ESTABLISHMENT OF THE DU.528 HUMAN LYMPHOHEMOPOIETIC STEM CELL LINE

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The occurrence of 'biophenotypic leukemias' with lymphoid and myeloid characteristics (1–5), together with cytogenetic or isozymic evidence of the clonal origin of myeloid, erythroid, megakaryocytic, and lymphoid cells in chronic myelogenous leukemia (6–9) suggest that leukemias may arise from pluripotent hematopoietic stem cells. However, leukemia-derived cell lines have not been established that are capable of producing mature cells of several lineages, i.e., that behave as functional multipotential stem cells. In general, leukemic cell lines show restricted patterns of differentiation, behaving like committed progenitors adhering imperfectly to early stages of a dominant program of differentiation, but with a defect that blocks maturation.

Two instances have recently been described in which an abrupt change in the lineage of origin of an acute leukemia suggested the involvement of a multipotential hematopoietic stem cell. In each patient, treatment with the adenosine deaminase (ADA)[1] inhibitor deoxycoformycin was associated with conversion from a T lymphoblastic to a promyelocytic phenotype (10, 11). One of these patients, a 16-yr-old boy with acute T lymphoblastic leukemia of 2 mo duration, was studied extensively in our laboratory (11). After a 4 d course of deoxycoformycin the phenotype of his leukemia cells changed over a 3 d period from T lymphoblastoid to promyelocytic, resulting in death from leukostasis. The T lymphoid-to-myeloid conversion was confirmed by histochemical, immunologic, and biochemical criteria, and was found to be complete in all involved tissues,

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[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; Ado, adenosine; dAdo, 2′-deoxyadenosine; dbcAMP, N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid; DU-HL60-3^B, biotinylated DU-HL60-3; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GCT, giant cell tumor; IL-2, interleukin 2; LCM, leukocyte-conditioned medium; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; NSE, nonspecific esterase; PAS, periodic acid-Schiff; PBS/BSA, phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide; PE-AV, phycoerythrin-avidin; PHA, phytohemagglutinin; PMA, phorbol-12-myristate-13-acetate; TCM, T cell–conditioned medium; 3A1*, fluoresceinated 3A1.

including thymus, at autopsy. The same abnormal karyotype was found in pre- and postconversion leukemic cells. These observations led us to suggest that ADA inhibition had directly induced the T lymphoid-to-myeloid transformation by altering the program of differentiation of a malignantly transformed stem cell (11). This possibility was of particular interest in view of the fact that a genetic deficiency of ADA results in selective absence of lymphoid lineages, a defect that could theoretically result from an effect of purine nucleosides on lymphohematopoietic stem cell differentiation. To pursue the questions raised by this remarkable response to treatment we have established a cell line from this patient's leukemia cells. In this report we describe properties of this cell line, which we have named DU.528.

Materials and Methods

Materials. Deoxyribonuclease I, *N*-hydroxysuccinimidobiotin, nitroblue tetrazolium (NBT), N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid (dbcAMP), phorbol-12-myristate-13-acetate (PMA), 5-azacytidine, 5-aza-2′-deoxycytidine, 2-aminoethylisothiouronium bromide hydrobromide, ethidium bromide, heparin, and 3′3′-diaminobenzidine were obtained from Sigma Chemical Co. (St. Louis, MO), as were staining kits for α-naphthyl butyrate esterase, naphthol AS-D chloroacetate esterase, periodic acid-Schiff, and myeloperoxidase. Alsever's solution and giant cell tumor (GCT)-conditioned medium were purchased from Gibco Laboratories (Grand Island, NY); purified phytohemagglutinin from Burroughs Wellcome Co. (Research Triangle Park, NC); HSI-LoSM medium from Hybridoma Sciences (Atlanta, GA); and Diff-Quik stain from Scientific Products, Inc. (Detroit, MI). TP-5, an analog of thymopentin, was provided by Dr. Gideon Goldstein, Ortho Pharmaceutical Corp. (Raritan, NJ), and 2′-deoxycoformycin (Pentostatin) by Warner Lambert-Parke Davis (Ann Arbor, MI).

Cell Growth and Culture Methods. Cells were routinely cultured in standard medium consisting of RPMI 1640 (Gibco Laboratories) supplemented with 10% horse serum and 10% heat-inactivated fetal calf serum 309 both from Gibco Laboratories, nonessential amino acids, 1 mM pyruvate, and 2 mM glutamine, under an atmosphere of 5% CO_2 in air at 37°C. Thawing medium consisted of RPMI 1640 containing 25% heat-inactivated fetal calf serum 309, penicillin/streptomycin, 20 U heparin/ml, and 1 mg/100 ml of deoxyribonuclease I. Cloning was performed by limiting dilution in standard growth medium (see Results). All experiments were performed with cultures in the logarithmic phase of growth. Cultures were deemed free of mycoplasma contamination by the inability of conditioned medium in which the cells had been grown to cause the conversion of deoxyadenosine to adenine (plus 5 μM deoxycoformycin to inhibit ADA).

Morphological, Cytochemical, Functional, and Cytogenetic Characterization. Standard differential counts were performed on cytocentrifuge (Shandon II; Shandon Southern Instruments Inc., Sewickley, PA) preparations of cells stained with the Diff-Quick modification of the Wright's-Giemsa technique (12). Cytochemical tests included staining for myeloperoxidase (MPO) (13), periodic acid-Schiff (PAS) (14), α-naphthyl acetate esterase (nonspecific esterase [NSE]) (14), and α-napthyl-butyrate esterase (14). NBT reduction was assessed as previously described (15). The percentage of cells synthesizing hemoglobin was determined by hemocytometer count after staining with benzidine (16). Rosette formation with sheep erythrocytes (E rosettes) was evaluated as described (17). Karyotyping was performed by standard methodology. Briefly, cultured cells were arrested with colchicine, treated with hypotonic KCl, and fixed with methanol/acetic acid (3:1). Giemsa-trypsin banding was performed as described (18). For each culture at least 50 spreads were examined to determine chromosome number. Complete karyotype analysis was performed on 10 spreads for the parent cell line and on 7–10 spreads for clones.

Analysis of Cell Surface Markers. Anti–human Leu-2b, anti–human Leu-3a + 3b, anti–human Leu-5, anti–human Leu-6, anti–human HLA-DR, anti–human Leu-M2, anti–human Leu-M3, anti–human transferrin receptor, and avidin-phycoerythrin conjugate

were obtained from Becton, Dickinson & Co. (Mountain View, CA); anti-B1 and anti-B2 from Coulter Electronics, Inc. (Hialeah, FL); anti-BA1 from Hybritech, Inc. (San Diego, CA); and affinity-purified, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG from Tago Inc. (Burlingame, CA). MY-10 monoclonal antibody (19) was kindly provided by Dr. Curt Civin, Johns Hopkins Oncology Center (Baltimore, MD). P3x63/Ag/8 mouse myeloma cell-induced mouse ascitic fluid, anti-TAC (anti-interleukin 2 [IL-2] receptor [20]), and 3A1 (21) monoclonal antibodies were provided by Dr. Barton Haynes, and the DU-HL60-3 (22) monoclonal antibody by Dr. Richard Metzgar, both of Duke University Medical Center. Terminal deoxynucleotidyl transferase immunoperoxidase kit (Supertechs, Inc., Bethesda, MD) was provided by Dr. Fred Bollum, Uniformed Services University of the Health Sciences.

10^6 Cells in standard medium were washed twice with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (PBS/BSA), and then incubated with 1:50 to 1:200 dilutions of monoclonal antibody for 30 min at 4°C in a microtiter (96 well, round bottomed) plate. After two washes with PBS/BSA, cells were incubated with affinity-purified, FITC-labeled goat anti-mouse IgG (1:100) for 30 min at 4°C. After two additional washes, cells were resuspended in PBS/BSA. Membrane staining was assessed immediately by either fluorescence microscopy or fluorescence-activated cell sorter (FACS) analysis of 10,000 cells per sample, performed on an Ortho 50 cytofluorograph (Ortho Pharmaceutical Corp.). Alternatively, the cells were fixed by the addition of 37% formaldehyde (10 μl/ml of cell suspension) and stored at 4°C in the dark for later FACS analysis. In each assay, background fluorescence was determined by incubating cells with P3x63/Ag/8 ascitic fluid (control murine myeloma protein), instead of specific primary antibody, and by incubation with FITC goat anti-mouse IgG alone. The percentage of nonviable cells in unfixed preparations was determined by a hemocytometer count of cells that stained with trypan blue or by FACS analysis of cells that stained with ethidium bromide (0.1 mg/ml, 5 min exposure).

Double parameter fluorescence analysis was used to determine whether two different membrane antigens, 3A1, a T cell marker, and DU-HL60-3, a myeloid marker, were both present on individual cells. All incubations were for 30 min at 4°C in the dark and cells were washed with PBS/BSA without azide. 10^6 Cells were washed twice and incubated with biotinylated DU-HL60-3 (DU-HL60-3^B, prepared as described [23]). After two washes the cells were counterstained with 100 μl of phycoerythrin-avidin (PE-AV) (1:100; Becton, Dickinson & Co.) Cells were again washed twice and then incubated with fluoresceinated 3A1 (3A1*) prepared by Dr. Barton Haynes. After two more washes the cells were resuspended and fixed with formaldehyde as described above. 10,000 cells per sample were analyzed for red (DU-HL60-3^B) and green (3A1*) fluorescence on an Epics 5 FACS (Coulter Electronics, Inc.). In each experiment, appropriate negative controls were used to establish gates for quantitating specific fluorescence in the red and green channels and for simultaneous expression of both red and green fluorescence. These controls included similarly processed cells sequentially incubated with (*a*) biotinylated goat anti-mouse IgG, PE-AV, and directly fluoresceinated P3x63/Ag/8 (double negative control); (*b*) 3A1* alone (green positive, red negative control); and (*c*) DU-HL60-3^B and PE-AV (red positive, green negative control).

Crude Growth Supplements. Media conditioned by human peripheral blood leukocytes (LCM) for 7 d in the presence of 1% (vol/vol) phytohemagglutinin and 10% heat-inactivated fetal calf serum 309 (PHA-LCM) (24), and media conditioned by human peripheral blood T cells (TCM) (obtained by E rosette separation) for 72 h in the presence of 1% PHA (PHA-TCM) (25), were prepared as described.

Results

Establishment and Cloning of the DU.528 Cell Line. Details of the patient's clinical course and phenotypic conversion have been described (11). Leukapheresis was performed at diagnosis, before treatment, when the total white count was >100,000/μl (>90% T lymphoblasts). After density gradient purification

cells were stored in the presence of 12% dimethylsulfoxide in liquid nitrogen. A vial (10^9 cells) was thawed by swirling for 2 min in a 37°C water bath, then diluted fourfold with thawing medium over 10–15 min. The culture was then further diluted in standard medium to 5×10^6 cells/ml. After 2 wk the culture was maintained at $0.8–2 \times 10^6$ cells/ml, and, after 10–12 mo, at densities as low as 2×10^5 cells/ml. After 18 mo in continuous culture, the cells were cloned by limiting dilution in standard medium. 190–200 wells of 96-well microtiter plates were set up at each of several cell densities (1,000 to 10 cells per well). Growth occurred in all wells at 1,000 and 500 cells per well; in 48 of 192 wells at 100 cells per well; in 5 of 191 wells at 50 cells per well; and in no wells at either 25 or 10 cells per well. Each of the five wells that yielded growth at the 50 cells per well density was expanded for further characterization.

Over the first 10 mo of culture in standard medium containing 10% horse serum and 10% heated fetal calf serum, the population doubling time of DU.528 cells gradually diminished from 7–10 d to 48–96 h (Fig. 1). On several occasions over a 2 yr period we have reevaluated the serum requirements of the cell line. Growth was not supported by medium containing 20% fetal calf serum alone; in 20% horse serum the cells grew at 70–80% of the rate in standard medium, for ~3 wk, but then gradually lost viability. Growth was not influenced by either of two commercial medium supplements (GCT, a source of colony-stimulating activity [26], and HSI-LoSM, a supplement designed to support growth of hybridoma clones) or by TP-5, a thymic hormone preparation. PHA-LCM, a

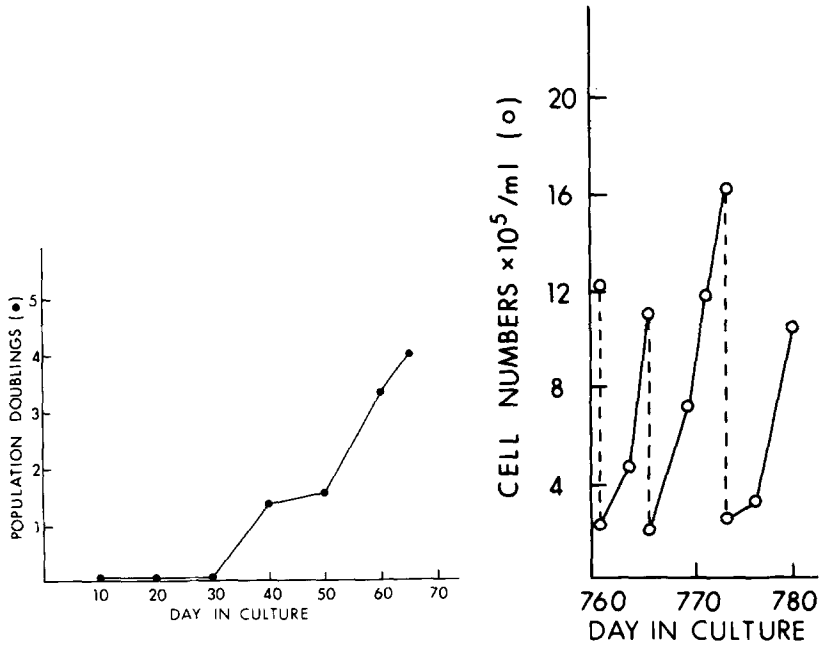


FIGURE 1. Growth curves for DU.528 during initial period in culture (*A*) and after 2 yr in continuous culture (*B*). (*A*) Growth characteristics during the initial period in culture. No increase in cell number was observed during the first month after thawing, after which a population doubling time of 10–14 d was observed. (*B*) Growth of the established cell line after 2 yr in culture, demonstrating a population doubling time of 48–72 h.

source of burst-promoting activity (24), increased the growth rate by ~30%. PHA-TCM, a crude preparation of T cell growth factor (25), was strongly growth inhibitory (>50% inhibition when present as a 5% addition to standard medium) (Fig. 2). The growth characteristics of DU.528 clones are similar to the parent line.

All cells examined from the DU.528 cell line and its clones showed the stemline karyotype: 46,XY,−14,+(1;14)(p33;q11),del(1)(p33),+del(1)(q11),del(13)(q14) (Fig. 3). This is the same karyotype found in the patient's leukemia cells, both at diagnosis and after phenotypic conversion (11) (the $13q^-$ marker was present in freshly obtained leukemia cells from the patient although it was not shown in the original publication). No cells showed a normal karyotype, which was present in the patient's mitogen-stimulated (nonmalignant) peripheral blood lymphocytes (11). In addition to the DU.528 cell line, derived from the patient's pretreatment leukemia cells, we have also established a long-term line from circulating leukemia cells obtained from the patient after phenotypic conversion. The karyotype of this latter cell line is identical to that shown in Fig. 3, and its characteristics are the same as those described below for DU.528.

Spontaneous, Multilineage Differentiation. When first placed in culture DU.528 cells had uniform T lymphoblastoid morphology (large, often cleft, nuclei; prominent nucleoli; scant, basophilic cytoplasm) (Fig. 4*A*). Consistent with this morphology, these cells did not stain for myeloperoxidase or nonspecific esterase (myeloid and monocytoid properties, respectively), but >90% of cells stained weakly for butyryl esterase in a cytoplasmic distribution with a fine, granular pattern characteristic of lymphoid cells. An occasional cell was PAS positive. This initial cell population reacted with the 3A1 monoclonal antibody, a pan T cell marker of undifferentiated T cell precursors in the embryonic thymus (21, 27, 28), but not with a monoclonal antibody (DU-HL60-3) raised against the HL60 promyelocytic cell line, which detects cells of the granulocyte/monocyte series (22) (Fig. 5*A*).

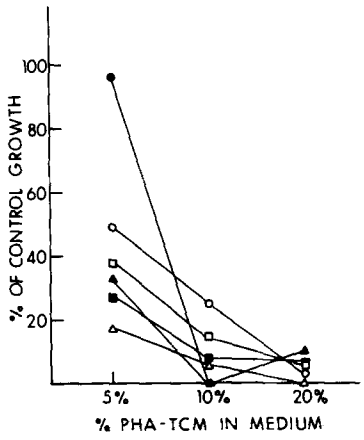


FIGURE 2. Effect of PHA-TCM on the growth of DU.528 cells. DU.528 cells were cultured in standard medium containing the indicated concentrations of PHA-TCM. The results are from one of four identical experiments conducted over 30 mo. A growth-inhibiting effect was observed on all occasions.

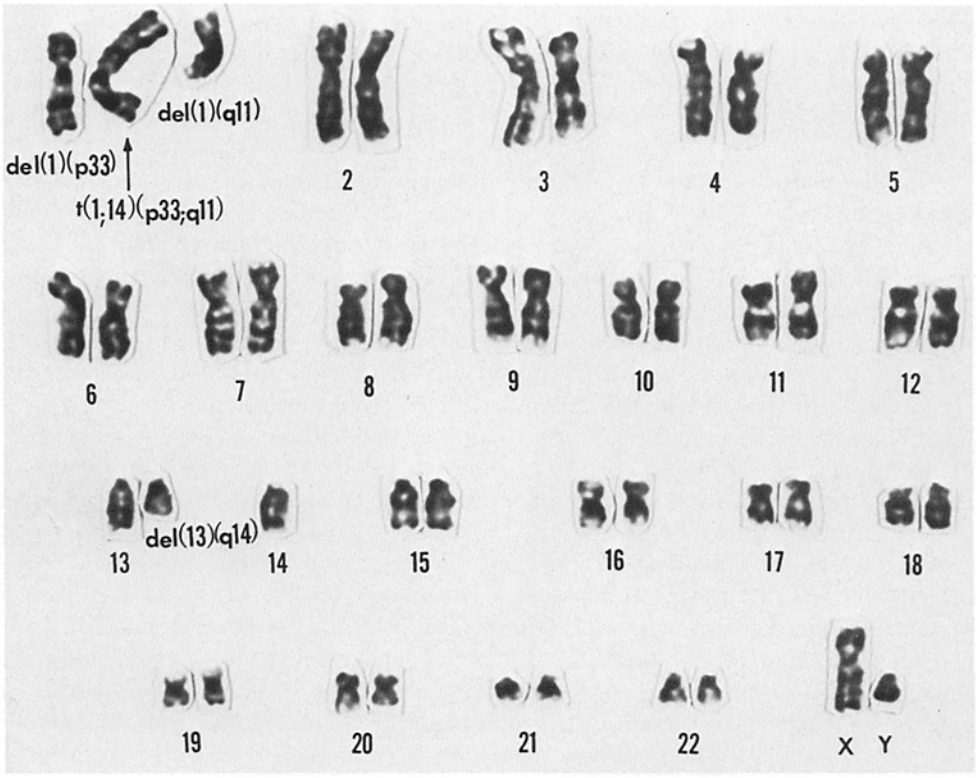
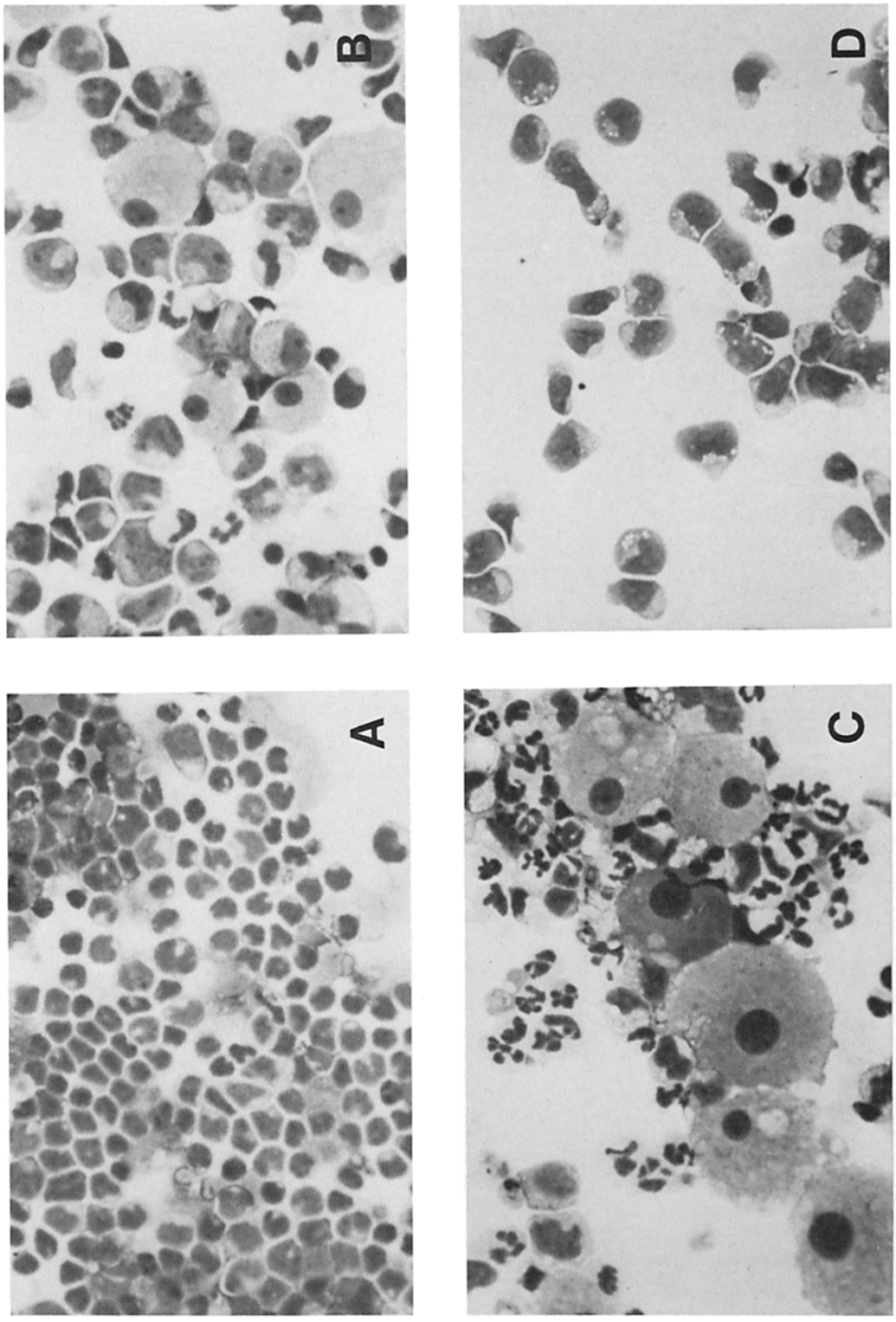


FIGURE 3. Karyotype of DU.528 cell line. The No. 1 chromosomes are replaced by two deleted No. 1 chromosomes and one product of a translocation between No. 1 and No. 14. One No. 13 is replaced by a deleted No. 13 chromosome. There are no other numerical or structural abnormalities. The arrangement shown is from an analysis performed on DU.528 cells that had been in culture continuously for >2 yr. Karyotypes were analyzed for four of the five clones of DU.528. Two clones (2 and 5) showed only the stemline karyotype; 50% of spreads from clone 3 had the stemline karyotype and 50% had the same karyotype with a new i(9q) replacing one copy of chromosome 9. A single spread from clone 4 had the stemline karyotype; all others contained an extra copy of chromosome 17 in addition to all the changes seen in the parent cell line.

During the 3rd wk in culture we noted the presence of many immature cells of indeterminate lineage and some cells with distinct myeloid morphology (Fig. 4*B*). By 2 mo the culture was composed primarily of myeloid cells, including mature segmented neutrophils and large mononuclear cells (Fig. 4*C*). The change in morphology was accompanied by acquisition of the DU-HL60-3 antigen, although most cells continued to express the 3A1 antigen (Fig. 5*A*). Over the next 2 wk terminally differentiated myeloid cells disappeared and were replaced by immature cells with blast morphology, which expressed 3A1 but not DU-HL60-3 (Figs. 4*D* and 5*A*). A second vial of cryopreserved cells from

FIGURE 4. Change in morphology of DU.528 cells during the first 10 wk in culture. Photomicrographs (×470) of Wright's-Giemsa-stained cytospin preparations made on the following days after thawing: day 1, *upper left*; day 30, *upper right*; day 55, *lower left*; day 64, *lower right*.


B
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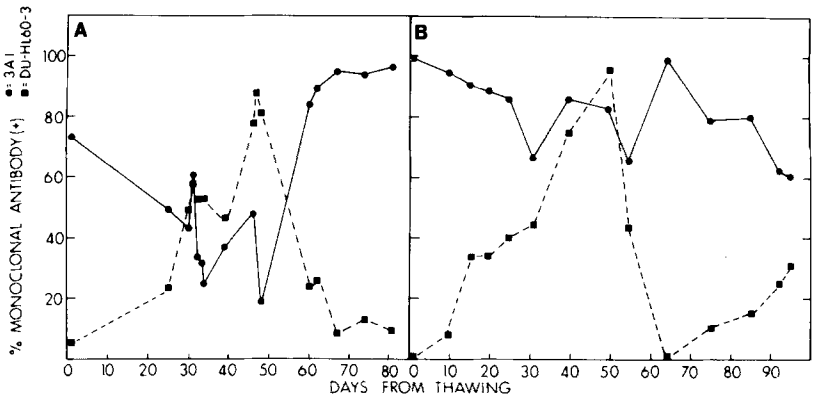

FIGURE 5. Spontaneous change in surface antigen expression on DU.528 cells during initial 10 wk in culture. (*A*) Results from analysis of the culture described in Fig. 4; (*B*) Results from similar analyses of a second culture established independently from cryopreserved cells obtained from the patient at time of diagnosis. On the indicated days after thawing, samples of the cultures were removed for FACS analysis to determine the percentages of cells that expressed the 3A1 (solid lines) and DU-HL60-3 (dashed lines) antigens.

diagnosis was thawed and studied more closely. A similar fluctuation in surface antigenic phenotype (Fig. 5*B*) and morphology (not shown) was observed.

The observed fluctuation in phenotype could have resulted from the presence of two distinct populations of cells, one lymphoid and the other a myeloid progenitor, or it could have resulted from differentiation of cells with an initially T lymphoblastoid phenotype along a myeloid pathway. The latter possibility was suggested by the finding on some occasions that both the 3A1 and DU-HL60-3 antigens were present on >50% of cells (e.g., days 40–50, Fig. 5*B*). Simultaneous expression of the T cell and myeloid markers on individual cells was confirmed by dual parameter immunofluorescence cytofluorographic analysis. In the experiment shown in Fig. 6, performed after 12 wk of culture, 17% of cells expressed both markers. At the time of thawing, <2% of these cells were found to simultaneously express both markers.

We have monitored the morphologic, histochemical, and surface antigenic characteristics of DU.528 cells at weekly or biweekly intervals during the first year in culture and at frequent intervals during the second year (Tables I and II, Fig. 7). The most striking property of the cell line is its heterogeneity. At most times cultures of DU.528 and each of its clones were composed predominantly of undifferentiated, lymphoblastoid cells. However, there were almost always some cells representing both lymphoid and nonlymphoid lineages at many stages of differentiation. At unpredictable intervals the parental DU.528 cell line and its clones underwent waves of spontaneous differentiation similar to the behavior observed during the first 2 mo in culture, although the intervals between these events became longer and the percentages of mature cells decreased. The percentage of cells simultaneously expressing the 3A1 and DU-HL60-3 surface antigens, determined by dual-label immunofluorescence cytofluorographic analysis, fluctuated from <1 to 84% in cultures of DU.528 sampled randomly over the first 2 yr in continuous culture, and has remained <5% during

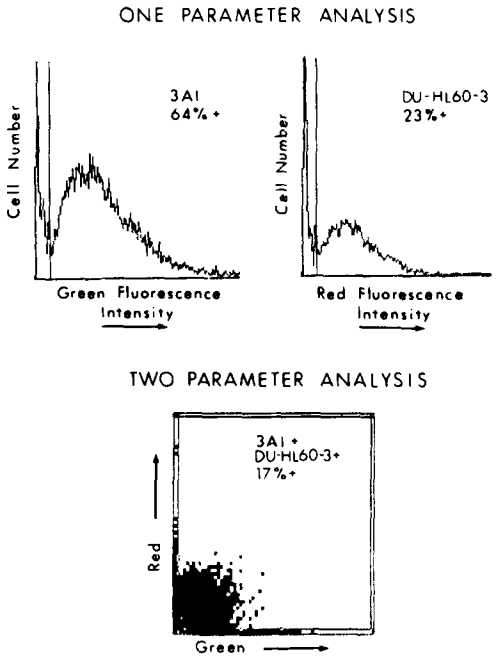

FIGURE 6. Simultaneous expression of T cell and myeloid surface antigens by DU.528 cells. Dual parameter analysis for expression of the 3A1 pan T cell antigen and DU-HL60-3 myeloid antigen was performed as described in Materials and Methods. The analyses shown were performed after 12 wk in culture. 64% of the cells expressed the 3A1 antigen, 23% the DU-HL60-3 antigen, and 17% expressed both markers. Similar analyses performed at various times during 2 yr of continuous culture revealed coexpression of these antigens by from <1 to 84% of cells.

TABLE I

Morphologic Classification of DU.528 Cell Line During Continuous Culture

Days in culture	Blast	Mature lymphocyte	Promyelocyte	Myelocyte and metamyelocyte	Band and segmented neutrophil	Eosinophil	Monocyte and histiocyte	Megakaryocyte	Nucleated erythrocyte
	%	%	%	%	%	%	%	%	%
2	95						5		
18	16	16		7	8	26	16	3	9
23	3	6	2	7	8	30	9	18	7
60	25	8		9	31	3	15	2	7
94	44	5		7	20		17	2	5
122	78	2		5			13		5
764	64	1		5	3	1	21		5
785	23	5	1	15	17	1	26		11
843	90	2	1	1			2		4

Cell classification was determined by differential count of at least 200 Wright's-stained cells, which were prepared from logarithmically growing cultures by cytocentrifugation. The data were chosen to illustrate the diversity present during the initial 3 mo and after >2 yr in continuous culture. Except for cells with a megakaryocytic morphology, which are no longer seen, all other cell types listed in the table continue to be found on some days.

the last half of the 3rd yr. DU.528 cells maintained continuously in culture for >2.5 yr show little evidence of spontaneous nonlymphoid differentiation.

The photomicrographs in Fig. 7, *A–C* were made from a single culture of DU.528 cells that had been in culture continuously for >2 yr. Fig. 7*A* shows a

TABLE II
Surface Antigens Expressed by DU.528

Monoclonal antibody	Specificity	Percent positive (range)	
		Undifferentiated culture	Differentiated culture
3A1	Pan T cell	39–99	48–98
Leu-2b, OKT8	Cytotoxic T cells	<1–6	8–23
Leu-3a, OKT4	Helper T cells	<1–7	8–63
Leu-4	Pan T cell	2–5	NT
Leu-5, OKT11	E rosette receptor	<1–8	10–82
Leu-6, OKT6	Thymocytes	<1–7	1–46
TR, 5E9	Transferrin receptor	26–84	69–84
HLA-DR, IA	B cells, monocytes	<1–6	2–84
MY-10	Hematopoietic progenitors	<1–39	NT
DU-HL60-3	Granulocytes	3–13	5–87
Leu-M2	Monocytes	3–10	3–24
Leu-M3	Monocytes, macrophages	<1–6	2–6
TAC	IL-2 receptor	2–38	43–90
TDT	T cell subset	<1–10	<1–10

Undifferentiated cultures contained a homogeneous population of undifferentiated blasts. Differentiated cultures are those in which >10% of cells had morphologic characteristics of erythroid, myeloid, or monocytoid lineages at various stages of maturity. NT, not tested. Each antibody was tested by FACS analysis on at least four occasions and analyses were performed on >60 occasions over 2 yr.

cluster containing a blast, a dyserythropoietic erythroid precursor, a segmented neutrophil, a lymphocyte, and a monocyte; Fig. 7*B*, several erythroid cells among numerous immature cells of indeterminate lineage; Fig. 7*C*, two neutrophils, a monocyte, and two undifferentiated blasts. Histochemical studies and functional characteristics were consistent with cell morphology. Thus, staining with benzidine demonstrated hemoglobin synthesis by erythroid cells (Fig. 7*D*); promyelocytic cells contained characteristic myeloperoxidase-positive granules (Fig. 7*E*) and monocytic cells stained with nonspecific esterase, which was not inhibited by fluoride (Fig. 7*F*). Typical E rosetting of T lymphocytes was observed (Fig. 7*G*). Spontaneous function of macrophages was strikingly demonstrated in DU.528 cultures by the finding of engulfed erythroid nuclei in histiocytic cells (erythrophagocytosis, a function of bone marrow macrophages in some pathologic conditions) (Fig. 7*H*).

At times when cultures of DU.528 appeared uniformly undifferentiated the predominant marker expressed was 3A1 (Table II, 'Undifferentiated' column). These cells also expressed the transferrin receptor, a lineage-nonspecific marker present on actively replicating cells (27, 29). Up to 40% of undifferentiated cells expressed the MY-10 antigen, which has been reported to occur on a small fraction of bone marrow cells enriched for myeloid-erythroid progenitor cells (19). Terminal deoxynucleotidyltransferase, a marker of T cell precursors in the thymic cortex as well as a minor population of bone marrow lymphoid progenitors (30), was detectable on <10% of DU.528 cells. <8% of these undifferentiated cells expressed the E rosette receptor or the T4 or T8 T cell differentiation

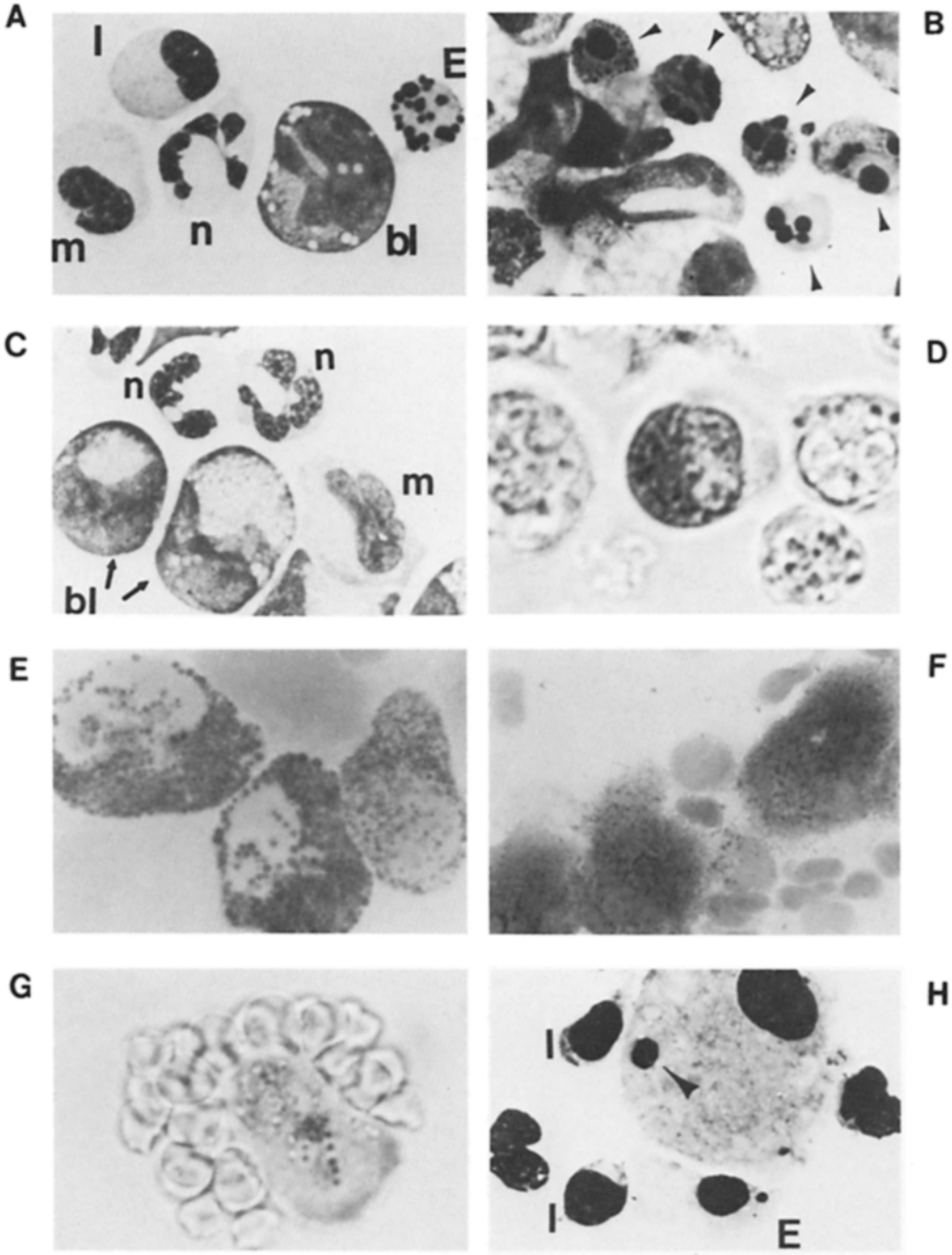

FIGURE 7. Diversity of hematopoietic cell types present in cultures of the DU.528 cell line. (*A–C*) Wright's-Giemsa-stained cytospin preparations (×1,000) of DU.528 clone 1. (*D*) Benzidine-stained DU.528 cell. (*E*) Promyelocyte cells in DU.528 culture stained from myeloperoxidase (granules). (*F*) Sheep erythrocyte–rosetted DU.528 cell. (*G*) Wright's-Giemsa-stained cytospin preparation of DU.528 culture (×1,000) showing a large mononuclear histiocytic cell containing an engulfed erythroid nucleus (arrow, 'erythrophagocytosis'). *bl*, blast; *l*, lymphocyte; *n*, neutrophil; *m*, monocyte; *E*, nucleated erythrocyte.

antigens, but up to 38% expressed the T cell growth factor (IL-2) receptor, as indicated by reactivity with the anti-TAC monoclonal antibody (20).

In cultures with >10% of cells showing myeloid or erythroid morphology (Table II, 'Differentiated' column), we observed a wider range of surface antigen expression. Reactivity with the DU-HL60-3 and Leu-M3 monoclonal antibodies, and expression of HLA-DR antigens was consistent with the presence of monocytes and macrophages. In addition, on many occasions a significant percentage of cells expressed some T cell differentiation antigens, including T6, T4, and T8. Expression of the T3 antigen, which correlates with expression of the T cell antigen receptor (31), was not seen. There was generally a greater frequency of anti-TAC–positive cells in these more differentiated populations than in uniformly undifferentiated cultures. Markers of B cells have not been extensively studied. The B cell differentiation antigens B1, B2, and BA1 (32) were present on 0–10% of DU.528 cells. Further assessment of the possibility of B cell differentiation and function is in progress.

Response to Inducers of Differentiation. The data summarized in Table III show the response of clone DU.528-3 to agents that have frequently been used to induce maturation of myeloid and erythroid leukemia cells and cell lines (phorbols, dibutryl cyclic AMP, 5′-azacytidine, and azadeoxycytidine). Each of these agents had a clear-cut effect, resulting in increases in the percentages of cells that expressed myeloperoxidase and nonspecific esterase (histochemical markers for granulocytes and monocytes, respectively), and in the ability to reduce NBT, a functional assay for mature granulocytes.

In several experiments, during the first 2½ yr in culture, we have asked whether the ADA substrates, adenosine (Ado) and 2′-deoxyadenosine (dAdo), in the presence of the ADA inhibitor deoxycoformycin, can induce undifferentiated DU.528 cells to express markers of myeloid differentiation. In a representative experiment, we monitored effects on the growth of clone DU.528-4 and on its expression of the DU-HL60-3 antigen and a DR class II histocompatibility antigen (Table IV, experiment A). As has been found in studies of a large

TABLE III
Induction of Myeloid Maturation in DU.528

Additions	Range tested	Maximum percent positive cells		
		NBT	MPO	NSE
	M			
None (control)	—	12	1	12
dbcAMP	10^{-6} to 10^{-4}	68	59	58
PMA	1.6×10^{-9} to 1.6×10^{-7}	50	84	70
5-Azadeoxycytidine	10^{-9} to 10^{-4}	5	62	46
5-Azacytidine	5×10^{-8} to 10^{-5}	43	23	49

NBT, reduction of nitroblue tetrazolium; MPO, myeloperoxidase; NSE, nonspecific esterase; dbcAMP, dibutyryl cyclic AMP; PMA, phorbol myristate acetate. Clone DU.528-3 cells, which were morphologically immature (60% 3A1-positive; 10% DU-HL60-3 positive), were incubated for 4 d in the presence of the compounds listed, over the concentration range indicated, and then evaluated histochemically as described in Materials and Methods.

TABLE IV

Effects of ADA Inhibition and ADA Substrates on Differentiation of DU.528

Additions*	Day of treatment	Total cells‡	DU-HL60-3		DR	
			Percent positive	Total cells§	Percent positive	Total cells§
Exp. A. DU528 clone 4						
None	0	1.8	2	0.4	1	0.2
None	6	18.7	8	14.6	3	5.0
Uri_{20}	6	18.4	8	14.0	5	8.6
dCF_5	6	17.1	13	21.9	5	8.9
plus Ado_5	6	15.8	10	15.6	6	10.1
plus Ado_{20}	6	3.5	60	21.3	49	17.4
plus $Ado_{20}Uri_{20}$	6	16.8	9	14.3	6	10.7
plus $dAdo_5$	6	11.5	18	21.1	8	8.8
plus $dAdo_{20}$	6	2.3	75	17.1	79	17.9
plus $dAdo_{20}Ado_5Uri_{20}$	6	5.3	62	32.9	84	44.5
Exp. B. DU528, uncloned						
None	6	14.3	1	1.3	1	1.3
$dCF_5dAdo_{20}Ado_5Uri_{20}$	6	4.2	48	20.2	60	25.0

Undifferentiated cultures (>95% blasts) were incubated in standard medium containing the indicated compounds. On days 0 and 6 cells were counted and the expression of DU-HL60-3 and HLA-DR antigens analyzed by FACS.

* Uri, uridine; dCF, deoxycoformycin; Ado, adenosine; dAdo, deoxyadenosine. Subscripts indicate concentration (μM).

‡ Cells/ml $\times 10^{-5}$.

§ Cells/ml $\times 10^{-4}$.

number of human and murine lymphoblastoid cell lines, deoxycoformycin alone is nontoxic in vitro, but it potentiates the toxicity of Ado and dAdo (33). In the absence of ADA substrates, deoxycoformycin had little effect on either growth or marker expression. In the presence of deoxycoformycin, growth-inhibiting concentrations (20 μM) of both Ado and dAdo induced 60 and 75% of cells, respectively, to express the DU-HL60-3 antigen (vs. <10% in controls), and 49 and 79% to express the DR antigen (vs. <5% in controls). Despite their inhibition of cell growth, Ado and dAdo treatment resulted in increases of 1.4- and 2-fold in the absolute number of cells expressing these markers. Uridine, which bypasses the block in de novo pyrimidine synthesis caused by low concentrations of Ado (33, 34), prevented the effects of 20 μM Ado on both cell growth and new marker expression. Of interest, the combination of 5 μM Ado plus uridine, which had no effect on growth or antigen expression, diminished slightly the growth-inhibiting effect of 20 μM dAdo. Under these conditions the treated cultures contained twofold and fivefold more DU-HL60-3- and DR-positive cells than controls. Even more striking effects were observed with parental DU.528 cells (Table IV, experiment B), which were in a more undifferentiated state at the time of this experiment.

The induction of expression of myeloid markers by Ado and dAdo was observed in 15 separate experiments conducted with DU.528 cells during the first 2½ yr in culture. However, with cells that have been maintained in continuous culture for >2.5 yr, we have not observed response to purines or to

conventional differentiating agents. As noted above, these cells also show little evidence of spontaneous differentiation. It thus appears that on prolonged continuous culture the line has lost the capacity for differentiation, or that we have selected for a proliferating, nondifferentiating population. Early passage cells continue to display the capacity for differentiation.

Discussion

Treatment of leukemias with pharmacologic and physiologic agents that induce differentiation in vitro is currently being considered, with the aim of overcoming the block in terminal maturation (35, 36). In addition to a temporal association between drug administration and change in phenotype, proof of an effect on differentiation, rather than selective cytotoxicity, requires evidence that cells with a new phenotype are derived from the leukemic clone. Demonstration of some direct action of the inducer on the target cell in vivo would also be desirable, as would reproduction of the differentiative response in reconstruction experiments with the target cells in vitro.

In the patient from whom the DU.528 cell line was derived we documented complete T lymphoid-to-myeloid transformation during a 7 d period beginning with the start of deoxycoformycin infusion (11). Over this period the leukemia cell count was stable, elevated in the 70,000–80,000 cells/μl range, kinetics that suggested a precursor-product relationship. We have demonstrated effective ADA inhibition and consequent biochemical abnormalities (dATP and S-adenosylhomocysteine accumulation) in leukemia cells during the period of conversion, and we have shown that pretreatment T lymphoblasts and post–deoxycoformycin treatment promyelocytes had the same abnormal karyotype. The presence of this karyotype in the DU.528 cell line and its clones, as well as in the cell line established from cells obtained after phenotypic conversion, establishes that these cell lines are also derived from the same neoplastic clone. For >2 yr in continuous culture these cells retained the capacity for proliferation (self-renewal) and for generation of progeny of (at least) three lineages: T lymphoid, granulocytic/monocytic, and erythroid. These characteristics provide strong support for our conclusion (11) that leukemia in this patient arose from a pluripotent stem cell capable of lymphoid and myeloid differentiation.

Leukemia cells from patients with acute myeloid leukemias have been found to coexpress various markers of myeloid, erythroid, and lymphoid lineages ('lineage infidelity') (37–40). Though we have not systematically evaluated this phenomenon, some degree of lineage infidelity is suggested by the coexpression of the 3A1 T cell and DU-HL60-3 myeloid antigens on DU.528 cells at some stages of differentiation. It is unclear whether lineage infidelity represents a pathologically disordered pattern of gene expression that results from malignant transformation, or whether transient expression of markers of all lineages is a normal characteristic of pluripotent stem cells. The mature morphology and functional characteristics of differentiated DU.528 cells suggests the operation of well-coordinated, lineage-specific programs of differentiation.

Proliferation and differentiation of DU.528 occur without the addition of factors other than serum. These processes may be truly autonomous or under some form of autocrine regulation. The latter term, in its usual context (41, 42),

refers to the control of tumor cell growth by factors produced by the tumor itself. In the special case of DU.528, cultures of which vary from almost entirely undifferentiated cells to mixed populations displaying a wide range of phenotypes, factors produced by a subpopulation of DU.528 cells of one lineage or stage of maturation may influence the differentiation of another subpopulation of immature cells with differentiative potential. On the other hand, differentiation of DU.528 cells may be a stochastic process, as has been shown to be the case in studies of in vitro differentiation of mouse and human bone marrow (43, 44). Efforts to examine these possibilities are in progress.

The characteristics of multipotential DU.528 cells are unclear, but they presumably have an undifferentiated morphology and express few lineage-specific markers. Recent reports (45, 46) have suggested that the first cells to appear in the embryonic mouse thymus, thought to be stem cells, express the receptor for T cell growth factor (IL-2) but neither the Lyt-2 (cytotoxic phenotype) or L3T4 (helper phenotype) antigens. Proliferation of these cells is IL-2 independent. The antigen expressed most consistently by undifferentiated DU.528 cells is 3A1, which is thought to be the first T cell marker expressed on prothymocytes in the embryonic human thymus (28). In some populations of undifferentiated DU.528 cells we observed cells with the phenotype $3A1^+$, anti-TAC^+ (IL-2 receptor), $T8^-$ (cytotoxic), $T4^-$ (helper). It is intriguing to speculate that these cells may be multipotential stem cells, rather than committed T cell progenitors. Studies in progress are aimed at isolating these cells to better characterize their surface antigens, arrangement of immunoglobulin and T cell receptor genes, their production of and response to proliferation- and differentiation-promoting factors, and their potential for multilineage colony formation.

Finally, we have shown that ADA substrates in the presence of deoxycoformycin induced the expression of markers of myeloid differentiation by T lymphoblastoid DU.528 cells. This finding supports our earlier proposal (11) that in vivo deoxycoformycin-induced lymphoid-to-myeloid conversion may have resulted from the effects of ADA substrates on stem cell differentiation, rather than from selective cytotoxicity. An extension of this view is that the pluripotent stem cell may be a direct target affected by genetic ADA deficiency. The DU.528 cell line may prove to be a useful model for examining the mechanisms by which purines and other factors influence lymphohematopoietic stem cell proliferation and differentiation.

Summary

We have established the DU.528 cell line from the pretreatment leukemia cells of a patient who underwent a T lymphoblastic-to-promyelocytic phenotype conversion during treatment with the adenosine deaminase inhibitor, deoxycoformycin. The cell line and clones obtained from it by limiting dilution have the same karyotype previously found in the patient's pretreatment T lymphoblasts and post–deoxycoformycin treatment promyelocytes. DU.528 cells in continuous culture for >2 yr display a predominant undifferentiated T lymphoblastoid phenotype. These cells spontaneously generate progeny of at least three lineages, T lymphoid, granulocytic/monocytic, and erythroid. The surface marker most consistently expressed by DU.528 cells in the undifferentiated state is the 3A1

antigen, which has been found on prothymocytes in the embryonic thymus. Some undifferentiated DU.528 cells also expressed the IL-2 receptor, but no other T cell differentiation antigens. Exposure of DU.528 cells to a variety of agents induced myeloid maturation; adenosine and deoxyadenosine, in the presence of deoxycoformycin, induced expression of myeloid differentiation antigens. Our results suggest that DU.528 is a lymphohematopoietic stem cell line and support the hypothesis that differentiation of pluripotent stem cells may be altered by genetic deficiency of adenosine deaminase. DU.528 cells may provide a useful model for examining factors that regulate stem cell proliferation and differentiation.

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References

1. Hull, M. T., and J. A. Griep. 1980. Mixed leukemia, lymphatic and myelomonocytic. *Am. J. Clin. Pathol.* 74:473.
2. Schroff, R. W., and K. A. Foon. 1982. Heterogeneity in a lymphoid tumor: coexpression of T and B surface markers. *Blood.* 60:373–380.
3. Perentesis, J., N. K. C. Ramsay, R. Brunning, J. H. Kersey, and A. H. Filipovich. 1980. Biphenotypic leukemia: Immunologic and morphologic evidence for a common lymphoid-myeloid progenitor in humans. *J. Pediatr.* 102:63.
4. Pui, C.-H., G. V. Dahl, S. Melvin, D. L. Williams, S. Peiper, J. Mirro, S. Murphy, and S. Stass. 1984. Acute leukemia with mixed lymphoid and myeloid phenotype. *Br. J. Haematol.* 56:121.
5. Paietta, E., J. P. Dutcher, and P. H. Wiernik. 1985. Terminal transferase positive acute promyelocytic leukemia: in vitro differentiation of a T-lymphocytic/promyelocytic hybrid phenotype. *Blood.* 65:107.
6. Nowell, P. C., and D. A. Hungerford. 1960. A minute chromosome in human chronic granulocytic leukemia. *Science (Wash. DC).* 132:1497.
7. Fialkow, P. J., R. J. Jacobson, and T. Papayannopoulou. 1977. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am. J. Med.* 63:125.
8. Fialkow, P. J., A. M. Denman, R. J. Jacobson, and M. W. Lowenthal. 1978. Chronic myelocytic leukemia. Origin of some lymphocytes from leukemic stem cells. *J. Clin. Invest.* 62:815.
9. Fauser, A. A., L. Kanz, K. J. Bross, and G. W. Lohr. 1985. T cells and probably B cells arise from the malignant clone in chronic myelogenous leukemia. *J. Clin. Invest.* 75:1080.
10. Murphy, S. B., S. Stass, D. Kalwinsky, and G. Rivera. 1983. Phenotypic conversion of acute leukemia from T-lymphoblastic to myeloblastic induced by therapy with 2′-deoxycoformycin. *Br. J. Haematol.* 55:285.
11. Hershfield, M. S., J. Kurtzberg, E. Harden, J. O. Moore, J. Whang-Peng, and B. F. Haynes. 1984. Conversion of a stem cell leukemia from a T-lymphoid to a myeloid phenotype induced by the adenosine deaminase inhibitor 2′-deoxycoformycin. *Proc. Natl. Acad. Sci. USA.* 81:253.

12. Thompson, S. W., and R. D. Hunt. 1966. Selected Histochemical and Histopathological Methods. Charles C Thomas Publisher, Springfield IL. 757–762.
13. Hanker, J. S., P. E. Yates, C. B. Metz, and A. Rustioni. 1977. A new specific, sensitive and non-carcinogenic reagent for the demonstration horseradish peroxidase. *Histochemical J.* 9:789.
14. Karpas, A., G. J. Hayhoe, J. S. Greenberger, C. R. Barker, J. C. Cawley, R. M. Lowenthal, and W. C. Moloney. 1977. The establishment and cytological, cytochemical and immunological characterization of human haemic cell lines: evidence for heterogeneity. *Leuk. Res.* 1:35.
15. Segal, A. W. 1974. Nitroblue-tetrazolium tests. Lancet. i:1248.
16. Garrett, C., and N. M. Kredich. 1981. Induction of hemoglobin synthesis by xylosyladenine in murine erythroleukemia cells. *J. Biol. Chem.* 256:12705.
17. Schiff, R. I., R. H. Buckley, R. B. Gilbertsen, and R. S. Metzgar. 1974. Membrane receptors and in vitro lymphocyte responsiveness in immunodeficiency. *J. Immunol.* 112:376.
18. Seabright, M. 1971. A rapid banding technique for human chromosomes. *Lancet.* ii:971.
19. Civin, C. I., L. C. Strauss, C. Brovall, M. J. Fackles, J. F. Schwartz, and J. H. Shapes. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J. Immunol.* 139:1.
20. Uchiyama, T., S. Broder, and T. A. Waldman. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature T cells. *J. Immunol.* 126:1393.
21. Haynes, B. F., G. S. Eisenbarth, and A. S. Fauci. 1979. Human lymphocyte antigens: production of a monoclonal antibody that defines functional thymus-derived lymphocyte subsets. *Proc. Natl. Acad. Sci. USA.* 76:5829.
22. McKolanis, J. R., M. J. Borowitz, F. L. Tuck, and R. S. Metzgar. 1984. Membrane antigens of human myeloid cells defined by monoclonal antibodies. *In* Leukocyte Typing. A. Bernard, L. Boumsell, J. Dausset, C. Milstein, and S. F. Schlossman, editors. Springer-Verlag, Heidelberg, Federal Republic of Germany. 387–394.
23. McFarland, E. J., R. M. Scearce, and B. F. Haynes. 1984. The human thymic microenvironment: cortical thymic epithelium is an antigenically distinct region of the thymic microenvironment. *J. Immunol.* 133:1241.
24. Aye, M. T., Y. Niho, J. E. Till, and E. A. McCulloch. 1974. Studies of leukemic cell populations in culture. *Blood.* 44:205.
25. Messner, H. A., C. A. Izaguirre, and N. Jamal. 1981. Identification of T lymphocytes in human mixed hematopoietic colonies. *Blood.* 58:402.
26. Abboud, C. N., J. F. DiPersio, J. K. Brennan, and M. A. Lichtman. 1980. Erythropoietic enhancing activity (EEA) secreted by the human cell line, GCT. *J. Supramol. Struct.* 13:199.
27. Haynes, B. F. 1981. Human T lymphocyte antigens as defined by monoclonal antibodies. *Immunol. Rev.* 57:127.
28. Harden, E. A., and B. F. Haynes. 1985. Phenotypic and functional characterization of human malignant T cells. *Sem. Hematol.* 22:13.
29. Huebers, H. A., and C. A. Finch. 1984. Transferrin: physiologic behavior and clinical implications. *Blood.* 64:763.
30. Bollum, F. J. 1979. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. *Blood.* 54:1203.
31. Romain, P. L., and S. F. Schlossman. 1984. Human T lymphocyte subsets. Functional heterogeneity and surface recognition structures. *J. Clin. Invest.* 74:1559.
32. Anderson, K. C., M. P. Bates, B. L. Slaughenhoupt, G. S. Pinkus, S. F. Schlossman,

and L. M. Nadler. 1984. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood.* 63:1424.

33. Kredich, N. M., and M. S. Hershfield. 1983. Immunodeficiency diseases associated with adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. *In* The Metabolic Basis of Inherited Disease. Fifth ed. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. Goldstein, and M. Brown, editors. McGraw-Hill Book Co., New York. 1157–1201.
34. Green, H., and T.-S Chan. 1973. Pyrimidine starvation induced by adenosine in fibroblasts and lymphoid cells: role of adenosine deaminase. *Science (Wash. DC).* 182:836.
35. Sachs, L. 1978. Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. *Nature (Lond.).* 274:535–539.
36. Koeffler, H. P. 1983. Induction of differentiation of human acute myelogenous leukemia cells: therapeutic implications. *Blood.* 62:709.
37. Marie, J. P., C. A. Izaguirre, C. I. Civin, J. Mirror, and E. A. McCulloch. 1981. The presence within single K562 cells of erythropoietic and granulopoetic differentiation markers. *Blood.* 58:708.
38. Smith, L. J., J. E. Curtis, H. A. Messner, J. S. Senn, H. Furthmayr, and E. A. McCulloch. 1983. Lineage infidelity in acute leukemia. *Blood.* 61:1138.
39. McCulloch, E. A. 1983. Stem cells in normal and leukemic hemopoiesis. *Blood.* 62:1.
40. Palumbo, A., J. Minowada, J. Erikson, C. M. Croce, and G. Rovera. 1984. Lineage infidelity of a human myelogenous leukemia cell line. *Blood.* 64:1059.
41. Sporn, M. B., and G. J. Todaro. 1980. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* 303:878.
42. Sporn, M. B., and A. B. Roberts. 1985. Autocrine growth factors and cancer. *Nature (Lond.).* 313:745.
43. Till, J. E., E. A. McCulloch, and L. Siminovitch. 1964. A stochastic model of stem cell proliferation, based on the growth of spleen forming cells. *Proc. Natl. Acad. Sci. USA.* 51:29.
44. Leary, A. G., M. Ogawa, L. C. Strauss, and C. I. Civin. 1984. Single cell origin of multilineage colonies in culture. Evidence that differentiation of multipotent progenitors and restriction of proliferative potential of monopotent progenitors are stochastic processes. *J. Clin. Invest.* 74:2193.
45. Ceredig, R., J. W. Lowenthal, M. Nabholz, and H. R. McDonald. 1985. *Nature (Lond.).* 314:98.
46. Raulet, D. H. 1985. Expression and function of interleukin-2 receptors on immature thymoctyes. *Nature (Lond.).* 314:101.