Cytologic Studies of Tumors. L. Clonal Proliferation of Four Stemlines in Three Hematopoietic Tissues of a Patient With Reticulosarcoma

By Yoshitaka Obara, Motomichi Sasaki, Sajiro Makino and Chikara Mikuni

A case of reticulosarcoma was found to have four chromosomally different stemlines in peripheral blood, lymph node and bone marrow. The four lines are recognized to be of monoclonal origin on the basis of the closely allied karyotypes characterized by trisomy for E16, E17 and E16, the loss of one A1, two F and two to four C elements in addition to M and R markers. The markers characterize three kinds of hematopoietic tissues: an M1, R1, R2 line occurs in lymph node, and an M1, M2, M3, R1 line in peripheral blood and M1, M4 and M1, M2, M3 lines in bone marrow. Cytogenetic variability of malignant cells was highest in peripheral blood. Information was provided to suggest that of the four stemlines, the lymph node line may be original, the peripheral blood line may be a derivative of the lymph node line and, finally, two marrow lines may have originated from the blood line or from one of the mutated cells slightly different from the marrow lines. The M markers were discussed in relation to malignant evolution of hematopoietic cells of this patient. The suggestion was made that the chromosomes of the patients with malignant hematopoietic disorders should be examined in different tissues.

Recent Advances in blood culture techniques for human cytogenetic studies have afforded an increasing appreciation of chromosomal pictures in hematopoietic diseases, with an extension of knowledge for an evaluation of etiological factors or causes of those disorders.21, 22

In some cases of reticulosarcoma and Burkitt's lymphoma rather specific chromosome anomalies have been described, though these have not been as consistent as the abnormality of the Ph1 chromosome in chronic myelogenous leukemia.2,13, 27 On the other hand, there are reports of some human tumors in which more than one stemline coexisted, and the mechanism of clonal evolution in the course of tumor development has been discussed.2, 7, 8 Currently available cytogenetic contributions are, thus, useful as basic criteria essential for understanding the pathogenetic features and for causal analysis of neoplastic disorders.

The present paper reports the occurrence of four different monoclonal stem-
lines in lymph node, peripheral blood and bone marrow of a patient with reticulosarcoma.

**MATERIALS AND METHODS**

Venous blood, bone marrow and lymph node samples for the chromosome study were collected during a period from April 19, 1969 to July 14, 1969.

Peripheral leukocytes and marrow cells of the patient were cultured according to a modified method of Moorhead et al.16 Two types of cultures were set up: one with the addition of phytohemagglutinin-M (PHA) at a final concentration of 0.02 ml./ml. culture medium and another without PHA. The cultures were incubated for approximately 72 hours at 37°C. After colchicine treatment during the final 1.5 hours of incubation, cells were harvested, treated with a KCl hypotonic solution for 15 minutes and fixed with acetic acid methanol (1:3).

In addition to the cultures, direct preparations of marrow cells were made according to Tjio and Whang28 with a minor modification. The marrow aspirates were incubated with NCTC-109 culture medium containing 25 per cent calf serum and 10⁻⁷ M colchicine for 2 hours at 37°C. A piece of biopsy specimen from the cervical lymph node of the patient was thoroughly minced with scissors and suspended in medium NCTC-109 supplemented with 25 per cent calf serum. After 72 hours of incubation at 37°C with and without PHA, cells were treated with colchicine at a final concentration of 10⁻⁷ M for 1.5 hours. The cells were fixed (1:3, acetic acid : methanol) following hypotonic treatment with KCl solution. Chromosome spreads were made by the flame-drying method and stained with Giemsa. Suitable metaphases were counted, photographed and analyzed in each sample.

**CASE HISTORY**

Female, age 47. Clinical examinations revealed that the patient had several tumors in her abdominal and chest regions at the end of October 1968. The diagnosis of malignant lymphoma was established through biopsy examinations and the patient was hospitalized at the Sapporo National Hospital on December 27, 1968. She had no fever or hemorrhagic tendencies. The liver, spleen and lymph nodes were scarcely enlarged. At the time of hospitalization, no tumor cells were detected in the peripheral blood despite the occurrence of more than 70 tumors, 10 to 20 mm. in diameter, under the skin of the abdominal and chest walls. These tumors completely disappeared following bleomycin treatment for 1 month. From mid-February she suffered from high fever and at the beginning of March, the enlargement of liver and spleen was remarkable. At the same time, the presence of 18 per cent tumor cells was noted in her peripheral blood. By May 9, 1969, the tumor cells decreased to 1 per cent with endoxan treatment. By mid-April, with the enlargement of cervical lymph nodes to pea-size, 18 per cent tumor cells were present in bone marrow. These cells were negative for peroxidase and PAS reactions. Histological examination favored the diagnosis of reticulosarcoma. On and after May 10, she received combined chemotherapy of vincristine, endoxan, 6-mercaptopurine and predonin (VEMP therapy) in lieu of bleomycin treatment. However, the tumor cells increased to 79 per cent in bone marrow by July 14 and to 93 per cent in the peripheral blood by July 22. Death occurred on July 27, 1969 in a state of leukemic reticulosarcoma. Figure 1 gives a summary of chemotherapy and the hematologic data of this patient.

**CYTOLOGIC FINDINGS**

The marker chromosomes noted in the samples of this patient were as follows: M₁, submetacentric larger than A₁ chromosome; M₂, submetacentric smaller than M₁ but larger than A₁; M₃, subtelocentric identical in length with A₃; M₄, metacentric or slightly submetacentric larger than A₁; R₁, ring chromosome possibly of C origin; and R₂, ring chromosome smaller than R₁.
**Fig. 1.**—A summary of the therapeutic and hematologic data of the patient under study.

**Fig. 2.**—Karyotype of lymph node cell showing M1, R1 and R2 markers. Note trisomy for E16, E17 and E18 and the loss of one A1, two C and two F chromosomes.

**Lymph Node**

On April 19, 1969, cells from an enlarged cervical lymph node were cultured with and without PHA in suspension for 3 days. In PHA-treated cultures, four out of 70 mitotic cells showed an abnormal chromosome constitution remarkable by the existence of M1, R1 and R2 markers. Also, these cells were characterized by trisomy for E16, E17 and E18, the loss of one A1, two F, and two to three C chromosomes (Fig. 2). One cell was found to have only M1 and R1 markers along with an extra B element in lieu of an A2 chromosome, in addition to the above abnormal constitution. In the cells without the markers, two cells showed structurally anomalous chromosomes: one had an acrocentric chromosome apparently identical in size with C elements in lieu of an A3 chromosome, while the other contained one chromosome similar in size and shape to E18 chromosomes in place of a C element. Most of the remaining 63 mitotic cells were karyotypically normal, except for several cells which were hyper- or hypodiploid and showed random addition or loss of chromosomes. An accurate percentage of stemline cells in the lymph node of the patient was...
Fig. 3.—Karyotype of peripheral blood cell showing M1, M2, M3 and R1 markers. Note trisomy for E16 and E17 and the loss of one A1, one A2, three C and two F chromosomes.

not obtained due to bacterial contamination of the culture without PHA. However, the stemline cells constituted 7 per cent of the PHA-treated culture.

Peripheral Blood

Chromosomes were studied twice in leukocyte cultures with and without PHA, on April 19 and June 28, 1969.

Examination on April 19. In the sample from PHA-treated cultures, the majority of mitotic cells was chromosomally normal: only one out of 56 cells had a karyotype with a heteromorphic A1 chromosome pair, most probably as a normal variation. We failed chromosomal examination in the non-PHA culture due to bacterial contamination.

Examination on June 28. The sample from PHA-treated cultures showed three abnormal cells with markers in 56 metaphases studied. Furthermore, the sample showed the occurrence of three hypotetraploid cells with 77–83 chromosomes containing tricentrics, dicentrics, acentrics, fragments, subteloacentrics and other abnormalities. Most probably, they had been produced prior to the doubling of the chromosome number.

The situation is quite different in the non-PHA culture. There was one predominant line (60%) carrying M1, M2, M3 and R1 markers. Those cells showed in addition to the above four markers, the following chromosome abnormalities: trisomy for E16 and E17, the loss of one A1, one A2, two F and two to four C chromosomes (Fig. 3). Considering the karyological similarity, they seem to be derived from the lymph node line. There were several mitotic cells with various abnormal chromosome constitutions. Most probably they are derivatives from the lymph node or peripheral blood lines (Table 1).

Bone Marrow

Direct preparations of marrow cells were made twice, on June 28 and July 14, 1969. In the June sample, 10 mitotic cells were studied and one of them was found to have 46 chromosomes which were characterized by two markers (M1 and M4), trisomy for E16, E17 and E18, the loss of one A1, two F and two
Table 1.—Summary of Data Showing the Variability of Marker Chromosomes in Different Tissues of a Reticulosarcoma Patient Under Study

<table>
<thead>
<tr>
<th>Date of Sampling</th>
<th>Tissue</th>
<th>PHA</th>
<th>Days In Vitro</th>
<th>Markers</th>
<th>Total Number of Cells Observed</th>
</tr>
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<tbody>
<tr>
<td>April 19, 1969</td>
<td>L.N.</td>
<td>+</td>
<td>3</td>
<td>*M1, R1, R2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1, R1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>65</td>
</tr>
<tr>
<td>April 19, 1969</td>
<td>P.B.</td>
<td>+</td>
<td>3</td>
<td>M1, M2, M3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1, M1</td>
<td>1</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>M1, R1, Ac</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>53</td>
</tr>
<tr>
<td>June 28, 1969</td>
<td>P.B.</td>
<td>-</td>
<td>3</td>
<td>M1 only</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1, R1, centric frag.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M1, M2, M3, R1</td>
<td>1</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>M1, M2, R2, C(?)</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>M1, M2, R2, frag.</td>
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<td></td>
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<td>*M1, M2, M3, B1</td>
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<td></td>
<td></td>
<td></td>
<td>M1, M2, centric frag.</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
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<td>3</td>
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<tr>
<td>June 28, 1969</td>
<td>B.M.</td>
<td>-</td>
<td>0</td>
<td>M1, M4</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>July 14, 1969</td>
<td>B.M.</td>
<td>-</td>
<td>0</td>
<td>*M1, M1</td>
<td>36</td>
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<td></td>
<td></td>
<td>*M1, M2, M3</td>
<td>9</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>M1, M3, R1, R2</td>
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<td></td>
<td></td>
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<td>4</td>
</tr>
</tbody>
</table>

M1, M2: large submetacentric markers; M3: subtelocentric marker; R1, R2: rings; Ac: acentric chromosome; L.N.: lymph node; PB.: peripheral blood; B.M.: bone marrow.

* Predominant cell lines in each tissue.

C elements, while the remaining nine were without markers. Such abnormal cells became predominant in the July sample; 35 out of 50 mitotic cells (70%) contained allied abnormalities (Fig. 4). There was one cell having M1, M1 and one Ph-like chromosome in addition to other unusual elements. Cells characterized by M1, M2 and M3 markers and trisomy for E16, E17 and E18, the loss of one A1, one A2, two F and two to four C elements occurred, forming another line. Furthermore, one cell contained M1, M3, R1 and R2 markers, similar in general appearance to those found in one abnormal cell from peripheral blood. There were four metaphases which showed a normal chromosome constitution. Marrow cultures with and without PHA showed an extremely low mitotic index, and there were six cells having no markers in both types of cultures.

It was found that one of the C group chromosomes, possibly No. 6, was characterized by a prominent constriction of the long arms near the centromere.
region in most cells with $M_1$ and $M_2$ markers (Fig. 4). The constriction was easily identified and morphologically distinguishable from the secondary constriction of No. 6 and No. 11 chromosomes as reported by Sasaki and Makino\cite{23} and that of No. 9 as described by Saksela and Moorhead.\cite{19} In general appearance, it is rather similar to that of No. 7 described by Sasaki and Makino.\cite{23} No answer was obtained in this study for the question as to whether this constriction is of artificial nature or an anomaly characteristic of the malignant marrow cells of this patient.

**Discussion**

Tumor cases with more than one stemline in the same host have been reported in humans and some other mammals,\cite{6,10,11,15} but the question has remained unanswered as to whether these lines are monoclonal or polyclonal in origin. Recently, with improved methods of chromosome spreading, evidence has been presented that in some instances of multi-stemline tumors, the karyotypes of different stemline cells contained a common marker or markers.\cite{2,7,17,18} This suggests that these lines are of monoclonal origin or derivatives of a pre-existing or still-existing stemline predecessor which has participated in the tumor formation. On the other hand, Gartler et al.\cite{8} described three cases of hereditary trichoepithelioma which showed marked intratumor variations of G6PD levels between different samples from the same tumor in the heterozygote. They interpreted the cases as indicating a multicellular origin of these tumors.

The present case of reticulosarcoma showed chromosomally distinct stemlines confined to each hematopoietic tissue: a line having $M_1$, $R_1$, $R_2$ markers in lymph node, a line characterized by $M_1$, $M_2$, $M_3$, $R_1$ markers in peripheral blood and two lines showing $M_1$, $M_4$ and $M_1$, $M_2$, $M_3$ markers in bone marrow. A plausible interpretation is that these lines were derived from a common line, because the karyotypes closely resemble each other, namely, the existence of
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the M1 marker and the loss of one A1, two F and two to four C elements, and trisomy for E16-E17.

The marrow observations are of particular interest, e.g., the cells with markers showed a rapid proliferation within a short period of time and yielded some cytogenetic evidence for evolutionary aspects of tumor cells. The marrow sample obtained in June showed the occurrence of the M1, M4 cells in a small proportion. In the July sample, however, the M1, M4 cells appeared in about 72% of the mitotic marrow cells, while another cell line having M1, M2, M3 markers showed 18 per cent (Table 1). It seems reasonable to consider that the M1, M2, M3 line was not derived from the M1, M4 line, but either from the M1, M2, M3, R1 stemline or from the M1, M2, M4 line in the peripheral blood cell population, and that the M1, M4 line originated from a new variant selected from certain clonal derivatives. In the June sample, the majority of the mitotic cells (60%) had M1, M2, M3 and R1 marker chromosomes in blood cultures without PHA, while the marrow cells did not show such a combination of markers (Table 1). On the other hand, the blood cultured with PHA on April 19 showed cells with no markers, whereas the lymph node cultured with PHA showed cells having M1, R1 and R2 (or M1, R1) markers in five out of 70 cells observed (Table 1).

From the above findings, a tentative explanation in favor of monoclonal development of stemlines in three hematopoietic tissues can be advanced and, (1) the original line of the four stemlines may be the M1, R1, R2 line occurring in lymph node, (2) the malignancy may be primarily related to the lymph node cells, and (3) that the descendants of the latter cells resulted in the formation of another line in the peripheral blood favorably adapted to its environment. Possibly, further mutated cells, such as those without ring markers, may constitute two lines in marrow. Table 1 summarizes the cytogenetic variability of the malignant hematopoietic cells in three different tissues from this patient. It is noteworthy that the extensive variability of cells with the markers was shown in the peripheral blood cultures rather than in lymph node or bone marrow. It is conceivable that certain environmental or physiological factors may be dissimilar in the three hematopoietic tissues, resulting in chromosomal changes different in the three tissues. For instance, differential responses of tumor cells to therapeutic agents in different tissues or different aspects of the population equilibrium in different disease stages, such as in remission and crisis could be factors which affect the karyotypic stability. It was reported in chronic myelogenous leukemia that, in the stage of remission, Ph-positive cells disappeared from peripheral blood but persisted in bone marrow,20,21 while in the blastic crisis many aneuploid cells occurred in both blood and marrow.24,25 Recently, we have encountered two malignant lymphoma cases which showed different chromosome constitutions in different tissues. Whether or not the higher variability in the stemline karyotype is a common feature of certain malignant lymphoid tumors is unknown at present, but our results still suggest the importance of chromosomal investigations in different tissues in many cases of hematopoietic disorders.

In the present case, the M1 marker was the most stable among the four lines
and the M₃ marker was relatively stable in peripheral blood. Reticulosarcoma and Burkitt's lymphoma have been reported to show cytogenetically allied abnormalities, such as the large submetacentric and/or subtelocentric markers and the loss of A group chromosomes.²,⁵,¹₃,¹⁴,²⁴,²⁷ It appears that morphologically, the marker M₁ found in the present case is similar to large submetacentric markers in three cases of reticulosarcoma reported by Atkin and Baker,² Chu et al.,⁵ and Kajii et al.¹⁴ Furthermore, the M₃ of our patient seems to be similar in appearance to the marker d₁ reported in a reticulosarcoma patient by Sasaki et al.,²¹ and to the marker M₂ in a reticulosarcoma patient by Atkin and Baker.² It seems, however, that subtelocentric and large submetacentric markers may not always be specific to reticulosarcoma cells, since markers of a similar type have been described in several other malignant diseases, such as in uterine cervical carcinoma by Atkin,¹ and Atkin and Brandao,³ in pleural effusion of metastatic cancer of the bladder by Spriggs et al.,²⁶ in carcinoma of the colon by Ishihara et al.¹² and Yamada et al.,³ in papillary adenocarcinoma by Atkin and Baker.²

We observed in the present case three cells with 77–83 chromosomes containing tricentrics, dicentrics, acentrics, fragments and other abnormalities in a PHA-treated blood culture (June 28, 1969). Of interest is that the patient was treated with bleomycin, together with VEMP therapy (Fig. 1). We have observed chromosome abnormalities of a similar nature in another two bleomycin-treated patients, one with Hodgkin's disease and another with mycosis fungoides. Most probably, these abnormal chromosomes observed in the PHA-treated blood culture may be produced through bleomycin therapy. The effect of bleomycin in connection with the induction of chromosome abnormality is a subject for further study. The work is now in progress in our laboratory.

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REFERENCES


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