IL-2, the fresh leukemic cells started to form clumps and clusters already after 24 h in culture and have been in IL-2-dependent continuous culture for 5 mo. Control cultures without growth factor showed no viable cells after 6 wk of culture. GM-CSF-dependent cellular subclones were obtained by limiting dilution from the GM-CSF-dependent leukemic cell cultures after 2 mo of continuous culture. Several GM-CSF-dependent cellular subclones were obtained, which all displayed the same phenotype as demonstrated in Fig. 2, Tables II and III. These are designated GM-CSF dependent leukemic cell lines, one of which is designated MN1.1.

Morphology and Cytochemistry of Fresh and Cultured Leukemic Cells. Representative morphologies of uncultured and cultured leukemic cells are shown in Fig. 1. A peripheral blood smear of fresh leukemic cells obtained at second relapse is shown in Fig. 1 A. Two small lymphoblasts with sparse, deeply basophilic cytoplasm and a high nuclear/cytoplasm ratio were observed. The nuclei were irregular and convoluted and displayed a rather dense, irregular chromatin structure with small nucleoli. Cytochemical stains were negative for MPO and NSE, but generally positive for TdT (Table II).

IL-3-cultured leukemic cells, GM-CSF-cultured leukemic cells, and the GM-CSF-

TABLE II
Cytochemistry and Morphology of Fresh and Cultured Leukemic Cells

Cytochemistry/Morphology	Fresh	IL-3 or GM-CSF	Growth factor independent	IL-2
MPO	0	69	0	0
NSE	0	4	0	0
TdT	44	3	23	>90
Lymphoblasts	100	0	0	0
Undifferentiated blasts	0	6	100	0
Granulated lymphocytes	0	0	0	100
Myelocytes	0	16	0	0
Segmented granulocytes	0	72	0	0
Monocytoid cells	0	6	0	0

Staining for MPO, NSE, and TdT and differential count of uncultured (fresh) peripheral blood leukemic blasts, from which the leukemic cell cultures were established, the IL-3 or GM-CSF bulk cultures or the GM-CSF-dependent subclone MN1.1, the growth factor-independent (leukemic cells initially cultured in the presence of IL-3 and subsequently weaned from this growth factor), and the IL-2-dependent leukemic cells.

dependent cell line MN1.1 (Fig. 1 B) demonstrated the presence of all stages of myeloid differentiation (Table II). Terminally differentiated neutrophils (72%), most of which were MPO⁺, along with a small percentage of monocytoid cells (6%) and undifferentiated blasts (6%) were present. The extent of myeloid maturation in the

TABLE III
Immunophenotype of Fresh and Cultured Leukemic Cells

Cluster designation/mAb	Fresh	IL-3 or GM-CSF	Growth factor independent	IL-2
CD2	92	7	94	98
CD3	96	3	97	89
CD4	7	60	0	0
CD5	94	NT	95	96
CD7	97	0	100	100
CD8	12	0	0	84
CD13	0	70	55	0
CD14	0	15	12	0
CD25	0	0	0	0
CD33	0	99	75	37
CD34	0	0	0	0
My8	0	85	0	0
WT31	25	0	36	84
Anti- δ	0	0	0	0
Anti-TryA	0	0	0	0
Leu-19	0	0	0	94

Phenotype of the uncultured (fresh) peripheral blood leukemic blasts, the IL-3, or GM-CSF bulk cultures or the GM-CSF-dependent subclone MN 1.1, the growth factor-independent, and the IL-2-dependent leukemic cells. Results are means obtained from two to five individual analyses. Immunophenotyping was carried out according to Materials and Methods. WT31 is the anti-TCR-1 (Becton Dickinson & Co.), anti- δ is the anti- δ TCS-1 (T-Cell Science Inc.) mAb. NT, not tested.

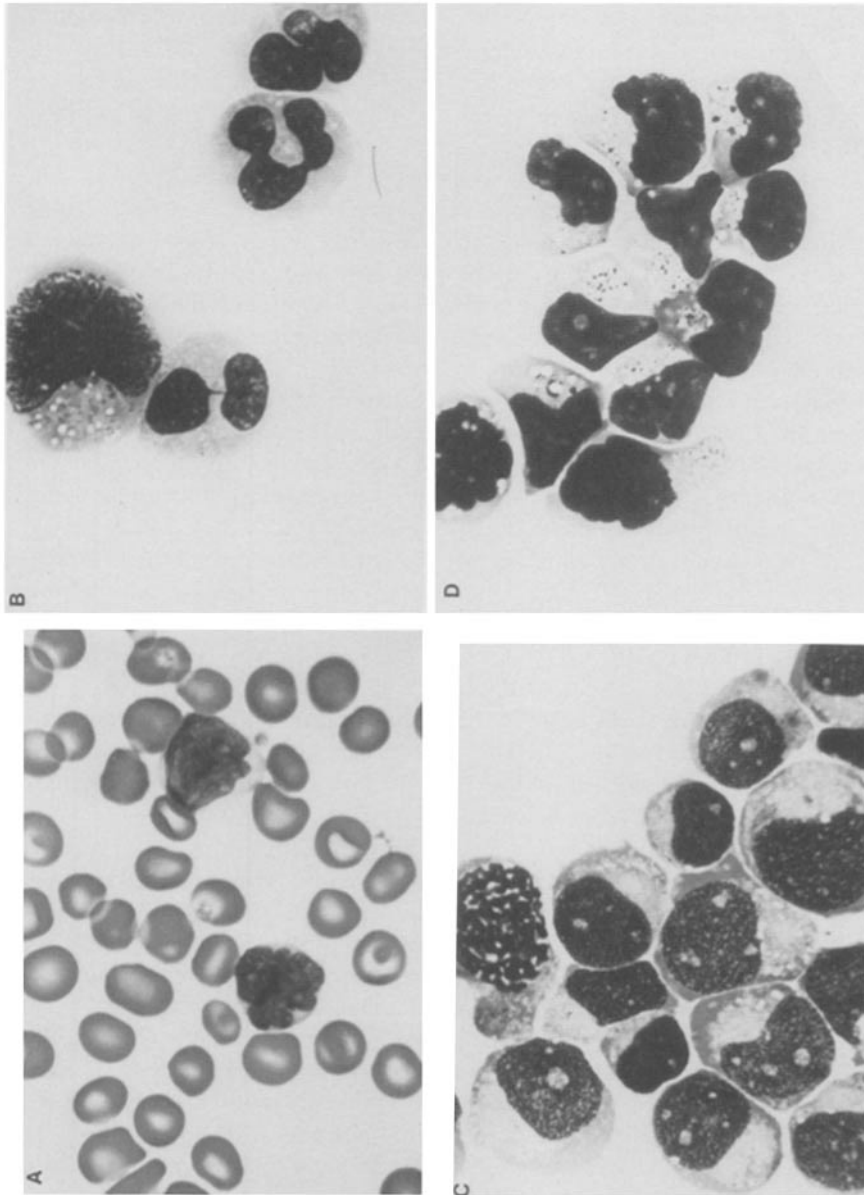


FIGURE 1. (A) Two lymphoblasts with convoluted nuclear outlines in blood smear made from specimen from which leukemic cell cultures were established. (B) Maturing neutrophils with nuclear hypobolubation from the IL-3-cultured specimen. (C) Leukemic cells with fine nuclear chromatin and prominent nucleoli from the growth factor-independent culture (leukemic cells initially cultured in the presence of IL-3 and subsequently weaned from this growth factor). (D) IL-2-dependent leukemic cells. The majority of these cells have distinct nucleoli and azurophilic granulation. (Fig. 1, A-D, Wright's Giemsa $\times 1,000$).

GM-CSF-cultured leukemic cells was subject to some variation. Furthermore, the degree of MPO expression as detected by cytochemical staining diminished with time in culture. Ultrastructural studies, however, demonstrated that virtually 100% of GM-CSF-cultured leukemic cells expressed high levels of MPO (data not shown).

Growth factor-independent leukemic cells (Fig. 1 C) displayed size heterogeneity and contained a deeply basophilic cytoplasm. The nuclei were of slightly irregular shape, sometimes indented, and had a condensed chromatin structure and two or more nucleoli. Mitotic figures were frequently seen. Cytochemical stains were negative for MPO, NSE (Table II), as well as SB and toluidine blue. 23% of the cells expressed TdT. The lack of MPO expression was confirmed ultrastructurally.

IL-2-cultured leukemic cells (Fig. 1 D) contained a lightly basophilic cytoplasm, azurophilic granules, and various degrees of vacuolization. Nuclei were of irregular shape, sometimes indented, and displayed up to four nucleoli. Cytochemically, all the cells were negative for MPO, NSE, and weakly positive for TdT (Table II). No parallel tubular arrays, typically seen in uncultured large granular lymphocytes (21), were demonstrated ultrastructurally in the IL-2-dependent leukemic cell cultures. However, they may be lost during in vitro culture (29).

Immunophenotype. The immunophenotypes of the fresh and the cultured leukemic cells are shown in Table III. The fresh leukemic cells were strongly positive for the T-lineage markers CD2, CD5, and CD7, as well as for the mature T lymphocyte antigen CD3, a phenotype consistent with acute T-lymphoblastic leukemia. Only 25% of the fresh leukemic cells stained positive for WT31, which binds to the CD3/TCR α/β chain heterodimer complex (30, 31). However, there was no evidence for the expression of the "second" TCR γ/δ chain heterodimer as assessed by staining with anti- δ (anti-TCS-1) and anti-Ti γ A (32). The cells were negative for CD4 and CD8 as well as B-lymphoid (CD10, CD19, CD20, CD24), precursor-associated (CD9, CD34), and myeloid antigens (CD13, CD14, MY 8, and CD33) (Fig. 2 A).

A high percentage of the IL-3- and GM-CSF-cultured leukemic cells expressed CD33 (Fig. 2 B), CD13, and MY8 (33). T-lineage markers (CD2, CD3, CD7) were expressed by a low percentage of cells in this culture. This phenotype was consistent with the myeloid morphology of cells grown under these conditions.

The growth factor-independent leukemic cell cultures displayed a mixed phenotype coexpressing T-lineage (CD2, CD3, CD7) and myeloid (CD13 and CD33) antigens. Two-color fluorescence demonstrated coexpression of CD7 and CD33 by 73.6% of the cultured leukemic cells (Fig. 2 C). Only 36% expressed WT31, they were negative for CD4, CD8, anti- δ (anti-TCS-1), and anti-Ti γ A. This pattern of T cell antigen expression was very similar to that of the fresh leukemic cells.

The IL-2-cultured leukemic cells were positive for CD2, CD7, and the CD3/TCR- α/β complex (WT31⁺). Additionally, they expressed the T suppressor/cytotoxic-associated antigen CD8, and Leu-19, which is characteristically found on fresh peripheral blood NK cells and can be acquired by normal peripheral blood lymphocytes when cultured in the presence of IL-2. Surprisingly, 37% of the cells expressed the myeloid precursor-associated antigen CD33 (Fig. 2 D).

Genotype. The clonal relationship of the fresh leukemic and cultured cells was addressed subsequently. Fig. 3 A-C, shows representative Southern blots of the IL-3-cultured leukemic cells (lane 1), fresh leukemic cells obtained from peripheral blood during second relapse (lane 2), germline source (lane 3), and IL-2-dependent leu-

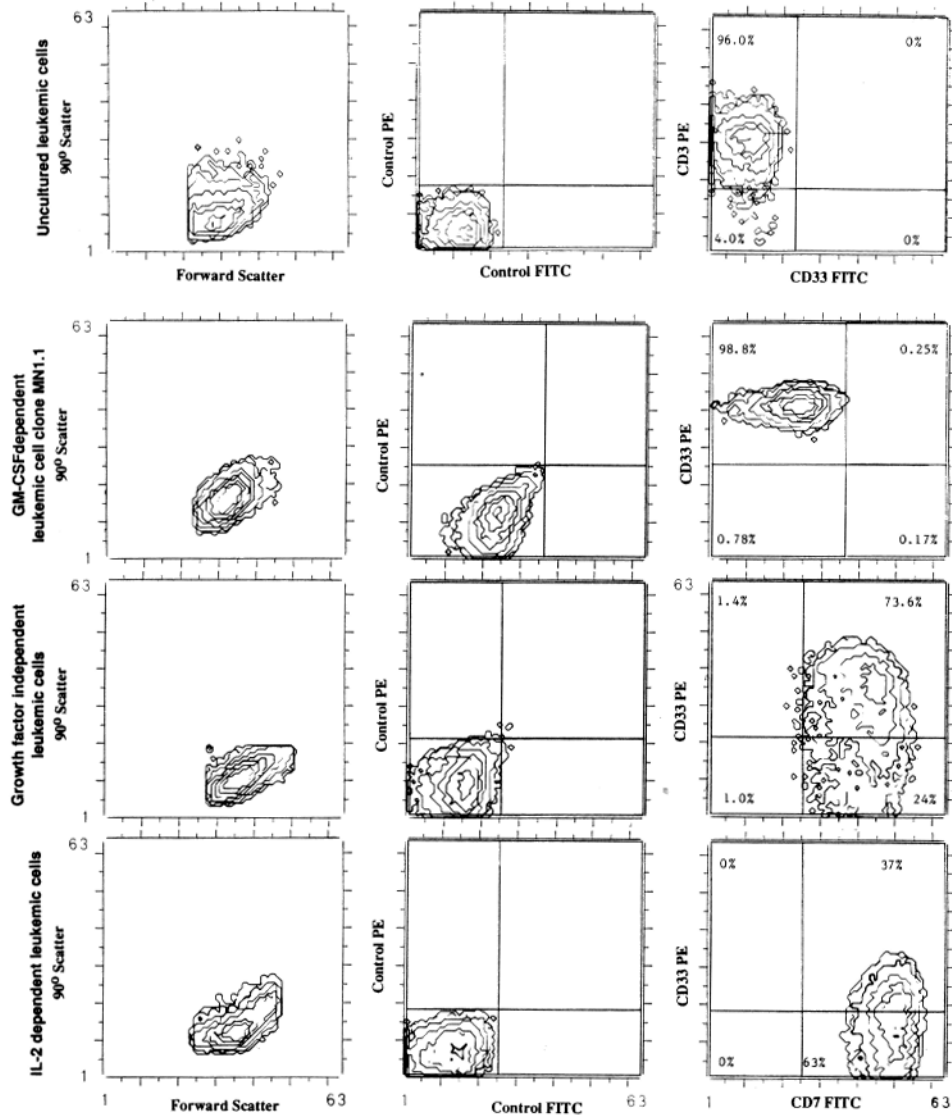


FIGURE 2. Expression of CD3 and CD33 on uncultured leukemic cells (A), and CD7 and CD33 on GM-CSF-dependent leukemic cellular subclone MN-1.1 (B), growth factor-independent (C), and IL-2-dependent leukemic cells (D). For each sample, forward and 90° scatter profile of gated cells are shown on a linear scale. The fluorescence intensity for FITC and PE is shown on a logarithmic scale, the lowest contour level was three cells. Percentages of cells in each quadrant are given. Staining and selection of controls was performed as outlined in Materials and Methods.

kemic cells (lane 4). Both the fresh and the IL-3-cultured leukemic cells displayed identical unique 11.5-kb Bam HI (Fig. 3 A) and 10.0-kb Xba I (Fig. 3 B) TCR- δ constant region rearranged bands with the p δ 7 probe. A faint germline band in the fresh leukemic sample was presumably due to the presence of nonleukemic cells.

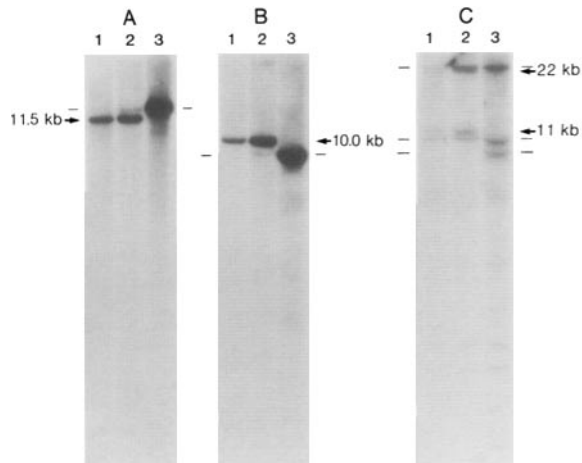


FIGURE 3. Southern blot analysis IL-3 cultured (lane 1), uncultured (lane 2) leukemic cells, and placenta (lane 3). High molecular weight DNA was digested with Bam HI (A) and Xba I (B and C) and probed with p δ 7 (A and B) and with pT γ 1 (C). Rearrangements are marked with arrows, germline bands with bars.

Identical unique rearrangements of the TCR- δ constant region were observed in growth factor-independent, GM-CSF- and IL-2-dependent cell cultures (data not shown). The uncultured and all cultured cells displayed an identical 1.0-kb Eco RI (Fig. 4 A) and 10-kb Bam HI (not shown) rearrangement of the J δ 1 region. The IL-3-cultured and fresh leukemic cells also displayed identical clonal rearrangements of the TCR γ chain gene (Fig. 3 C). A single identical TCR β chain gene rearrangement was observed in uncultured, IL-2- and GM-CSF-dependent, and growth factor-independent leukemic cells, as shown in Fig. 4 B. The immunoglobulin heavy chain gene and the break point cluster region (*bcr*) were found in the germline configuration in all cultured and the uncultured leukemic cells (data not shown).

Thus, leukemic cells cultured under various conditions displayed morphological features of at least two different lineages, but showed identical rearrangements of

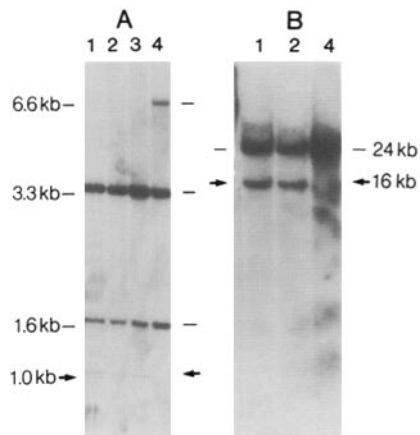


FIGURE 4. Southern blot analysis of IL-2-dependent (lane 1), uncultured (lane 2), GM-CSF-dependent (lane 3) leukemic cells, and placenta (lane 4). High molecular weight DNA was completely digested with Eco RI (A) and Bam HI (B) and probed with the TCR- δ cDNA probe 0-240/38 (A) and the TCR- β probe T β (B). Rearrangements are marked with arrows, germline bands with bars.

the TCR- β , - γ , and - δ genes to the fresh leukemic cells. These data clearly demonstrated that the fresh leukemic cells and the different leukemic cell cultures were derived from the same malignant clone.

Karyotype. Cytogenetic analyses of the GM-CSF-dependent leukemic cell culture, the IL-3-cultured leukemic cells, and the growth factor-independent leukemic cell cultures identified six closely related abnormal clones. All had an interstitial deletion of part of the long arm of chromosome 6 and a three-break translocation involving the long arm of chromosome 1 and short arms of chromosomes 10 and 12 (Table IV). A G-banded karyotype of one metaphase cell from the GM-CSF culture demonstrating these abnormalities is shown in Fig. 5. These findings provided further evidence that the cultured cells arose from a common malignant precursor.

Functional Studies. To determine the functional characteristics of the morphologically terminally differentiated neutrophilic granulocytes of the GM-CSF-cultured cells, the oxygen radical production of either unstimulated or stimulated (PMA or FMLP) cells was assessed by measuring the NBT reduction (Table V). Freshly obtained peripheral blood granulocytes served as a positive control and the growth factor-independent leukemic cell cultures as a negative control. 20% of PMA, but none of the FMLP-stimulated GM-CSF-cultured leukemic cells reduced NBT. Growth factor independent leukemic cell cultures displayed no oxygen radical synthesis in response to stimuli.

Since IL-2-dependent leukemic cells expressed a phenotype similar to cytotoxic lymphocytes (see Table III), NK and LAK activity were tested. In a chromium-release assay, the IL-2-dependent leukemic cells exerted a high lytic activity against both the NK-sensitive K562 and the NK-resistant HL-60 targets, while the myeloid GM-CSF-dependent and the undifferentiated growth factor-independent leukemic cells were devoid of lytic activity against either target cell line (Fig. 6).

TABLE IV
Cytogenetic Studies of Cultured Leukemic Cells

Cultures	Number of metaphases analyzed		G-banded karyotypes
	Total	Per clone	
Growth factor independent	10	7	47, XY, + 19, del (6)(q15q25), t(1;10;12)(q25;p13;p13)
		2	47, XY, + 19, del (6)(q15q25), dir dup (17)(q11.2->q23), t(1;10;12)(q25;p13;p13)
		1	48, XY, + 19, del (6)(q15q25), + del (6)(q15q25), t(1;10;12)(q25;p13;p13)
GM-CSF	10	2	48, XY, + 17, + del (6)(q15q25), t(1;10;12)(q25;p13;p13)
		8	49, XY, - 7, + 13, + 19, + del (6)(q15q25), t(1;10;12)(q25;p13;p13), + der (7) t (7;17)(q36;21)
IL-3	10	5	47, XY, + 19, del (6)(q15q25), t(1;10;12)(q25;p13;p13)
		2	48, XY, + 19, del (6)(q15q25), + del(6)(q15q25), t(1;10;12)(q25;p13;p13)
		3	49, XY, + 17, + 19, + del (6)(q15q25), t(1;10;12)(q25;p13;p13)

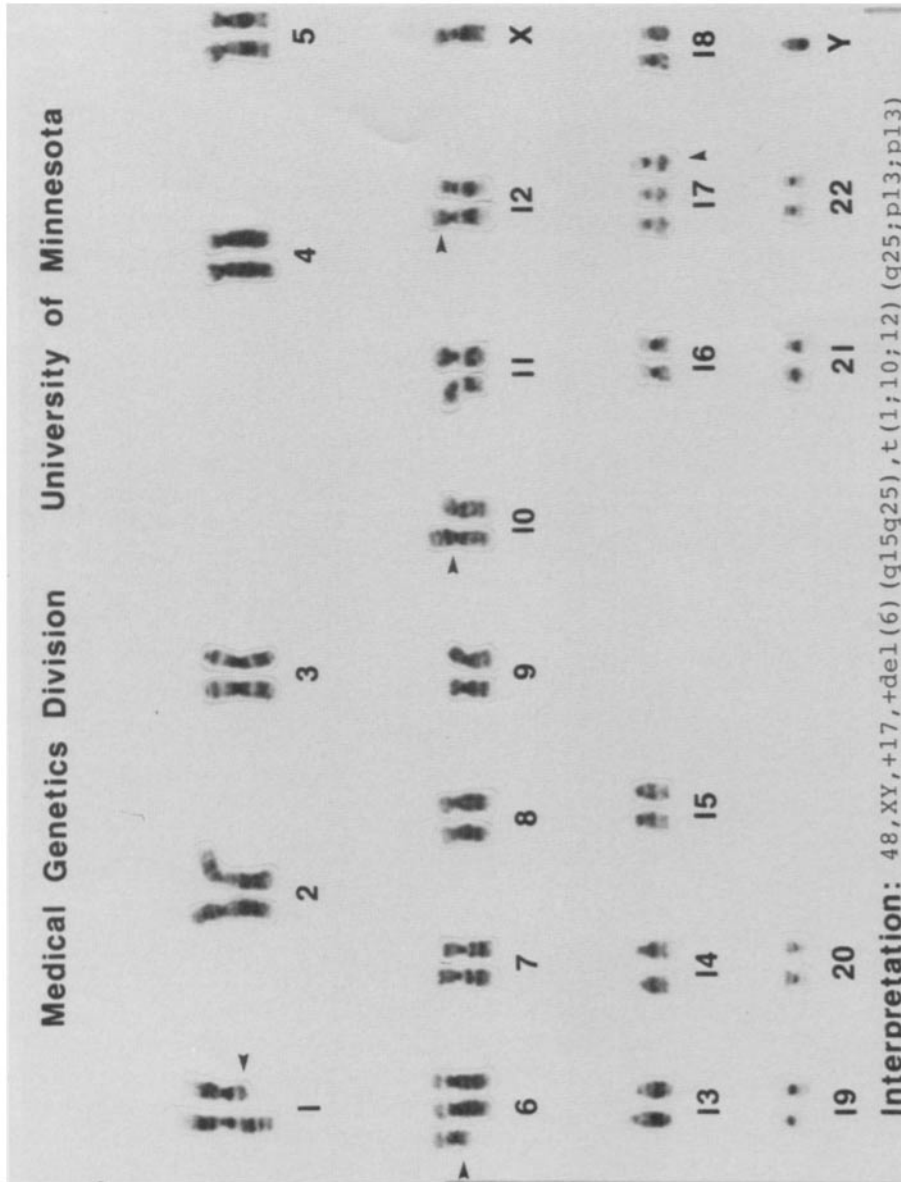


FIGURE 5. Wright's G-banded metaphase cell from the GM-CSF culture showing the deletion of the long arm of chromosome 6 and the 1;10;12 translocation.

TABLE V
Oxygen Radical Production of Cultured Leukemic Cells

Stimulus	Control PMN	GM-CSF	Growth factor independent
None	12	0	0
PMA	90	20	0
FMLP	90	0	0

Oxygen radical production was assessed by measuring the reduction of NBT in unstimulated or PMA (5 ng/ml) or FMLP (10^{-7} M)-stimulated cells. Cells were stained with Wright-Giemsa and scored for black-blue formazan deposits. Control PMN, polymorphonuclear cells from a healthy donor; GM-CSF, GM-CSF-dependent leukemic cell culture.

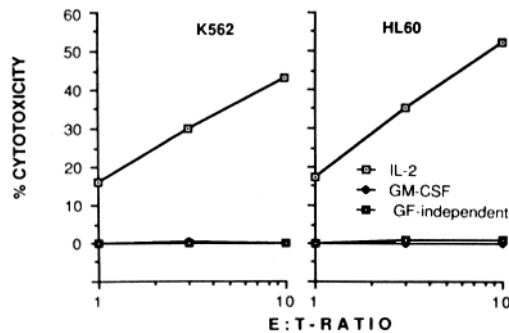


FIGURE 6. Percent cytotoxicity of the IL-2-dependent leukemic cell culture (IL-2), the GM-CSF-dependent cell culture (GM-CSF), and the growth factor-independent leukemic cell culture (GF-independent) against the NK-sensitive target cell line K562 and the NK-resistant target cell line HL-60 in a chromium-release assay. Mean values of triplicates are given, SEM was <5%. Representative data of one experiment which were reproduced in two additional experiments are shown.

Discussion

Multilineage Differentiation Potential of MT-ALL. To the knowledge of the authors, this is the first report of a mature predominantly CD3⁺ WT31⁺ T-ALL with multilineage differentiation potential. At presentation, the leukemic cells displayed the typical morphological and cytochemical characteristics of an acute lymphoblastic leukemia and predominantly expressed a phenotype consistent with mature CD3⁺ TCR- α/β -expressing T lymphocytes (30-32). This mature phenotype was in contrast to the immature phenotype of the previously reported multipotential T-ALL DU.528 (9, 10) and the T-lymphoblastic lymphoma (11), which expressed CD2, CD5, and CD7 (consistent with an early thymocyte) and CD7, HLADR, and TdT (consistent with a prothymocyte) (12-14) respectively. The uncultured tumor cells of these patients did not express a CD3/TCR complex on the cell surface (9-11). Our studies demonstrate by morphological, phenotypic, and functional criteria, multilineage differentiation of leukemic MT-ALL blasts. Three different leukemic cell cultures were derived from the original leukemic blasts:

(a) Myeloid and monocytic cells of various differentiation stages including terminally differentiated functional neutrophilic granulocytes arose in the presence of IL-3 and GM-CSF. In contrast to the DU.528 cell line (9, 10), no erythroid cells, megakaryocytes, eosinophils, or basophils were observed in these cultures.

(b) Mixed lineage cells in those cultures, that were started with IL-3 and subsequently weaned from this growth factor, displayed an undifferentiated blast mor-

phology and coexpressed T lineage-associated antigens (CD2,CD3,CD7,WT31) with the panmyeloid antigen CD13, and the early myeloid antigen CD33, which is expressed on a subset of CFU-GEMM, BFU-E, and CFU-GM and their progeny (reviewed in reference 33).

(c) Granulated lymphocytes developed in the presence of IL-2 with characteristic morphological (azurophilic granules), phenotypic (acquisition of CD8 and Leu-19), and functional features (NK and LAK activity) of MHC-unrestricted cytotoxic lymphocytes (34). Strikingly, coexpression of the T cell antigens CD2,CD3,CD5,CD7,CD8, WT31 together with the CD33 antigen was observed in the IL-2-dependent leukemic cultures.

Although coexpression of myeloid and lymphoid, as well as T and B cell antigens, has been observed in a small percentage of acute leukemias (35), coexpression of the mature T lymphocyte antigen CD3 and WT31 in association with the immature myeloid antigens CD33 and CD13 has not been reported to our knowledge. The possibility that this process represented a rare T cell blast crisis of a chronic myelogenous leukemia was essentially ruled out by the lack of cytogenetic or molecular evidence (*bcr* rearrangement) of Philadelphia chromosome formation. Two alternative hypotheses for the coexpression of antigens that are normally found on cells belonging to different lineages have been proposed: Normal precursor cells at early stages of differentiation and before definite commitment to a specific lineage may display a certain degree of "lineage promiscuity" (8) with coexpression of antigens associated with different lineages. Thus, a differentiation event normally occurring in a few precursors may be immortalized by malignant transformation and become detectable after clonal expansion. The expression of mature T cell antigens (CD3, WT31) with immature myeloid antigens (CD33 and/or CD13) in the present case, however, is more consistent with the concept of "lineage infidelity" as proposed by Smith et al. (36). They suggest that transformational events in leukemias may be associated with aberrant genetic programming and the expression of "inappropriate" gene products. However, it is currently unknown whether a normal counterpart bearing this mixed lineage phenotype exists. Our results therefore warrant further studies to search for coexpression of antigens associated to the T lymphocyte and the myeloid lineages in nonleukemic cells.

Genotypic and Cytogenetic Evidence for the Derivation of Cultured and Uncultured Leukemic Cells from the Same Malignant Clone. Unique TCR- δ , - β , and - γ gene rearrangements unambiguously proved that the uncultured and the various cultured leukemic cells displaying characteristics associated to different lineages were derived from the same malignant clone.

Recently, the human TCR- δ gene, which encodes a protein that is expressed as a heterodimer with the protein product of the TCR- γ gene, has been identified and characterized (25-27, 37-41). Identical unique monoallelic rearrangements of the TCR- δ constant region were detected in all cultured and the uncultured leukemic cells. A restriction fragment length polymorphism was ruled out by use of different restriction enzymes for this analysis and by demonstration of a germline band in contaminating nonleukemic cells of the patient. The rearrangement of the TCR- δ constant region detected by conventional Southern analysis after Bam HI and Xba I digests may be due to recombinatorial events involving TCR- α J regions 3' of the TCR- δ region as described in RPMI 8402 (40). Additional Southern analysis re-

vealed an identical monoallelic rearrangement of the TCR- δ J1 region. The repertoire of the TCR- δ gene rearrangements is currently unknown. However, the 1.0-kb Eco RI/12.0-kb Bam HI rearrangement of MT-ALL was detected in only 3 of 100 leukemias and cell lines studied (Griesinger, F., submitted for publication). This essentially rules out the possibility that this J δ 1 rearrangement was fortuitously identical in the different MT-ALL cultures studied. An identical monoallelic rearrangement of the TCR- β gene and biallelic rearrangements of the TCR- γ gene in all cultured and the uncultured leukemic cells provided further proof for their clonal identity. The molecular genetic findings of derivation from a single clone were consistent with the cytogenetic results obtained from the IL-3- and GM-CSF-dependent, as well as the growth factor-independent leukemic cells.

Induction of Proliferation and Differentiation of Leukemic Cells by Recombinant Human Growth Factors. Leukemic cells in these studies responded to different recombinant physiological growth factors with proliferation and ordered differentiation. However, the question of which subset of leukemic cells was capable of in vitro differentiation and/or whether the different leukemic cultures were the result of the growth of minor leukemic populations remains to be addressed.

IL-3 and GM-CSF are involved in the regulation of proliferation and differentiation of normal and leukemic myeloid precursor cells (4-7, 42-51). Some effects of IL-3 and GM-CSF on murine nonleukemic T and B cell precursors have been proposed (52, 53). Recently, a GM-CSF- and IL-3-dependent precursor T cell leukemic cell line T-ALL 101 has been described (54), which did not, however, show evidence of myeloid differentiation or maturation. In the leukemic cell cultures described herein, differentiation and maturation along the myeloid cell lineage occurred only in the presence of IL-3 and GM-CSF, but not in the presence of IL-2. These data suggest that leukemic cells derived from the malignant clone and apparently committed to T cell differentiation were susceptible to IL-3 and GM-CSF and responded with a genetic program similar to normal hematopoietic precursor cells. In contrast to the DU-528 cell line, erythroid, megakaryocytic, basophilic, and mast cell elements (10) were absent in the IL-3 and GM-CSF cultures of MT-ALL. This suggests a restricted differentiation potential of this leukemia or, alternatively, the requirement of additional factors for multilineage differentiation that may be provided by 5637-conditioned medium used in the DU.528 cultures.

IL-2 stimulates T cell differentiation and proliferation and induces cytotoxic activity in NK cells, T lymphocytes, and some T cell leukemias (34, 55-62). MT-ALL cultured in the presence of IL-2 developed functional, morphological, and phenotypic features similar to non-MHC-restricted cytotoxic lymphocytes. This suggests that a genetic program, similar to normal cytotoxic lymphocytes (34), was activated in the leukemic cells by IL-2. To our knowledge, this is the first T-ALL to display such IL-2-induced cytotoxic activity without the morphological or phenotypic characteristics of "LGL"-leukemia (58) or of a "LAK cell precursor" ALL (62).

Although we were able to demonstrate the derivation of the IL-3-GM-CSF- and IL-2-dependent and the growth factor-independent leukemic cells from the same malignant clone, the exact pathway of differentiation from the original fresh leukemic cells to the different leukemic cell cultures is currently unknown. It is possible that undetectably small leukemic populations with myeloid and cytotoxic characteristics obtained a proliferative advantage in vitro in the presence of the appropriate

growth factor. Alternatively, cells with multilineage differentiation potential might have differentiated into the described cells of the different cell lineages. Whether such multilineage differentiation results from activation of normal genetic differentiation programs or through abnormal programs marking leukemic transformation remains an area of further studies.

Summary

We report an acute T-lymphoblastic leukemia with a predominantly mature CD3⁺CD7⁺WT31⁺ phenotype that was induced to differentiate into different cell lineages by various recombinant human growth factors. In the presence of IL-3 or GM-CSF, the leukemic cells gave rise to myeloid and monocytic cells including terminally differentiated, partially functional, segmented neutrophilic granulocytes as assessed by morphologic, cytochemical, immunophenotypic, and functional criteria. In the presence of IL-2, leukemic granulated lymphoid cells exhibiting MHC-unrestricted cytotoxicity and expressing a CD2⁺CD3⁺CD5⁺CD7⁺CD8⁺CD33⁺WT31⁺Leu19⁺ phenotype arose. Leukemic cell cultures initiated with IL-3 yielded growth factor-independent cells with a mixed lineage phenotype and morphologic and cytochemical evidence of immature blasts. These were T lymphocyte and myeloid surface antigen (CD2,CD3,CD5,CD7,CD13,CD33,WT31) positive. Identical rearrangements of the constant region of the TCR- δ gene and of the joining regions of the TCR- β , - γ , and - δ genes were observed in the fresh and all cultured leukemic cells, indicating that they were derived from the same malignant clone. Consistent with the molecular genetic data, the cytogenetic analyses of the GM-CSF-, IL-3-cultured and the growth factor-independent leukemic cells showed the presence of multiple, closely related abnormal clones, all of which had an interstitial deletion of part of the long arm of chromosome 6 and a complex 1;10;12 translocation.

In conclusion, these data demonstrate the involvement of a multipotent leukemic precursor cell in this predominantly mature CD2⁺CD3⁺CD5⁺CD7⁺WT31⁺ T-ALL. This multipotent leukemic precursor may be susceptible to various growth factors and respond with ordered differentiation and maturation.

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