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# Flow Cytometric Detection of Proliferative Cells in Leukemias

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**We studied the proliferative activity of leukemic cells obtained from the peripheral blood and bone marrow of 34 patients; 30 with acute leukemia and 4 with chronic myelogenous leukemia in blastic crisis. Flow cytometry was performed using monoclonal antibody against DNA polymerase $\alpha$. Since fresh and frozen cells showed virtually identical DNA polymerase $\alpha$-positive populations and flow cytometric histograms, 52 cryopreserved samples (25 from peripheral blood and 27 from bone marrow) were used in this study. The DNA polymerase $\alpha$-positive population ranged from 20.4% to 84.7% in peripheral blood, and from 6.5% to 92.1% in bone marrow. A positive correlation ($r=0.76$, $P<0.01$) was found between DNA polymerase $\alpha$-positive populations in peripheral blood and bone marrow from the same patient. This suggests that the DNA polymerase $\alpha$-positive population in the bone marrow can be estimated from that in peripheral blood. No relationship was observed between the positive population and the response to chemotherapy. Statistical analyses for all cases showed no relationship between the DNA polymerase $\alpha$-positive population and either the tumor cell count or time to reach a nadir. However, a negative correlation was observed between the positive population in bone marrow samples and the time to reach a nadir ($r=-0.64$, $P<0.05$) in those patients who achieved a complete response. In addition, in the cases of acute non-lymphocytic leukemia who did not respond to chemotherapy, a positive correlation was observed between the tumor cell count in bone marrow and the DNA polymerase $\alpha$-positive population ($r=0.93$, $P<0.01$). Thus, the method described here provides a simple and time-efficient means of detecting the proliferative activity of leukemic cells, which is a useful parameter in the treatment of leukemia.**

Key words: Flow cytometry — DNA polymerase $\alpha$ — Cellular proliferation — Leukemia

Knowledge of the proliferative fractions in tumors is important in estimating the prognosis of malignant tumors. Recent development of monoclonal antibodies against proliferation-associated antigens, such as pol $\alpha$,[4] PCNA and Ki-67, has made it possible to estimate the proliferative activity of malignant tumors immunologically.[1-4] In leukemias, flow cytometric analysis appears to be the best method available for quantifying the growth fractions of leukemic cells.[5-7] In the present study, we used pol $\alpha$ as the marker for proliferative cells, since 1) cells in all phases of the cell cycle have been shown to possess pol $\alpha$[8-10] and 2) it appears to possess clear biological significance in the replication of chromosomal DNA in eukaryotic cells.[11-13] In addition, the proliferative cell index determined from the pol $\alpha$-positive population has been shown to be well correlated to histological and nuclear grades which influence the prognosis of endometrial carcinoma.[14] The object of this study was to measure the proliferative fractions of leukemic cells from patients with acute leukemia and CML-BC by flow cytometry. Statistical analysis was performed to determine whether pol $\alpha$-positive populations correlated with either the response to chemotherapy or the disease prognosis.

## MATERIALS AND METHODS

**Patients** We studied 34 patients with leukemia; 18 men and 16 women aged 16 to 76 years old (median 46). The

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[4] Abbreviations used: pol $\alpha$, DNA polymerase $\alpha$; PCNA, proliferative cell nuclear antigen; FAB classification, French-American-British Cooperative Group classification; ANLL, acute non-lymphocytic leukemia; ALL, acute lymphoblastic leukemia; M1, acute myeloblastic leukemia without maturation; M2, acute myeloblastic leukemia with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; M5, acute monocytic leukemia; M6, erythroleukemia; L1, acute lymphoblastic leukemia with predominantly small cells; L2, acute lymphoblastic leukemia with predominantly large cells; CML-BC, chronic myelogenous leukemia in blastic crisis; RPMI 1640, Rosewell Park Memorial Institute 1640; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PFA, paraformaldehyde; FITC, fluorescein isothiocyanate; FITC-anti pol $\alpha$ Ab, FITC-conjugated monoclonal antibody against pol $\alpha$; CR, complete response or complete remission; PR, partial response; NR, no response.

subtypes of the 25 cases of ANLL were 9 M1; 6 M2; 3 M3; 5 M4; 1 M5 and 1 M6. Four patients had CML-BC and 5 had ALL; 1 L1 and 4 L2. The subtypes were classified according to the FAB classification.[15] These patients' profiles appear in Table I. Patients with acute leukemia had more than 70% leukemic blasts in their peripheral blood or bone marrow at the time of sampling.

**Cells** Peripheral blood and bone marrow blood were collected in heparinized tubes. Mononuclear cells were separated by the Ficoll-Hypaque gradient centrifugation method. They were cryopreserved in liquid nitrogen in RPMI 1640 with 10% FCS (Gibco, Frankfurt, Germany) and 10% DMSO (Wako, Tokyo) under standard conditions until assay. Frozen cells were reconstituted at 37°C, washed with PBS (Nissui, Tokyo) and then tested. Samples of both cryopreserved and fresh leukemic cells were available in only 4 cases. The cryopreserved cells were tested for viability using trypan blue staining after reconstitution. Samples with a viability of more than 80% were used in the study. Twenty-five peripheral blood samples and 27 bone marrow samples were available for testing (Table I). Peripheral blood lymphocytes obtained from healthy volunteers served as the negative control, while exponentially growing MOLT4 cells (Jap-

Table I. Hematological Parameters of 34 Leukemia Patients

| Diagnosis[a] | Patient number | Age | Sex | Tumor cells | | Time to nadir (day) | Response to chemotherapy[c] |
|---|---|---|---|---|---|---|---|
| | | | | Peripheral blood ($\times 10^9$/liter) | Bone marrow ($\times 10^{10}$/liter) | | |
| ANLL-M1 | 1 | 54 | M | 74.8 | 80.7 | 33 | CR |
| | 2 | 43 | F | 257.5 | 95.8 | 17 | NR |
| | 3 | 72 | M | 8.6 | 1.6[b] | — | — |
| | 4 | 27 | F | 172.0 | 89.3 | 16 | NR |
| | 5 | 16 | M | 18.6 | 17.5 | 24 | CR |
| | 6 | 30 | M | 5.6[b] | 26.6 | 17 | CR |
| | 7 | 38 | F | 193.9 | 47.6[b] | 17 | NR |
| | 8 | 76 | M | 1.6[b] | 28.9 | 20 | PR |
| | 9 | 43 | M | 137.1 | 82.3 | 19 | PR |
| ANLL-M2 | 10 | 59 | M | 2.0[b] | 8.3 | 19 | CR |
| | 11 | 41 | F | 3.1 | 3.8[b] | 24 | CR |
| | 12 | 51 | F | 96.0 | 34.2[b] | 24 | NR |
| | 13 | 35 | F | 2.1 | 50.3 | 23 | CR |
| | 14 | 37 | M | 35.2 | 73.2 | — | — |
| | 15 | 47 | M | 3.5 | 15.9 | 13 | CR |
| ANLL-M3 | 16 | 20 | F | 81.0 | 64.8 | — | — |
| | 17 | 40 | M | 31.5 | 60.6 | — | — |
| | 18 | 38 | M | 0.4[b] | 47.7 | 17 | NR |
| ANLL-M4 | 19 | 27 | F | 35.6 | 11.0 | 17 | CR |
| | 20 | 37 | F | 30.3 | 15.2 | 28 | CR |
| | 21 | 68 | F | 49.6[b] | 10.8 | 19 | PR |
| | 22 | 51 | M | 4.1[b] | 7.9 | 15 | CR |
| | 23 | 20 | F | 157.5 | 63.5 | 12 | CR |
| ANLL-M5 | 24 | 42 | F | 164.3 | 76.1 | 18 | NR |
| ANLL-M6 | 25 | 70 | F | 24.0[b] | 11.5 | — | — |
| CML-BC | 26 | 42 | M | 27.9 | 12.0[b] | 18 | CR |
| | 27 | 74 | M | 11.9 | 19.0[b] | 26 | PR |
| | 28 | 50 | F | 23.2 | 2.7 | 22 | PR |
| | 29 | 41 | M | 27.0 | 4.8 | 26 | PR |
| ALL-L1 | 30 | 18 | F | 23.0 | 76.4 | 11 | CR |
| ALL-L2 | 31 | 41 | F | 2.2[b] | 24.0 | 29 | CR |
| | 32 | 55 | M | 45.8 | —[b] | 13 | — |
| | 33 | 49 | M | 2.9[b] | 14.9 | 12 | NR |
| | 34 | 36 | M | 5.3 | 17.4 | 27 | CR |

*a*) FAB classification (Ref. 14).
*b*) Materials not tested.
*c*) CR, complete remission or complete response; PR, partial response; NR, no response; —, not evaluable.

anese Cancer Research Resources Bank, Tokyo) were used as the positive control.

**Fixation and staining** Cells were washed three times with cold PBS and fixed in 2% PFA on ice for 45 min, and then washed three times in cold PBS. Cells were mixed with FITC-anti pol $\alpha$ Ab (diluted 1:10), and then incubated on ice for 45 min. Cells were then washed three times with cold PBS. Monoclonal antibody directed against calf thymus pol $\alpha$ was produced as described by Masaki *et al.*[1] FITC-labeled antibody containing 2 mg/ml IgG was obtained from Medical and Biological Laboratories Co., Ltd., Nagoya.

**Flow cytometric analysis** We used a CYTORON (Ortho, Mass., USA) equipped with a 15 mW argon laser with an excitation wavelength at 488 nm. Fluorescence was analyzed from 515 to 530 nm (FITC) using the Ortho software. Fluorescence was amplified logarithmically and a positive threshold was determined with the negative control cells such that those cells showed less than 0.5% and the positive population showed at least 0.1%. For each sample, negative control cells were prepared without treatment with monoclonal antibody. At least $2\times10^4$ cells were used in each experiment.

**Clinical parameters and statistical analyses** We analyzed the relationship between clinical parameters, such as the blast cell count in the peripheral blood and bone marrow blood, the number of days from the onset of chemotherapy to the nadir, and the response to chemotherapy, and parameters related to proliferative activity, such as the positive population of pol $\alpha$. The nadir is the point where the minimum leucocyte count is observed during the chemotherapeutic course. Statistical analyses were performed by calculating Spearman's correlation coefficients.[16]

**Chemotherapy** All patients received induction chemotherapy with a multi-drug combination or with a single agent. Among 25 patients with ANLL, 16 patients were treated with enocitabine and daunorubicin or aclarubicin,[17] and 7 patients received mitoxantrone and cytosine arabinoside.[18] Patient #3 received vincristine and prednisolone, but further chemotherapy could not be performed due to complicating psychosis. Patient #25 suffered from erythroleukemia and received citarabine ocfosfate. Four patients with ALL received doxorubicin, vincristine and prednisolone,[19] and one patient received ranimustine and etoposide. There were 4 patients with CML-BC; 2 patients were treated with vincristine and prednisolone, 1 with mercaptopurine and 1 with cytosine arabinoside. Response to chemotherapy was judged after the first induction course. Leucocyte counts at the nadir were less than 1000/$\mu$l in all evaluated cases. Six patients could not be evaluated because of early death.

## RESULTS

**Fresh and frozen cells** Four specimens from three individuals were observed as both cryopreserved and fresh leukemic cells. The positive population was virtually identical in both samples (Fig. 1).

**Pol $\alpha$-positive population** The percentage of lymphoblastic cell line MOLT4 cells in the logarithmic growth phase ranged from 90.0% to 98.8% (median 94.2%).

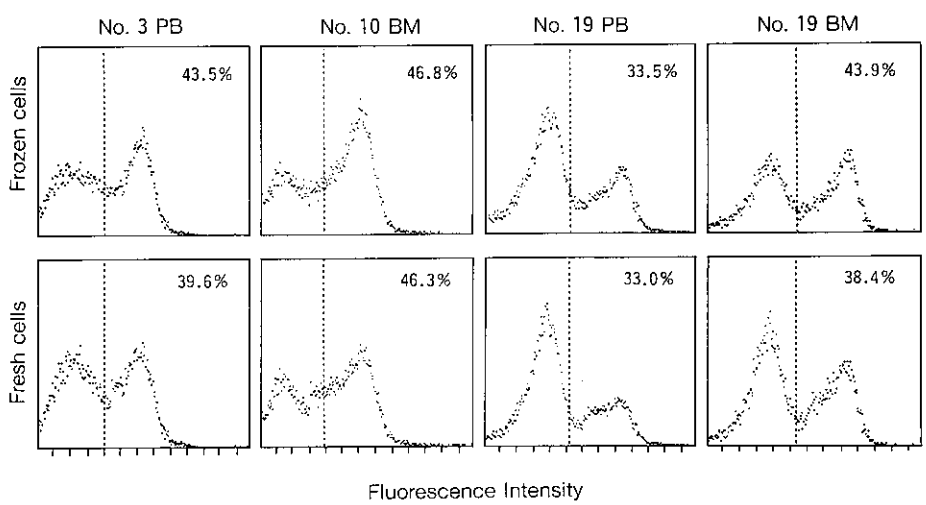


Fig. 1. Flow cytometric histograms of DNA polymerase $\alpha$ staining of 4 samples from 3 individuals (cases 3, 10 and 19) for whom both frozen and fresh cell samples could be tested. The profiles of frozen cells appear in the upper row and those of fresh cells appear in the lower row. Data in each histogram show the positive population (%). PB, peripheral blood; BM, bone marrow.

Peripheral blood lymphocytes from three healthy volunteers showed about 2% positive staining. In all cases, the pol $\alpha$-positive fraction of leukemic cells ranged from 20.4% to 84.7% (median 59.9%) for the peripheral blood cells and from 6.5% to 92.1% (median 57.2%) for bone marrow cells. A wide distribution of the pol $\alpha$-positive population was characteristic in both peripheral blood and bone marrow. The pol $\alpha$-positive fraction for each subtype of leukemia is shown in Table II.

**Correlation between findings in peripheral blood and bone marrow** In 18 of 34 cases, both peripheral blood and bone marrow samples were available for measurement. The proportion of the population that was pol $\alpha$-positive in the bone marrow showed a markedly positive correlation to that in the peripheral blood, with a correlation index of 0.76 ($P<0.01$, Fig. 2).

**Relationship to response to chemotherapy** The pol $\alpha$-positive population of each group was divided according to the response to chemotherapy (Fig. 3). In peripheral blood, the pol $\alpha$-positive population ranged from 20.4% to 83.5% (median 61.4%) for patients with CR, from 40.4% to 83.3% (median 54.9%) for patients with PR, and from 27.6% to 84.7% (median 59.9%) for patients with NR. In the bone marrow, the values ranged from 6.5% to 92.1% (median 58.1%) for patients with CR, from 30.9% to 88.9% (median 61.7%) for patients with PR, and from 9.9% to 82.4% (median 57.2%) for patients with NR. No differences were observed between these 3 groups.

**Relationship to hematological parameters** Statistical analyses using the results of all cases showed that there was no relationship between the pol $\alpha$-positive population and either the initial blast cell count or the time to reach the nadir (data not shown). However, a negative correlation ($r=-0.64$, $P<0.05$) was observed between the pol $\alpha$-positive population of bone marrow samples and the time to reach the nadir in those patients who achieved a complete response (Fig. 4). A similar correlation ($r=-0.51$, $P<0.05$) was observed in all of the cases of ANLL (Fig. 5). In the ANLL cases who did not respond, a positive correlation ($r=0.93$, $P<0.01$) was observed between the tumor cell count in bone marrow and the pol $\alpha$-positive population (Fig. 6).

Table II. DNA Polymerase $\alpha$-Positive Population in 34 Leukemia Patients

| Patient number[a] | Positive population (%) | |
|---|---|---|
| | PB | BM |
| 1 | 20.4 | 6.5 |
| 2 | 84.7 | 72.2 |
| 3 | 43.5 | — |
| 4 | 59.9 | 82.4 |
| 5 | 56.5 | 58.1 |
| 6 | — | 64.3 |
| 7 | 34.0 | — |
| 8 | — | 30.9 |
| 9 | 44.9 | 61.7 |
| 10 | — | 46.8 |
| 11 | 75.8 | — |
| 12 | 27.6 | — |
| 13 | 79.4 | 71.1 |
| 14 | 24.3 | 27.6 |
| 15 | 53.8 | 44.8 |
| 16 | 38.5 | 56.3 |
| 17 | 78.6 | 89.2 |
| 18 | — | 43.5 |
| 19 | 33.5 | 43.9 |
| 20 | 57.5 | 32.1 |
| 21 | — | 40.6 |
| 22 | — | 59.6 |
| 23 | 83.5 | 78.4 |
| 24 | 62.6 | 57.2 |
| 25 | — | 34.7 |
| 26 | 83.4 | — |
| 27 | 40.4 | — |
| 28 | 64.9 | 79.3 |
| 29 | 83.3 | 88.9 |
| 30 | 75.6 | 92.1 |
| 31 | — | 26.6 |
| 32 | 82.0 | — |
| 33 | — | 9.9 |
| 34 | 61.4 | 74.4 |
| Total | 59.9 (20.4–84.7) | 57.2 ( 6.5–92.1) |

*a*) Patient numbers are the same as those in Table I. PB, peripheral blood; BM, bone marrow.

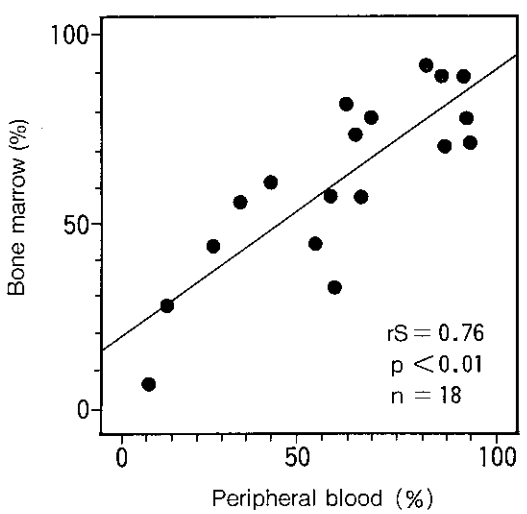


Fig. 2. Correlation between the DNA polymerase $\alpha$-positive populations in peripheral blood and bone marrow. Samples of both peripheral blood and bone marrow were available from 18 patients. rS, correlation index.

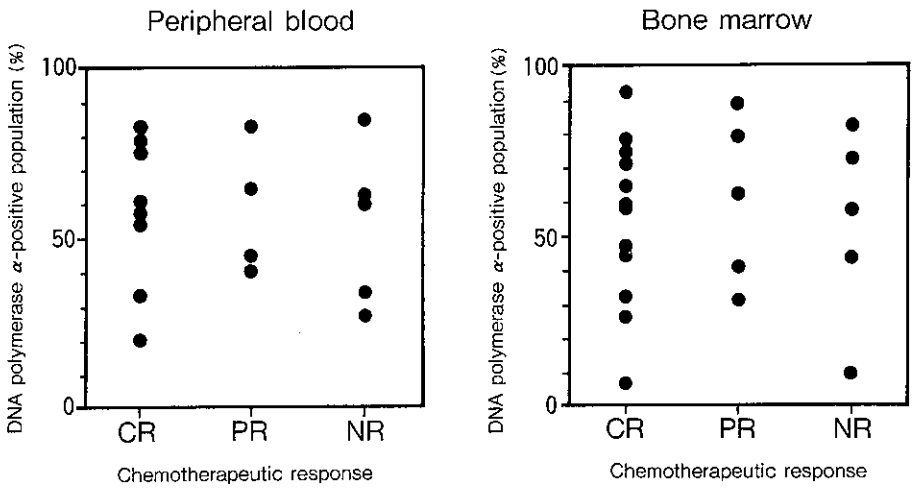


Fig. 3. DNA polymerase $\alpha$-positive population and the response to chemotherapy. CR, complete response or complete remission; PR, partial response; NR, no response.

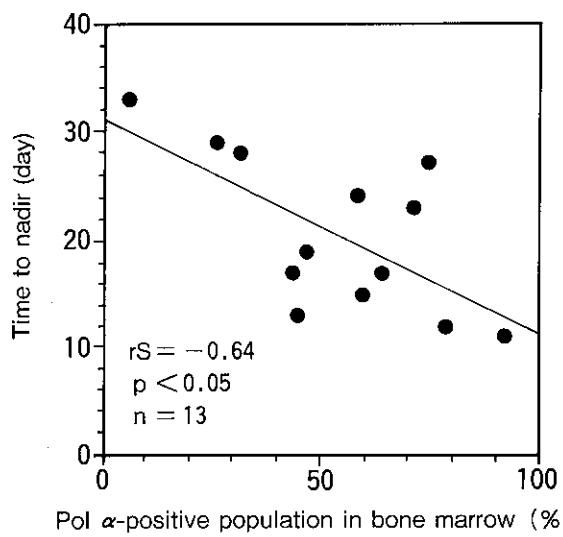


Fig. 4. Relationship between the DNA polymerase $\alpha$-positive population in bone marrow and time to reach the nadir in patients with complete remission. rS, correlation index.

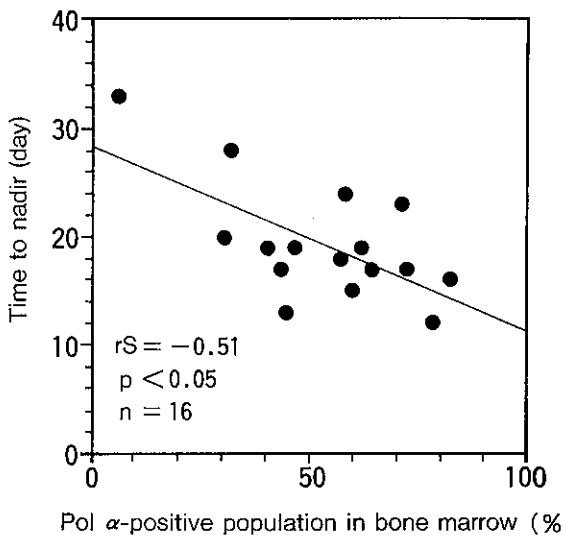


Fig. 5. Relationship between the DNA polymerase $\alpha$-positive population in bone marrow and time to reach the nadir in patients with ANLL. rS, correlation index.

## DISCUSSION

In this study, FITC-labeled monoclonal antibody against pol $\alpha$ was used as a flow cytometric marker for identifying proliferative cells, since pol $\alpha$ is the chief enzyme involved in DNA replication.[11-13] In addition, since pol $\alpha$ is present in cells in all phases of the cell cycle except for resting G0 cells,[8-10] the percentage of pol $\alpha$-positive cells can provide an index of the fraction of proliferative cells.[2, 14]

First, we measured the pol $\alpha$-positive populations in fresh leukemia cells and frozen cells obtained from three individuals to determine the effect of freezing in liquid nitrogen on the parameters. Since the cryopreserved and fresh samples showed similar values (Fig. 1), we judged that both types of samples could be used to evaluate cellular proliferation.

Second, we measured the pol $\alpha$-positive populations of 52 cryopreserved samples. The values obtained exceed the thymidine labeling indices (1.0% to 20.0% in ALL) that have been previously reported.[20] However, the higher values obtained in this study seem reasonable since the $^3$H-thymidine labeling index reflects only those cells in the S phase of the cell cycle.[21, 22] The distribution

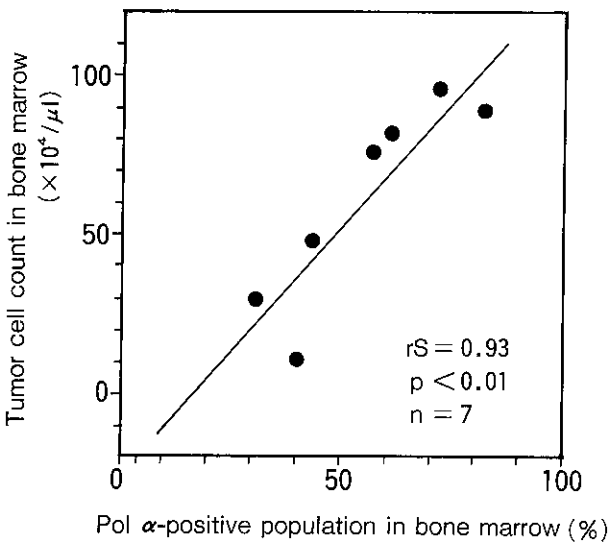


Fig. 6. Relationship between the DNA polymerase $\alpha$-positive population and the tumor cell count in bone marrow in seven ANLL patients without complete remission. rS, correlation index.

pattern of the pol $\alpha$-positive fraction is quite different from those obtained with anti Ki-67 or anti PCNA antibodies.[5] The higher labeling index of PCNA reported by others,[5,6] as compared to that of pol $\alpha$, may be explained by our finding that cells in shallow G0, as well as cycling cells, still express PCNA.[23]

Third, we estimated the relationship between leukemic growth in bone marrow and peripheral blood from the same patients by measuring leukemic cells from both sources. A strong positive correlation ($r=0.76$, $P<0.01$) was observed between the pol $\alpha$-positive populations from these two sources (Fig. 2). These findings suggest that leukemic growth in peripheral blood is active when such growth is also observed in bone marrow; the reverse is surely true. This also suggests that the proliferative activity of leukemic cells in bone marrow can be estimated qualitatively from that in peripheral blood. Our finding may support previous results showing that the durations of the S-phase of leukemic cells from peripheral blood and bone marrow were very similar.[24] Thus, it is worthwhile to measure the pol $\alpha$-positive population of leukemic cells from peripheral blood even when a bone marrow sample is not available.

Finally, we investigated whether the pol $\alpha$-positive population correlates to clinical parameters such as the tumor mass, the time to reach the nadir and the response to chemotherapy. When we considered all of the leukemia cases in this study, no correlation was observed between the pol $\alpha$-positive population and the response to chemotherapy. The statistical analyses also revealed the lack of a correlation between the positive population and either the tumor mass or the time to reach the nadir. However, in patients who achieved complete remission, a negative correlation ($r=-0.64$, $P<0.05$; Fig. 4) was observed between the time to reach the nadir and the pol $\alpha$-positive population in bone marrow. This means that patients whose leukemia cells in bone marrow showed higher pol $\alpha$-positive populations reached the nadir quickly, while those with lower pol $\alpha$-positive populations reached the nadir more slowly. Since a similar correlation ($r=0.51$, $P<0.05$; Fig. 5) was observed in all of the ANLL cases regardless of their response to chemotherapy, the general conclusion might be drawn that patients who show high pol $\alpha$-positive populations reach the nadir soon after the onset of chemotherapy. There was a positive correlation ($r=0.93$, $P<0.01$) between the tumor mass in bone marrow and the pol $\alpha$-positive population in non-responsive cases of ANLL. Unfortunately the reverse correlation was not observed in CR cases of ANLL. However, there was a tendency for the cases with a larger tumor mass and a higher pol $\alpha$-positive population to achieve a complete remission.

We are now attempting to develop a method of two-color analysis which would enable us to display the DNA histogram and pol $\alpha$ simultaneously, since measuring the S-phase fraction should provide additional prognostic information.[25-29] We believe that further analysis of a large patient population should provide valuable information for the treatment of leukemia.

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## REFERENCES

1) Masaki, S., Shiku, H., Kaneda, T., Koiwai, O. and Yoshida, S. Production and characterization of monoclonal antibody against 10S DNA polymerase $\alpha$ from calf thymus. *Nucleic Acids Res.*, **10**, 4703–4713 (1982).
2) Mushika, M., Miwa, T., Suzuoki, Y., Hayashi, K., Masaki, S. and Kaneda, T. Detection of proliferative cells in dysplasia, carcinoma *in situ*, and invasive carcinoma of the uterine cervix by monoclonal antibody against DNA polymerase $\alpha$. *Cancer*, **61**, 1182–1186 (1988).
3) Benjamin, D. R. Proliferating cell nuclear antigen

(PCNA) and pediatric tumors: assessment of proliferative activity. *Pediatr. Pathol.*, **11**, 507–519 (1991).
4) Gerdes, J., Dallenbach, F., Lennert, K., Lemke, H. and Stein, H. Growth fractions in malignant non-Hodgkin's lymphomas (NHL) as determined *in situ* with the monoclonal antibody Ki-67. *Hematol. Oncol.*, **2**, 365–371 (1984).
5) Ito, M., Tsurusawa, M., Zha, Z., Kawai, S., Takasaki, Y. and Fujimoto, T. Cell proliferation in childhood acute leukemia. *Cancer*, **69**, 2176–2182 (1992).
6) Giordano, M., Danova, M., Pellicciari, C., Wilson, G. D., Mazzini, G., Fuhrman Conti, A. M., Franchini, G., Riccardi, A. and Manfredi Romanini, M. G. Proliferating cell nuclear antigen (PCNA)/cyclin expression during the cell cycle in normal and leukemic cells. *Leuk. Res.*, **15**, 965–974 (1991).
7) Shibata, M., Hirono, Y., Takahashi, M. and Kaneda, T. Expression of DNA polymerase $\alpha$ and Leu3a molecules in growing and saturated cultures of human leukemic cells: phenotype analysis of proliferative cells by flow cytometry. *Jpn. J. Cancer Res.*, **80**, 1083–1088 (1989).
8) Bensch, K. G., Tanaka, S., Hu, S.-Z., Wang, S.-F. and Korn, D. Intracellular localization of human DNA polymerase $\alpha$ with monoclonal antibodies. *J. Biol. Chem.*, **257**, 8391–8396 (1982).
9) Nakamura, H., Morita, T., Masaki, S. and Yoshida, S. Intracellular localization and metabolism of DNA polymerase $\alpha$ in human cells visualized with monoclonal antibody. *Exp. Cell Res.*, **151**, 123–133 (1984).
10) Brown, M., Bollum, F. J. and Chang, L. M. S. Intracellular localization of DNA polymerase $\alpha$. *Proc. Natl. Acad. Sci. USA*, **78**, 3049–3052 (1981).
11) Weissbach, A., Baltimore, D., Bollum, F., Gallo, R. and Korn, D. Nomenclature of eukaryotic DNA polymerases. *Science*, **190**, 401–402 (1975).
12) Weissbach, A. Eukaryotic DNA polymerases. *Ann. Rev. Biochem.*, **46**, 25–47 (1977).
13) Hubscher, U. DNA polymerases in prokaryotes and eukaryotes: mode of action and biological implications. *Experimentia*, **39**, 1–5 (1983).
14) Mushika, M., Shibata, K., Miwa, T., Suzuoki, Y. and Kaneda, T. Proliferative cell index in endometrial adenocarcinoma of different nuclear grades. *Jpn. J. Cancer Res.*, **80**, 223–227 (1989).
15) Bennett, J. M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D. A. G., Gralnick, H. R. and Sultan, C. Proposals for the classification of the acute leukaemias. *Br. J. Haematol.*, **33**, 451–458 (1976).
16) Spearman, C. The proof and measurement of association between two things. *Am. J. Psychol.*, **15**, 72–101 (1904).
17) Ohno, R. Recent progress in the treatment of adult acute leukemia. *Acta Haematol. Jpn.*, **52**, 1287–1293 (1989).
18) Wakita, A., Murase, T., Uchida, T., Fujiwara, Y., Tanaka, M. and Ohkita, T. Mitoxantrone and conventional-dose cytosine arabinoside for relapsed and refractory acute leukemia. *Jpn. J. Cancer Chemother.*, **19**, 653–657 (1992) (in Japanese).
19) Clarkson, B., Ellis, S., Little, C., Gee, T., Arlin, Z., Mertelsmann, R., Andreeff, M., Kempin, S., Koziner, B., Chaganti, R., Jhanwar, S., McKenzie, S., Cirrincione, C. and Gaynor, J. Acute lymphoblastic leukemia in adults. *Semin. Oncol.*, **12**, 160–179 (1985).
20) Look, A. T., Roberson, P. K., Williams, D. L., Rivera, G., Bowman, W. P., Pui, C.-H., Ochs, J., Abromowitch, M., Kalwinsky, D., Dahl, G. V., George, S. and Murphy, S. B. Prognostic importance of blast cell DNA content in childhood acute lymphoblastic leukemia. *Blood*, **65**, 1079–1086 (1985).
21) Meyer, J. S. and Bauer, W. C. *In vitro* determination of tritiated thymidine labeling index (LI). *Cancer*, **36**, 1374–1380 (1975).
22) Dombernowsky, P., Bichel, P. and Hartmann, N. R. Cytokinetic analysis of the JB-1 ascites tumour at different stages of growth. *Cell Tissue Kinet.*, **6**, 347–357 (1973).
23) Mushika, M., Sakakibara, T. and Kaneda, T. Comparative study of immunostaining of trophoblastic epithelium with monoclonal antibodies to three proliferation-associated antigens, DNA polymerase $\alpha$, Ki-67, and PCNA/cyclin. *Igaku no Ayumi*, **161**, 437–438 (1992) (in Japanese).
24) Raza, A., Yasin, Z. and Grande, C. A comparison of the rate of DNA synthesis in myeloblasts from peripheral blood and bone marrows of patients with acute nonlymphocytic leukemia. *Exp. Cell Res.*, **176**, 13–19 (1988).
25) Dean, P. N. A simplified method of DNA distribution analysis. *Cell Tissue Kinet.*, **13**, 299–308 (1980).
26) Barlogie, B., Spitzer, G., Hart, J. S., Johnston, D. A., Buchner, T., Schumann, J. and Drewinko, B. DNA histogram analysis of human hemopoietic cells. *Blood*, **48**, 245–258 (1976).
27) Dolbeare, F., Gratzner, H., Pallavicini, M. G. and Gray, J. W. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA*, **80**, 5573–5577 (1983).
28) Gratzner, H. G. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science*, **218**, 474–475 (1982).
29) Dow, L. W., Chang, L. J. A., Tisiatis, A. A., Melvin, S. L. and Bowman, W. P. Relationship of pretreatment lymphoblast proliferative activity and prognosis in 97 children with acute lymphoblastic leukemia. *Blood*, **59**, 1197–1202 (1982).