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Expression of DNA Polymerase α and Leu3a Molecules in Growing and Saturated Cultures of Human Leukemic Cells: Phenotype Analysis of Proliferative Cells by Flow Cytometry

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A flow cytometric method to analyze phenotypes of proliferative cells was developed using human leukemic cell line MOLT 4. A nuclear protein, DNA polymerase α (pol α), was selected as a marker for proliferative cells, and Leu3a molecule as a cell-surface antigen phenotype marker of the cells. The procedure involved the simultaneous use of fluorescein-conjugated anti-pol α antibody, developed by us, and commercially available phycoerythrin-conjugated anti-Leu3a antibody. The optimal fixative for both proteins was phosphate-buffered 2% paraformaldehyde. The pol α-positive population in logarythmically growing MOLT 4 cells was estimated, by flow cytometry, to be ca. 95%. A sharp flow cytometry histogram with a strong pol α-linked fluorescence was observed. On the other hand, the pol α-positive population in the saturated culture was ca. 70%, with weaker pol α-linked fluorescence. Thus, the population of pol α-positive cells and the amount of pol α in cells was dependent on the cell density of the culture. In contrast, ca. 90% Leu3a-positive populations with similar flow cytometry histograms were seen in either growing or saturated states, suggesting that expression of Leu3a was independent of cell density. The flow cytometric method using fluorescein isothiocyanate-conjugated anti-pol α antibody is useful for detecting proliferative fractions of free tumor cells, such as leukemic cells. Furthermore, analysis of the phenotype of the proliferative or non-proliferative cells became easier by simultaneous labeling with antibodies against pol α and phenotype-specific proteins.

Key words: Flow cytometry — DNA polymerase α — Leu3a — Leukemic cells

DNA polymerase α (pol α)[4] is one of the essential enzymes in the replication of chromosomal DNA in eukaryotic cells.[1-3] Recent analysis using monoclonal antibody to this enzyme showed that this enzyme is localized in nuclei in G1, S and G2 phases, and in cytoplasm in the M phase of the cell cycle, but that it is not found in resting cells.[4] Taking advantage of these characteristics, we previously reported the proliferative activity of solid tumors of cervical carcinoma and endometrial adenocarcinoma by immunological staining of pol α.[5, 6] This activity was shown by the proliferative cell index (PCI), which was the percentage of pol α-positive cells in the tumor. Recently, Namikawa *et al.*[7] and Campana and Janossy[8] reported successful detection of surface antigens of proliferative lymphocytes *in situ* in malignant tissues by double- or triple-color-labeling methods. In contrast to solid tumors, flow cytometry has advantages over the immunohistochemical approach in the study of proliferative activity and/or phenotypes of proliferative cells of free tumor cells, such as leukemic cells. For this reason, we developed the flow cytometric method for simultaneous detection of nuclear protein pol α and a surface antigen phenotype marker of cells. In this experiment, we selected Leu3a (CD4) as the surface antigen because it is an important marker of helper T cells. Thus, the present study involved the combined use of FITC-conjugated anti-pol α antibody developed in our laboratory, and commercially available PE-conjugated anti-Leu3a antibody.

MATERIALS AND METHODS

Conjugation of fluorescent dyes to monoclonal antibodies
A hybridoma clone, CL-22-2-42B, secreting a mouse monoclonal anti-pol α antibody was used.[9] The hybridoma cells (5×10^7 to 10^8 cells per individual) were injected into the peritoneal cavities of F1 mice (C57BL/6×BALB/c) and ascites were obtained. The IgG fraction was prepared by ammonium sulfate precipitation, followed by DEAE-Sepharose chromatography. Fluorescent dyes, FITC and PE, were conjugated to anti-pol α antibodies according to the methods of Riggs *et al.*[10] and of Oi *et al.*,[11] respectively, with some modifications. PE-conjugated anti-Leu3a (CD4) was purchased from Becton Dickinson (Mountain View, Calif.).

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[4] The abbreviations used are: pol α, DNA polymerase α; FITC, fluorescein isothiocyanate; PE, phycoerythrin; FCM, flow cytometry; PFA, paraformaldehyde; PLP, periodate-lysine-paraformaldehyde; PBS, 10 mmol/liter sodium phosphate buffer (pH 7.4) containing 0.15 mol/liter NaCl.

Cell culture and DNA labeling Human T cell leukemia cell line, MOLT 4, was grown in Falcon 3013 culture bottles in RPMI 1640 medium containing 10% fetal calf serum and the medium was changed at 3-day intervals. For the experiments in this study, cell density at the initiation of culture (day 0) was fixed at 1×10^5 cells/ml in a Falcon 3024 bottle. The cells became saturated on day 4 and were then re-seeded at 1×10^5 cells/ml. During the next 4 days of culture, small aliquots of cells were harvested occasionally for analyses. The term "cells in a saturated state" used in the text indicates day 4 cells with a saturated cell density of ca. 1.5×10^6 cells/ml. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Viability was checked by the trypan blue dye exclusion test. Cells were labeled with ^{3}H-thymidine (5 μCi/ml) for 1 h in a CO_2 incubator. After washing of the cells with cold PBS, trichloroacetic acid-insoluble materials were collected on a GF/C filter. Radioisotope activity was counted with a liquid-scintillation counter in a toluene-based scintillation fluid.

Conditions for immunocytochemistry Cells (10^6) were washed 3 times with cold phosphate-buffered saline (PBS). The resultant cell pellet was tapped, then flushed with 1 ml of appropriate fixative and stood on ice for 30 min with occasional mixing. After 3 washes with cold PBS, cells were treated with either 20 μl of FITC-conjugated anti-pol α antibody (Lot Y2) or 20 μl of PE-conjugated anti-Leu3a antibody on ice for 30 min. The anti-pol α antibody was diluted to 1:40 by PBS containing 10% normal goat serum. For double color staining, FITC-conjugated anti-pol α antibody and PE-conjugated anti-Leu3a antibody were sequentially added. Finally, cells were washed 3 times with cold PBS. The same cell preparations were divided into two parts, one for immunofluorescence microscopy and the other for flow cytometry. Fixatives used were as follows: 2 or 4% PFA in PBS, 2 or 4% PLP (2 or 4% PFA, 10 mmol/liter $NaIO_4$ and 75 mmol/liter lysine) in 50 mmol/liter sodium phosphate buffer (pH 7.4), acetone, ethanol or methanol. PFA and PLP solutions were neutralized with NaOH before making the buffered solution.

Preparation of plasma clot Cells (1×10^6) were resuspended in 100 μl of normal human plasma anti-coagulated with sodium citrate. Then 10 μl of 0.25 mol/liter $CaCl_2$ was added to the cell suspension, and the mixture was allowed to stand for a while at 37°C. The plasma clot containing MOLT 4 cells thus obtained was then fixed with 2% PLP overnight at 4°C. The fixed plasma clot was embedded in OCT compound (Miles Inc., Elkhart, Ind.) and frozen in a dry ice-ethanol bath. Plasma-clot specimens were stored at −80°C until use.

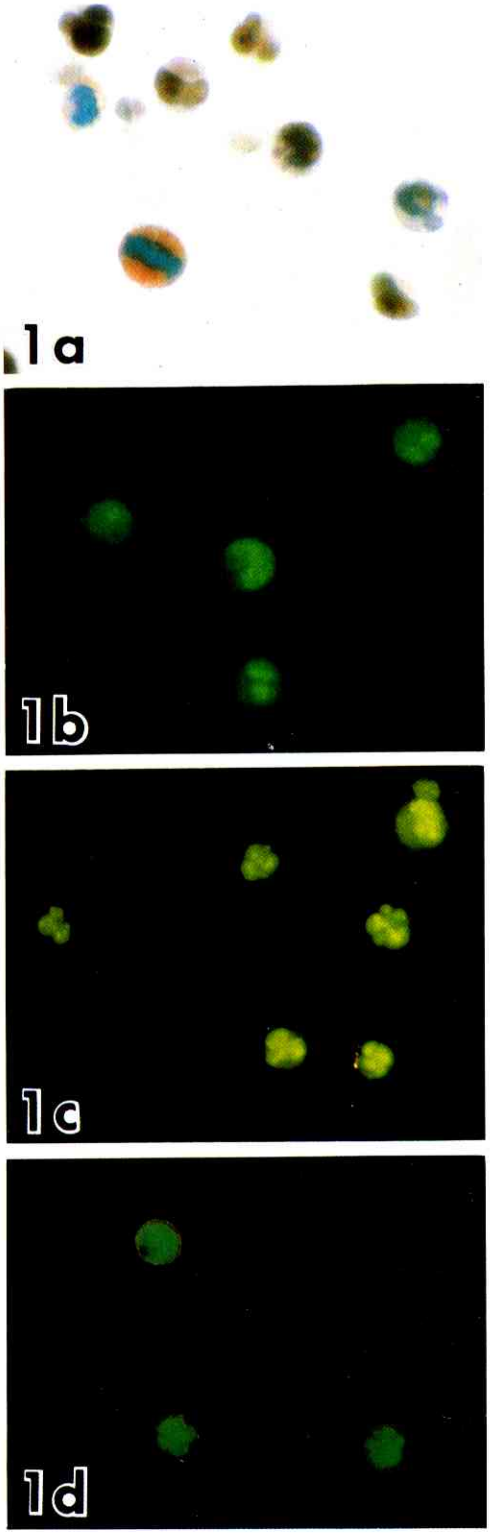
Fluorescence microscopy and flow cytometry An Olympus model BHS-RFK microscope equipped with a UVFL ×40 oil immersion objective lens was used for immunofluorescence microscopy. A Spectrum III analyzer (Ortho Diagnostic Systems) was used for flow cytometry. Data were analyzed on an NEC PC-9801 Vm computer using the DS-1 program developed by Ortho Diagnostic Systems for two-color analysis.

Measurement of DNA histogram MOLT 4 cells in each experiment were fixed with 70% ethanol at −20°C. After washing of the fixed cells with PBS, they were treated with 1 mg/ml RNase at 37°C for 30 min. RNase was washed out with PBS, then 0.1 mol/liter glycine-NaOH (pH 10.0) containing 0.1% Triton X-100 was added, and the mixture was allowed to stand for 30 min. Cells were then treated with propidium iodide (50 μg/ml in 3.8% sodium citrate containing 0.1% Triton X-100) for 10 min at 4°C. Fluorescence intensity was measured by a Cytoron (Ortho Diagnostic Systems) and then analysis of the DNA histogram was performed using Dean's program.[12]

RESULTS

Optimal fixative for simultaneous detection of Leu3a and pol α In the standard method for studying the surface phenotype of lymphocytes by flow cytometry or fluorescence microscopy, intact cells are usually reacted with an appropriate antibody without fixation. Unfortunately, pol α could not be detected without fixation because of its general nuclear localization.[4–6] Consequently, we first tried to obtain a common fixative(s) for both pol α and Leu3a using MOLT 4 cells in the growing phase. Three fixatives, acetone, ethanol and methanol, were appropriate for Leu3a; however, pol α was not detected using cell preparations fixed with any of these agents, in agreement with a previous report.[4] The remaining 4 fixation conditions (2 or 4% PFA, and 2 or 4% PLP) preserved the antigenicity of both Leu3a and pol α. To evaluate 2% PFA fixation, we measured the Leu3a-positive population of MOLT 4 and normal human peripheral blood lymphocytes by flow cytometry. Similar values Leu3a-positive population were obtained either with or without 2% PFA fixation: 85% of MOLT 4 cells and 30–45% of normal human peripheral lymphocytes were Leu3a-positive in both cases. Thus we concluded that 2% PFA is a mild and simple fixative for simultaneous detection of Leu3a and pol α.

Staining patterns of pol α and Leu3a To establish the precise localization of pol α, we used the cryostat sections (5 μm). The exponentially growing MOLT 4 cells (day 2 cells) were divided into 2 groups, one for plasma-clot preparation and another for fixation with PFA in suspension. Figure 1a shows the nuclear localization of pol α, because convoluted nuclei which are characteristic for MOLT 4 cells were positively stained by anti-pol α antibody; however, in the case of mitotic cells, pol α was



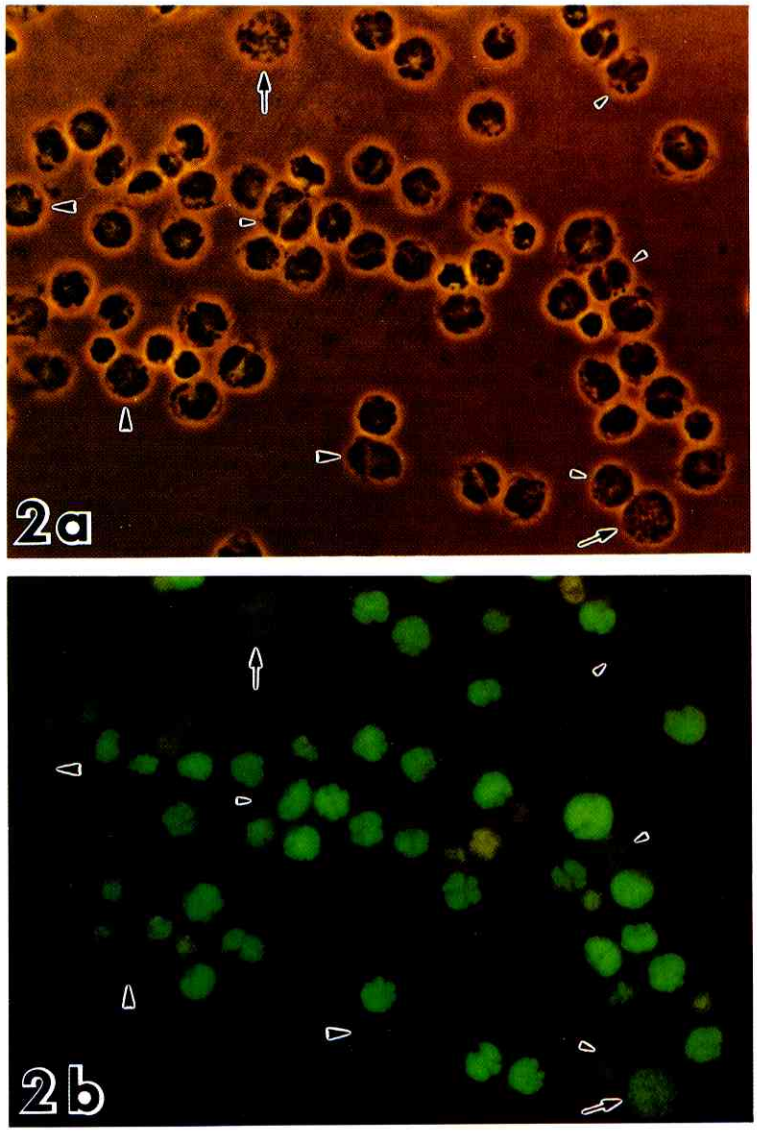


Fig. 1. Immunostaining of growing MOLT 4 cells with monoclonal anti-DNA polymerase α antibody (×650). (a) Cryostat sections (5 μm) of PLP-fixed plasma clot containing MOLT 4 cells were stained by the indirect immunoperoxidase method. Nuclei were counterstained with methyl green. Single staining with FITC-conjugated anti-pol α antibody (b) and PE-conjugated antibody (c). (d) Double staining with FITC-conjugated anti-pol α antibody and PE-conjugated anti-Leu3a antibody. PFA-fixed MOLT 4 cells in suspension were used in (b), (c) and (d).

Fig. 2. DNA polymerase α localization of MOLT 4 cells in the saturated state. Day 4 cells were stained using FITC-conjugated anti-pol α antibody. (a) Photograph taken under phase contrast (×540). (b) Fluorescence photograph of the same field as (a). Large arrowheads indicate cells negatively stained; small ones indicate cells with cytoplasmic pol α, with or without nuclear pol α. Arrows indicate mitotic cells.

found in cytoplasm, agreeing with previous results.[4,6] The nuclear localization of pol α was also demonstrated using PFA-fixed free cells. Staining patterns of Fig. 1b (green color) and Fig. 1c (yellow color) were obtained with FITC-conjugated or PE-conjugated anti-pol α antibody, respectively. Double staining with a combination of FITC-conjugated anti-pol α and PE-conjugated anti-Leu3a antibodies revealed green nuclei (pol α-positive) and orange surface membrane (Leu3a-positive) in Fig. 1d. In the case of MOLT 4 cells in the saturated state, two populations were seen; one was weakly pol α-positive, and the other was negative. Among the pol α-positive populations, the major population was intranuclear pol α-positive and the minor one possessed cytoplasmic pol α with or without nuclear pol α (Fig. 2).

Evaluation of pol α and/or Leu3a-positive populations by flow cytometry In a case of single staining of pol α, ca. 84–90% of growing MOLT 4 cells were detected as pol α-positive. Both FITC-conjugated and PE-conjugated anti-pol α antibodies worked well, resulting in a similar magnitude of positive population. The total pol α-positive population did not alter in the double-staining experiment. This population was composed of 69% pol α^+ Leu-3a$^+$ cells and 15% pol α^+Leu3a$^-$ cells. The remaining 16% of cells was pol α-negative. Most of the pol α-negative population was Leu3a-positive. A similar value of Leu3a-positive population (83%) was detected both in the double- and single-staining procedures.

Expression of pol α and Leu3a molecules in cells in growing and saturated states We examined whether expression of pol α and Leu3a altered in relation to cell density during cell culture. In this experiment, MOLT 4 cells from saturated culture (day 4) were used. This cell population was composed of 48% cells in G0/G1, 42% in S and 10% in G2/M phases (Table I). On day 0, cells were diluted to the initial cell density of 1×10^5 cells/ml. Cells were cultured for 4 days and a small aliquot was harvested every day (Table I). As shown in Table II, the pol α-positive population rapidly increased to its maximum value (95%) on day 1. The DNA histogram showed that day 1 cells were composed of 36% cells in G0/G1, 55% cells in S and 9% in G2/M (Table I). In accordance with these changes, thymidine incorporation by the cells was rapidly increased on day 1. The maximum pol α-positive value was maintained on day 2 but

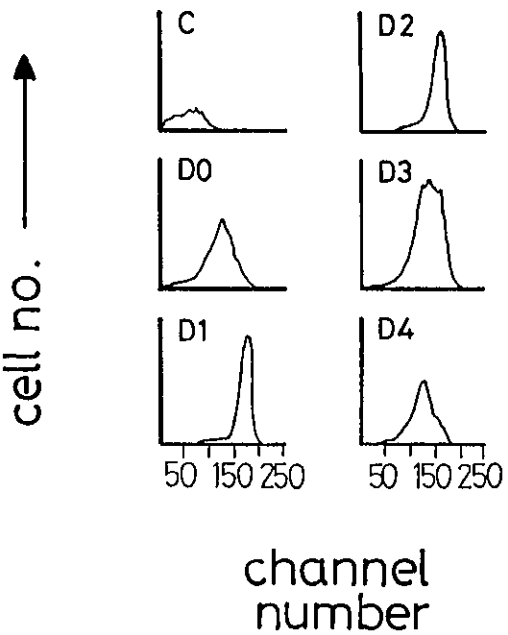


Fig. 3. Distribution of DNA polymerase α-linked fluorescence. FCM histograms were obtained using the same cell preparations as in Table I by labeling with FITC-conjugated anti-pol α antibody. A control datum without the antibody treatment is shown in C.

Table I. Population of MOLT 4 Cells in Typical Phases of the Cell Cycle[a]

Cell culture	G0/G1	S	G2/M	DNA synthesis	Viability	Cell density ($\times10^{-5}$)
day 0	48%	42%	10%	1381 cpm (100%)[b]	92%	12.0[a]
1	36	55	9	2223 (161)	93	2.0
2	35	58	7	2527 (183)	94	4.1
3	36	58	6	1835 (133)	97	6.9
4	46	38	17	901 (65)	89	15.3

a) MOLT 4 cells in saturated culture (day 4 cells) with a cell density of 12×10^5 cells/ml were diluted to 1×10^5 cells/ml on day 0. Cell aliquots were taken from the culture bottle every 24 h. DNA histogram analysis and flow cytometric analysis (Table II) were performed as described in "Materials and Methods." The coefficient of variation in cell cycle analysis ranged between 2.1 and 2.4.

b) The amount of ^{3}H-thymidine uptake per 10^5 cells. The values are represented as percentages of that of day 0 cells, defined as 100%.

Table II. Flow Cytometric Analysis of MOLT 4 Cells with Double Labeling of FITC-conjugated Anti-pol α Antibody and PE-conjugated Anti-Leu3a Antibody

Cell culture[a]	Green (FITC)/Orange (PE)				Total pol α^+	Total Leu3a$^+$
	−/−	−/+	+/−	+/+		
day 0	4.0%	27.0%	10.0%	59.0%	69.0%	86.0%
1	0.6	4.3	8.1	86.9	95.0	91.2
2	1.3	2.9	9.6	86.1	95.7	89.0
3	1.0	10.8	6.0	82.1	88.1	92.9
4	4.6	22.5	8.1	64.8	73.0	87.3

a) Samples were the same as those in Table I.

was followed by a gradual decrease; pol α-positive populations of 88% and 73% were obtained on days 3 and 4, respectively (Table II). The FCM histograms of pol α-linked fluorescence intensity are shown in Fig. 3. It is reasonable, because of our experimental design, that the histogram of day 0 and that of day 4 were essentially the same. Two points should be noted: 1. The broad histogram of day 0 became sharp on day 1. 2. The sharp histogram of day 2 became broader as culture progressed. The histograms tell us that the sharp histograms of days 1 and 2 arose from high pol α-linked fluorescence intensity at around the 160 channel. Subsequently, a peak with low intensity (at around the 120 channel) appeared and increased its size in proportion to the decrease in the peak with strong intensity (see D4 of Fig. 3), where ca. 30% of the cells had lost pol α (Table II). On the other hand, the shape of the FCM histogram for Leu3a seemed not to alter (data not shown) and the total Leu3a-positive population changed only slightly, from 86% to 93% (Table II). However, a substantial change in the subpopulation of pol α^-Leu3a$^+$ cells was observed. This population was 3–4% in the early logarithmic growing phase; however, it increased to more than 20% in the saturated state.

DISCUSSION

We firstly looked for a common fixative for both pol α and Leu3a molecules. As fixatives, 2–4% PFA and 2–4% PLP in phosphate buffer (pH 7.4) were suitable for pol α, so we examined whether they were appropriate for Leu3a. With or without 2% PFA fixation, the same magnitude (30–45%) of Leu3a-positive population of normal human lymphocytes was observed in our flow cytometric experiments. Furthermore, as these values agreed well with others[13] using intact cells, 2% PFA was finally judged to be an optimal fixative for both pol α and Leu3a molecules. Secondly, we studied the localization of pol α and its positive population in the growing phase and the saturated state of MOLT 4 cells. Intranuclear localization of pol α was detected in growing cells, except those in the M phase. This agrees with the finding in colon cancer cell lines by Alama *et al.*[14] and with our previous results.[5,6] Flow cytometric analysis revealed that almost all the cells in the early logarithmic growing phase were pol α-positive; the population was ca. 95%. The pol α-linked fluorescence made a sharp FCM histogram with strong intensity. In contrast, a decreased pol α-positive population of ca. 70% was found in the saturated state. The fluorescence intensity became weak. This tendency agrees well with that found in colon cancer cell lines.[14] The major localization of pol α was still nuclear and a minor population with cytoplasmic localization was seen. The remaining 30% of cells were totally pol α-negative. Thirdly, we examined the kinetic behavior of pol α and Leu3a in terms of their positive populations, using MOLT 4 cells in the saturated culture. With the commencement of cell proliferation, the pol α-positive population rapidly increased from 70% to 95% during the first day of culture. In addition, the broad histogram of pol α-linked fluorescence intensity changed to a sharp one with strong intensity. These changes were accompanied with a substantial increase of cells in the S phase and a decrease of cells in G0/G1. Gradually, reverse changes were observed from day 2 to day 4. This tendency was quite reproducible. Thus, expression of pol α was dependent on cell density. In contrast to pol α, the Leu3a-positive population and the shape of the Leu3a-linked fluorescence peak did not change during cell culture. Consequently, the expression of Leu3a in MOLT 4 cells seems independent of cell density. Furthermore, it can be suggested that abundant pol α, initially located in nuclei of growing cells, was down-regulated when the cell approached the saturated state, and was finally metabolized in the resting phase. The thirty percent pol α-negative population found in the saturated state might be the cells in the deep resting phase, and non-mitotic cells possessing cytoplasmic pol α alone might be cells approaching the resting phase. Some researchers have suggested the cyclic migration of pol α between nuclei

and cytoplasm[3, 14]; however, a further critical experiment is needed to test this hypothesis. According to the previous study, all cells entering the cell cycle can be expected to express pol α. However, the pol α-positive population of growing cells never reached 100%; it always ranged between 90% and 95%. As most of the remaining pol α-negative population was Leu3a-positive, this was not an experimental artifact. It is not clear why 5%–10% of the population of growing cells was pol α-negative. One possible explanation for this finding is that this population represents some quiescent state (Q) located in G1 phase or other quiescent state(s) in S or G2, which are different from classical G0 described by Drewinko *et al.*[15] The flow cytometric method developed here should be useful to detect proliferative fractions of the free tumor cells in a short time period. Consequently, this method may surpass the autoradiographic technique. Furthermore, it enabled us to demonstrate phenotypes of proliferative or non-proliferative fractions. We are now involved in analyzing phenotypes of proliferative cells using fresh human leukemia cells.

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