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Profile of cell cycle in hemopoietic malignancy by DNA/RNA quantitation using 7AAD/PY

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Key words**:** cell line, cell kinetics, DNA/RNA quantitation, 7AAD/PY, Flow cytometry.

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Abstract We analyzed the surface phenotypes and cell cycle of twenty two hemopoietic cell lines using 7AAD/PY based on the cellular DNA/RNA content. Populations of G1a, G1b, S, and G2+M, DNA index (DI), and the RNA index of S phase (SRI) were calculated by means of DNA/RNA dot plots. Two new parameters were extracted from the cell cycle profiles, namely, the nucleic acid index of the S phase (NI) and the coefficient of variations in the RNA at S phase (SCV). DNA/RNA dot plots of cell lines revealed four characteristic profiles of the cell cycle defined with the calculated NI and SCV. These were Type 0 (small NI, large SCV), Type I (small NI, small SCV), Type II (large NI, small SCV) and Type III (large NI, large SCV). Type 0 included four stem cell lines: a t(1;19) leukemia, two Ph^1 positive ALL and a biphenotypic crisis of CGL. Type I included five ALL cell lines: three T-ALL and two common B-ALL. Type II contained ten myeloid cell lines: five AML and five myeloid crisis of CGL and Type III consisted of three relatively immature lymphoma cell lines: two Burkitt's lymphoma and a follicular center lymphoma cell line. Calculated (NI / SCV(%)) were as follows: Type 0 2.27 ± 0.19 / 16.7 ± 3.7; Type I 2.20 ± 0.30 / 11.1 ± 0.7; Type II 3.64 ± 0.52 / 11.8 ± 1.0 and Type III 3.60 ± 0.53 / 17.5 ± 1.9. Cell cycle analysis of blasts using 7AAD/PY combined with surface phenotyping may yield important and rapid information for the classification of hematopoietic malignancy within 2 hours of patient admission.
Key words: cell line**,** cell kinetics, DNA/RNA quantitation, 7AAD/PY, Flow cytometry

Introduction

Diagnosis and a therapeutic strategy for treating acute leukemia and other hemopoietic malignancies should be decided as soon as possible after patients are admitted. FAB classification of acute leukemia [1] is commonly used for this using Giemsa stain morphology, cytochemical detection of peroxidase and esterase activity in situ, and flow cytometric surface phenotyping [2]. The increasingly sophisticated techniques to identify hemopoietic lineages of leukemic cells, paradoxically, have involved clinical hematologists in a complex feature called "lineage infidelity" or "lineage promiscuity", where several acute leukemias have multilineage characteristics [3, 4, 5, 6]. Chromosome analysis and molecular biology [7, 8] are also useful for the correction and accuracy of the diagnosis of acute leukemia, and usually require more than two days after the initiation of chemotherapy. A correct diagnosis should be confirmed within hours.

Detailed cell cycle analysis using AO (acridine orange) was established in 1980 by Darzynkiewicz [9, 10] based on cellular DNA/RNA quantitation, which allowed the growth status of cells and the mechanism of bioactivity of growth factors to be more accurately understood. For example, growth factors have been classified as competence factors which force G_0 population to enter G_{1A} phase, progression factors (G_{1A} to G_{1B}) and differentiation factors (G_{1A} to G_{1D}) based on their effects on the cell cycle. For example, mitogens are competence factors and

interleukin-2 is a progression factor for lymphocytes [11, 12]. The ability to correlate the effect of a growth factor on the cell cycle will help elucidate the complex biological events induced by single or multiple growth factors. However, AO has major problems. The AO procedure requires harsh treatment of cells with detergent, and the emission spectrum of AO is sufficiently broad so that cells cannot be simultaneously stained with another fluorochrome for surface phenotyping. AO can only be applied to homogeneous samples such as cell lines and high percentage leukemias. Another major problem of AO arises from the fact that these procedures are based on the equilibrium of AO molecules with DNA versus RNA [13]. This problem results in technical difficulty associated with applying this technique for many investigators.

We established a new cell cycle method, 7AAD/PY, which enables simultaneous analysis of the surface phenotype and DNA/RNA quantitation [14]. This technique also preserves cell morphology. Here, we describe the analysis and calculation protocols using this methodology simulating cell lines established from hemopoietic malignancy as a model for the diagnosis and classification of acute leukemias and lymphomas.

Materials and methods

DNA/RNA staining reagents and monoclonal antibodies.

7-amino-actinomycin-D (7AAD) [15, 16] was purchased from Sigma Chemical Company, St. Louis, MO. Pyronin Y (PY) [17] was purchased from Polysciences, Warrington, PA. Each DNA/RNA dye was dissolved in PBS at 20 times the final concentration, and 50 μL of dye was added to 1 x 10^6 cells in 1 mL.

FITC-conjugated anti-Leu-3a (CD4), Leu-4 (CD3), Leu-5b (CD2), CALLA (CD10), Leu-9 (CD7), Leu-M1 (CD15), control IgG1 and PE-conjugated anti-Leu2a (CD8), Leu-12 (CD19), HLA-DR, Leu-16 (CD20), HPCA-2 (CD34), Leu-M9 (CD33), IL-2R (CD25), Leu-14 (CD22), Leu-19 (CD56), Leu-M3 (CD14), and control IgG1 were purchased from Becton Dickinson Immunocytometry Systems, Mountain View, CA. FITC-conjugated anti-IOB6 (CD38), IOP36 (CD36), P2 (CD41) and PE conjugated anti-Glycophorin A were purchased from Immunotech, Westbrook, ME. FITC-conjugated anti-OKT6 (CD1) and PE-conjugated anti-OKB7 (CD21) were purchased from Ortho Diagnostic Systems, Raritan, NJ. FITC-conjugated anti-MCS-2 (CD13) and Mikβ1 (CD122) were purchased from Nichirei, Tsukiji, Tokyo, Japan.

Cells and culture conditions.

Cell lines were mostly provided by the Japanese Cancer Research Resources Bank (JCRB: Tsukiji, Tokyo, Japan). Some cell lines were originally established in our laboratory: WK93, WH94, HT93 and TK91 by Dr. K. Kishi, OH94, SN-Ow1 and Oto by Dr. S. Maruyama, C2F8 and B4D6 by Dr. T. Furukawa, and YS-1 by Dr. K. Nagai. ALL/MIK [21], MEG-O1 [25], CMK11-5 [26], and MR-87 [29] were donated by Dr. M. Okabe, Dr. M. Ogura, Dr. T. Sato, and Dr. J. Okamura, respectively (Table 1). Cells were maintained in RPMI-1640 (Gibco Laboratories, Grand Island, NY) medium containing 100 U/mL penicillin G sodium (PCG; Biowhittaker, Walkersville, MD), 100 μg/mL streptomycin sulfate (SM; Biowhittaker), and 10% heat-inactivated fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan) at 37^{O}C in 5% CO_2 in humidified air. Exponentially growing cells were harvested for cell cycle analysis.

Heparinized human peripheral blood was obtained from healthy donors. Peripheral blood lymphocytes (PBL) were isolated by Lymphoprep (1.077, Nycomed Pharma AS, Oslo, Norway) density centrifugation. Light density mononuclear cells were washed with medium and analyzed for cell cycle as a standard with which to calculate DI and SRI of cell lines.

7AAD/PY method

Up to 1 x 10^6 cells were suspended in 1 ml of nucleic acid staining solution (NASS; 0.15 M NaCl in 0.1 M phosphate-citrate buffer supplemented with 5 mM sodium EDTA (Sigma) and 0.5% bovine serum albumin (Sigma; BSA, fraction V), pH 6.0) containing 0.004% saponin (from quillaja bark, Sigma), 50 μL of a 400 μM 7AAD solution (final 20 μM) was added and the cells were incubated at RT for 30 min. After incubation with the DNA dye, cells were cooled on ice for at least 5 min, then 50 μL of a 100 μM PY solution (final 5 μM) was added. The cell suspension was incubated for an additional 10 min on ice and analyzed.

Flow cytometry

Cell fluorescence was analyzed with a FACScan™ Flow Cytometer (Becton-Dickinson, Mountain View, CA). The cells were excited with a single, 488 nm argon laser, analyzed for simultaneous green (FL1; 525 nm), orange (FL2; 570 nm), and red (FL3; >650 nm) fluorescence emission, and 10,000 events were collected. The data were analyzed on Lysis II™ and CellFit™ software (Becton-Dickinson). To quantify DNA content and RNA content of cells using 7AAD/PY, the DNA index (DI) and the RNA index (RI) were calculated using histograms of DNA and RNA as follows :

DI = (peak channel number of 2N cells on a DNA histogram) / (peak channel number of resting lymphocytes on the same DNA histogram)

RI = (mean channel number of a subset of cells on a RNA histogram after DNA gate) / (mean channel number of resting lymphocytes on a RNA histogram)

RI should correlate with DI in hyper diploid cell lines. To neutralize the influence of DI to ascertain the correlation between RI and cell lineage, the nucleic acid index of S phase (NI) was calculated as follows :

NI = (RI of S phase) / DI

To demonstrate the range of the RNA quantity, the coefficient of variation of RNA quantity in S phase (SCV) was also calculated as follows :

SCV(%) = 100 x (standard deviation of RNA channel number in S phase) / (mean RNA channel number in S phase)

Results

Surface marker phenotyping

Twenty two exponentially growing cell lines were harvested and analyzed for two-color surface phenotyping using FITC and PE-conjugated control mouse IgG1 and monoclonal antibodies against twenty four lineage markers, CD1, CD2, CD3, CD4, CD7, CD8, CD10, CD19, CD20, CD21, CD22, CD25, CD13, CD14, CD15, CD33, CD34, CD36, CD38, CD41, CD56, CD122, HLA-DR, and glycophorin A (GPA). The phenotypes of the twenty two cell lines are summarized in Table 1. Cell lines discussed in this article included three T-ALL (Molt3, Molt4, CCRF-CEM), two common B-ALL (WK93, OH94), two Ph^1+ ALL (ALL/MIK, SN-Ow1), one t(1;19) ALL (WH94), two AML (KG-1, HT93), one Ph^1+ AML (MR-87), one erythro leukemia (HEL), one megakaryoblastic leukemia (CMK11-5), one biphenotypic crisis of CGL (YS-1), five myeloid crisis of CGL (C2F8, B4D6, TK91, Meg-O1, K562), two Burkitt's lymphoma (Raji, Daudi), and a follicular center lymphoma cell line (Oto).

Cell cycle analysis using 7AAD/PY

Cells were stained serially with 7AAD and PY, then the cell cycle was analyzed. Normal human peripheral blood lymphocytes were also stained with 7AAD/PY to calculate the DNA index (DI), the RNA index in S phase (SRI), the nucleic acid index of S phase (NI), and the coefficient of variation of RNA quantity in S phase (SCV) as shown in Figure 1. In brief, G1, S and G2M populations were calculated using a DNA histogram and RFIT™ program on CellFit™ software (Becton-Dickinson). G1 peak channel numbers of PBL (X) and a cell line (Y) were measured on DNA histograms (panels C and D) using RFIT™ software. DNA index (DI) = Y / X. RNA histograms of PBL (panel E), the cell line without an analytical gate (panel F), and after a gate set at S phase using the DNA histogram of panel D on CellFit™ software (panel G) are also analyzed. The G1a population, defined by Darzynkiewicz [9] as a subpopulation of G1 which has the RNA amount less than the minimal RNA quantity of S-phase fraction, was measured using an RNA histogram and manual set statistical analysis program on CellFit™ software referring to DNA/RNA dot plots to search for the minimal RNA quantity of S-phase cells, i.e., rising point of S fraction on RNA/DNA dot plot, and G1b = G1 - G1a (panel F). The nucleic acid index in S phase (NI) was calculated to eliminate the influence of DI to SRI, and SCV was calculated to measure the width of the RNA distribution in S phase as described in Materials and methods and Figure 1. Mean RNA channel numbers of PBL on panel E (U) and S phase of the cell line on panel G (V) and coefficient of variations of RNA channel numbers of the S phase on panel G were calculated using manual set statistical analysis program for cell cycle calculation on CellFit™ software. RNA index of S phase (SRI) = V / U. Nucleic acid index (NI) = SRI / DI.

The NI measurements suggested a small (around 2.2) and a large NI group (around 3.6) as shown in Table 2. The SCV measurements also suggested a small (around 11%) and a large SCV subset (around 17%). Therefore, four types were defined in terms of cell growth: Type 0 (small NI, large SCV), Type I (small NI, small SCV), Type

II (large NI, small SCV), and Type III (large NI, large SCV). Type 0 included stem cell leukemias (t(1;19) leukemia, Ph^1+ ALL and biphenotypic crisis of CGL). Type I included uniphenotypic T- or B- ALL. Type II included AML and myeloid crisis of CGL, whereas relatively immature lymphoma (Burkitt's lymphoma and a follicular center lymphoma) were classified into Type III. Typical cell growth patterns of cell lines are shown in Figure 2, and the distribution of NI and SCV(%) of the four types are summarized in Figures 3 and 4. As shown in Figure 2, typical Type 0 cell line has relatively short range of RNA on G1a population, usually the same as that of G1b, which indicates Type 0 cell line synthesizes relatively small amount of RNA before S-phase entrance. Meanwhile, the type has very wide range of RNA on S. Type I ALL cell line has relatively narrow range of RNA quantity on G1a as well. In contrast, this type has narrow range of RNA on S fraction. Type II AML cell line has narrow range of RNA on S as well. On the other hand, this type has very long G1a line on RNA/DNA dot plot. This fact indicates Type II AML synthesizes relatively large amount of RNA before beginning DNA synthesis. Type III lymphoma also synthesizes large amount of RNA on G1, and the RNA range on S-phase is very wide and, as a result, the shape of Type III cell line on RNA/DNA dot plot looks very similar to Type 0. The Type 0 cell lines and Type III lines are clearly distinguishable by means of measured NI as shown in Figures 3 and 4. The NI and SCV of four types of cell lines were as follows: Type 0, NI = 2.27 $\pm$ 0.19 and SCV = 16.7 $\pm$ 3.7 % (n=4); Type I, NI = 2.20 $\pm$ 0.30 and SCV = 11.1 $\pm$ 0.7 % (n=5); Type II, NI = 3.64 $\pm$ 0.52 and SCV = 11.8 $\pm$ 1.0 % (n=10) and Type III, NI = 3.60 $\pm$ 0.53 and SCV = 17.5 $\pm$ 1.9 % (n=3).

Discussion

We established a new cell cycle method, 7AAD/PY, which allows the simultaneous analysis of the surface phenotype and DNA/RNA quantitation [14]. Here, we also demonstrated the cell cycle progression of subsets of activated lymphocytes. CD4+ and CD20+ cells entered early G1 (G1T) on day 1, progressed into late G1 (G1B), S and G2+M on day 2, and appeared to be waiting to re-enter into G1B on days 3 and 4. In contrast, almost half of the CD8+ cells quickly entered G1B on day 1, and progressed through the cell cycle until day 4. A portion of the CD8+ cells did not enter the cell cycle by day 4. Activation markers were expressed in accordance with the cell cycle. IL2R was expressed in early G1 (G1T), HLA-DR was expressed at the very beginning of G1 without a detectable increase in the RNA quantity, and TfR was expressed in late G1 (G1B) just before the initiation of DNA synthesis.

We performed preliminary studies to apply 7AAD/PY to studies of de novo acute leukemias and lymphomas, and cell lines. The cell kinetics of de novo acute lymphocytic leukemia showed Type I growth; the G1 population entered S after relatively low grade RNA synthesis. AML showed Type II growth; the G1 population entered S after relatively high grade RNA synthesis. Type I and Type II growth showed a relatively narrow range of RNA quantity in S phase. In contrast, de novo stem cell leukemia such as Ph^1+ ALL and t(1;19) leukemia showed Type O growth which had wide range of RNA (data not shown). We started screening the cell cycle of leukemia cell lines using 7AAD/PY to simulate the calculation protocols to demonstrate the characteristic cell kinetics of Type I, Type II and Type O. The analysis of hemopoietic cell lines in this paper revealed another profile of cell growth named Type III, which has large NI and large SCV that included two Burkitt's lymphoma and a follicular center lymphoma cell line which may be summarized as relatively immature CD10+ lymphoma cell lines. The calculated NIs and SCVs(%) were 2.27 $\pm$ 0.19 and 16.7 $\pm$ 3.7 in four Type 0 cell lines, 2.20 $\pm$ 0.30 and 11.1 $\pm$ 0.7 in five Type I cell lines, 3.64 $\pm$ 0.52 and 11.8 $\pm$ 1.0 in ten Type II cell lines and 3.60 $\pm$ 0.53 and 17.5 $\pm$ 1.9 in three Type III cell lines, respectively. The 7AAD/PY staining and this calculation program may be able to diagnose acute leukemia within 2 hours of admission, more accurately when combined with surface phenotyping and conventional staining cytology in situ.

AO, another cell cycle analysis method based on DNA/RNA quantitation, is also useful for the differential diagnosis of acute leukemia [32, 33, 34], and the data presented here are compatible with their results. However, AO has several major problems and difficulties as discussed in the Introduction, so the method has not been widely applied by hematologists and researchers. Our method, 7AAD/PY, has solved most of the problems associated with AO. The 7AAD/PY method has the advantages of detailed cell cycle

analysis based on cellular RNA/DNA combined with surface phenotyping, a simple and reproducible procedure, preservation of light scattering properties, and a low coefficient of variation of the G1 peak. This method is also applicable for simultaneous three color analysis of two surface phenotypes and DNA quantitation using FITC- and PE-conjugated monoclonal antibodies and 7AAD [35].

Andreeff et. al. [32] and Maddox et. al. [34] showed that the RNA index of AML (20.6 ± 4.0) is significantly higher than that of ALL (11.2 ± 2.0). In these articles, they defined RNA index as following; RNA index = (mean RNA content of G0+G1 cells of sample) x 10, divided by the mean RNA content of normal control lymphocytes. This calculation protocol may overestimate the RNA index expected when the clone has the hyperdiploid DNA quantity. For example, the RNA index of Molt3 calculated with this protocol is about twice as much as that of WK93, because the DI of Molt3 is almost twice of that of WK93. So that, the ratio of the RNA index to DI may show the lineage of the clone more accurately than RNA index itself. Another issue is that the mean RNA level of G0+G1 is influenced by the ratio of populations of G0 and G1, which is another parameter of cell cycle. Then we have chosen NI, instead of RI, to show the lineage of clone more accurately. We also have chosen the coefficient of variation, instead of standard deviation, of RNA in S phase to eliminate the influences of mean RNA amount and the setting of flow cytometry instruments.

The reason why type 0 stem cell lines and type III immature lymphoma lines have wider distribution of RNA in S phase than type I ALL lines and type II AML lines, which was intensively studied by Andreeff and Maddox using AO, is not known. Type 0 stem cells have narrow range of RNA quantity in G1a phase, in contrast, wide spectrum of RNA synthesis potentiality on G1 phase, hence high SCV, which may indicates potency to decrease or increase RNA synthesis in G1 when the cells committed to lymphoid or myeloid lineage, respectively. Hence, it is suggested that the widely distributed S phase RNA in stem cell lines tends to shift to type I or type II when the clone is committed. In contrast, lineage committed Type I and Type II cell lines, in other words, homogeneously potentiated RNA synthesis in each type, may have narrow range of RNA quantity in S fraction and very characteristic profiles on RNA/DNA dot plot. On the other hand, Type III lymphomas are relatively immature CD10 positive lymphoid cells, intermediate lymphoma between common ALL and mature CD10 negative lymphoma. In our observations, CD10 negative mature B lymphoma cells harvested from patients with non Hodgkin's lymphoma showed type II growth (unpublished data). Moreover, some of discrete subpopulations of PHA-activated peripheral blood lymphocytes showed Type II growth [14]. Hence, the high SCV of Type III lymphomas may, again, indicates the potency to shift to more differentiated Type II lymphomas. In other words, the wide range of RNA of CD10 positive lymphoma may indicate that the clone belongs to the intermediate stage of lymphoid differentiation between CD10 positive lymphoblast and mature B-cell (Figure 5).

Cell cycle analysis is also important for predicting the effectiveness of chemotherapy [36, 37] and the prognosis of disease in a clinical setting, especially for lymphoid malignancy [38, 39, 40], as well as to analyze in the vitro effects of biological effectors such as chemotherapy drugs, growth factors and other biological modifying factors [11, 12, 41, 42, 43]. Cell cycle analysis of blasts using 7AAD/PY may yield other important information about classification, chemotherapy effectiveness and the prognosis of acute leukemias.

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Table 1. Review of 22 cell lines.

Diagnosis (a)	Cell line	Positive phenotypes (CD number) (b)	Lineage (c)	Reference (d)
T-ALL	Molt3	1/7/38	T	[18]
	Molt4	1/2/7/38/34	T	[19]
	CCRF-CEM	4/7/8/38	T	[20]
cALL	WK93	7/10/19/20/22/DR/38/	B	NP
	OH94	10/19/20/21/22/25/DR/38	B	NP
Ph1+ ALL	ALL/MIK	10/19/20/22/DR/38/34	B	[21]
	SN-Ow1	10/19/20/21/22/DR/38/13/15/33	BM	NP
t(1;19)	WH94	10/19/22/DR/38	B	NP
AML	KG-1	25/DR/38/34/13/33	M	[22]
	HT93	34/33/56	M	[31]
	CMK11-5	4/7/13/33/36/41	Meg	[26]
	MR-87	2/4/7/DR/33/56	M	[29]
	HEL	13/33/36/41	E	[23]
CGL BC	YS-1	10/19/20/22/DR/38/13	BM	NP
	C2F8	4/25/DR/33/36	EMeg	[24]
	B4D6	21/33/36	EMeg	[24]
	TK91	34/13/33	Meg	NP
	MEG-O1	21/13/33/36/41	Meg	[25]
	K562	20/15/33/41/122	EMeg	[30]
Burkitt	Raji	10/19/20/21/22/DR/38	B	[27]
	Daudi	10/19/20/21/22/DR/38/33	B	[28]
FCL	Oto	10/19/20/22/DR	B	NP

(a) Clinical diagnosis of the patients from whom the cell lines were established. cALL, common B-ALL; t(1;19), biphenotypic t(1;19) leukemia; Ph1+AML, Ph1 positive bcr negative AML; EL, erythro-leukemia; CGL BC, chronic granulocytic leukemia in blastic crisis; Burkitt, Burkitt's lymphoma; FCL, follicular center lymphoma. (b) Surface-phenotypically positive CD numbers of each cell line are shown. Glycophorin A was negative on every cell lines. DR, HLA-DR. (c) Lineages of cell lines. T, T-cell; B, B-cell; BM, biphenotypic B-cell and myeloid; M, myeloid; E, erythroid; Meg, megakaryocytic; and EMeg, erythro-megakaryocytic. (d) NP, not published.

Table 2. Cell cycle analysis of cell lines calculated using 7AAD/PY.

Cell line	G1a(%)	G1b(%)	S(%)	G2M(%)	DI	SRI	NI	SCV(%)	type
Molt3	29.4	18.3	40.8	11.5	2.04	4.01	1.97	10.5	I
Molt4	33.7	19.4	39.O	7.8	1.89	4.36	2.31	10.2	I
CCRF-CEM	31.7	30.9	32.O	5.4	1.88	3.7O	1.97	11.4	I
WK93	52.3	19.O	17.6	11.O	1.04	2.11	2.03	11.1	I
OH94	14.9	14.5	48.2	22.4	1.03	2.83	2.74	12.3	I
ALL/MIK	45.9	27.8	22.5	3.8	1.21	2.57	2.12	15.5	0
SN-Ow1	22.6	40.2	27.O	10.2	0.97	2.38	2.45	14.7	0
WH94	28.6	2.5	55.5	13.5	1.35	3.35	2.48	19.6	0
KG-1	41.1	27.4	24.6	6.9	1.01	3.84	3.8O	12.8	II
HT93	40.4	52.8	8.9	7.8	1.07	3.12	2.92	11.O	II
CMK11-5	35.6	35.2	21.O	8.2	2.04	6.88	3.37	11.7	II
MR-87	36.4	18.O	41.2	4.4	0.93	3.94	4.24	9.1	II
HEL	22.2	32.1	33.3	12.4	1.44	5.27	3.66	11.9	II
YS-1	24.5	43.1	23.7	8.7	1.05	2.14	2.04	16.8	0
C2F8	34.5	41.2	19.1	5.2	1.61	4.82	2.99	12.O	II
B4D6	34.4	25.8	34.8	4.9	1.54	5.22	3.39	12.5	II
TK91	35.8	36.6	20.4	7.2	1.64	5.37	3.27	12.3	II
MEG-O1	28.1	41.3	24.8	5.8	2.35	10.13	4.31	12.2	II
K562	21.5	18.O	46.4	14.1	1.4	6.21	4.44	12.3	II
Raji	23.3	17.O	46.1	13.6	1.07	4.59	4.29	20.2	III
Daudi	33.7	9.2	39.4	17.7	0.94	3.32	3.53	16.O	III
Oto	19.7	38.1	31.8	10.4	0.98	2.93	2.99	16.4	III

DI, DNA index; SRI, RNA index of S phase; NI, nucleic acid index of S phase and SCV, coefficient of variations in the RNA at S phase.

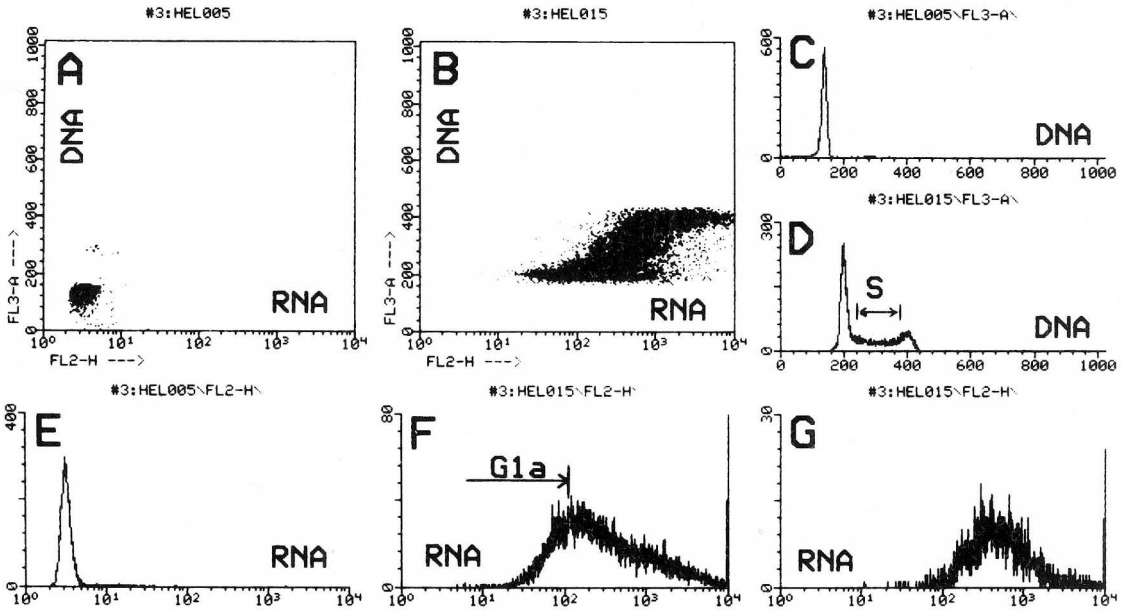

Fig 1. Cell cycle calculation protocols using 7AAD/PY. Peripheral blood lymphocytes (PBL) from healthy donors (panel A) and exponentially growing HEL cells (panel B) were harvested, serially stained with 7AAD and PY, then analyzed. RNA/DNA dot plots are shown in panels A and B. G1, S, and G2M populations of HEL were calculated using a DNA histogram (panel D) on CellFit^{TM} and RFIT^{TM} software (Becton-Dickinson). G1 peak channel numbers of PBL (140; X) and HEL (201; Y) were measured on DNA histograms (panels C and D) using RFIT^{TM} software. DNA index (DI) = Y / X = 201/140 = 1.44. RNA histograms of PBL (panel E), HEL without an analytical gate (panel F), and after a gate set at S phase using the DNA histogram of panel D (panel G) on CellFit^{TM} software are also shown. The G1a population was measured using the RNA histogram (panel F) referring RNA/DNA dot plot (panel B), that is, G1a is a population whose RNA amount is less than the minimal RNA quantity of S-phase population. The G1b was calculated as "G1b = G1 - G1a". Mean RNA channel numbers of PBL on panel E (131; U) and S phase HEL on panel G (690; V) and coefficient of variations of RNA channel numbers of S phase HEL on panel G (11.9 %) were also calculated using manual set statistical analysis program for cell cycle calculation on CellFit^{TM} software. RNA index of S phase (SRI) = V / U = 690 / 131 = 5.27. Nucleic acid index (NI) = SRI / DI = 3.66.

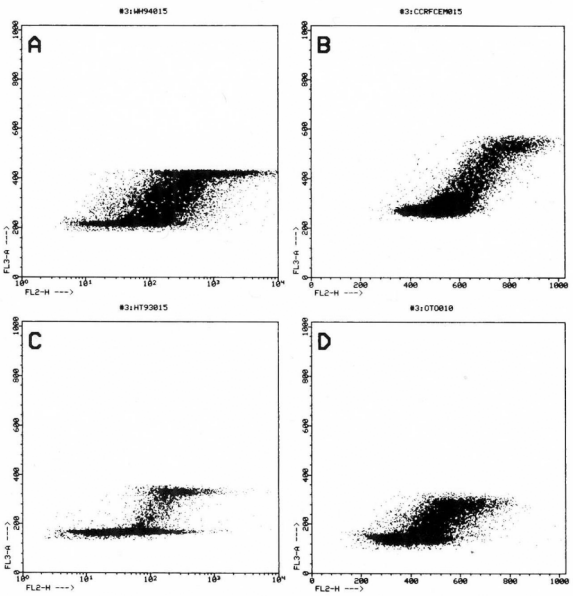

Fig 2. Features of the four types of cell growth. RNA/DNA dot plots of WH94 (panel A), CCRF-CEM (panel B), HT93 (panel C), and Oto (panel D) are demonstrated. The nucleic acid indices (NI) and coefficient of variations of RNA channel numbers of S phase population (SCV %) were measured as shown in Figure 1. WH94 showed relatively low NI (2.48) and high SCV (19.6%); wide range of RNA amount in S-phase, Type 0; CCRF-CEM low NI (1.97) and low SCV (11.4%); narrow range of RNA in S, Type I; HT93 high NI (2.92) and low SCV (11.0%), Type II and Oto high NI (2.99) and high SCV (16.4%), Type III.

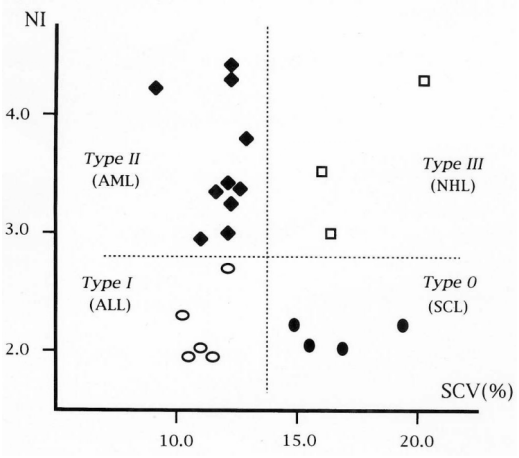

Fig 3. Distribution of NI and SCV(%) of cell lines. The NIs and SCVs of 22 lines shown in Table 2 are plotted. Type 0 (SCL, stem cell leukemia) included biphenotypic t(1;19) leukemia, Ph^1+ ALL and biphenotypic crisis of CGL. Type I (ALL) included uniphenotypic T- or B- ALL. Type II (AML) included AML and myeloid crisis of CGL. Type III (NHL, non Hodgkin's lymphoma) included Burkitt's lymphoma and a follicular center lymphoma.

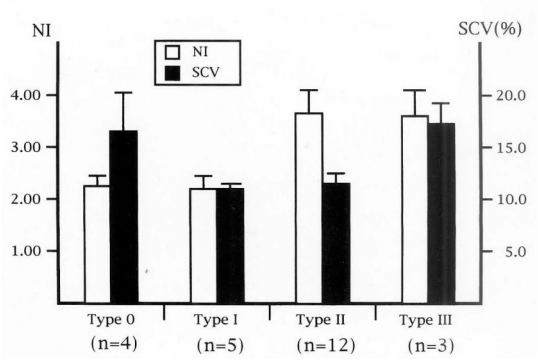

Fig 4. NIs and SCVs of four types of cell growth. The cell cycle in 22 lines was analyzed using 7AAD/PY. The NIs and SCVs were calculated as shown in Figure 1 and Table 2. The NI and SCV of each type were as follows: Type 0, NI = 2.27 $\pm$ 0.19 and SCV = 16.7 $\pm$ 3.7 % (n=4); Type I, NI = 2.20 $\pm$ 0.30 and SCV = 11.1 $\pm$ 0.7 % (n=5); Type II, NI = 3.64 $\pm$ 0.52 and SCV = 11.8 $\pm$ 1.0 % (n=10) and Type III, NI = 3.60 $\pm$ 0.53 and SCV = 17.5 $\pm$ 1.9 % (n=3).

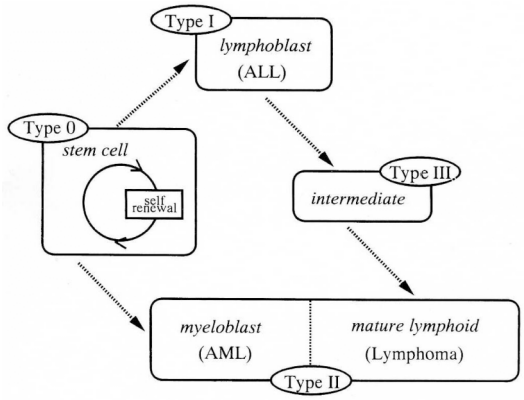

Fig 5. Schematic lineage commitment and differentiation. Type 0 stem cells have narrow range of RNA quantity in G1a phase, in contrast, wide spectrum of RNA synthesis potentiality on G1 phase, hence high SCV, which may indicates potency to decrease or increase RNA synthesis in G1 when the cells committed to lymphoid or myeloid lineage, respectively. Type III lymphomas are relatively immature CD10 positive lymphoid cells, intermediate lymphoma between common ALL and mature CD10 negative lymphoma. Cell cycle analysis of de novo lymphomas showed Type II growth (data not shown). Hence, the high SCV of Type III lymphomas may, again, indicates the potency to shift to more differentiated Type II lymphomas.