Chapter V

Clinical applications of flow karyotyping in myelocytic leukemia by stimulation of different subpopulations of cells in blood and bone marrow samples

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5.1 Abstract

Examples are presented in which normal as well as abnormal chromosome distributions could be obtained from the same individual by means of bivariate flow karyotyping. Selective stimulation of T-lymphocytes obtained by E-rosetting from the blood of a patient with acute myelocytic leukemia resulted in a normal flow karyogram. The specific stimulation of myelocytic leukemia cells with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) yielded flow karyograms displaying the leukemia-associated chromosome abnormalities. The resulting flow karyograms could be used to discriminate between homologue differences, which appear normally in virtually every individual, and leukemia-associated chromosomal aberrations. In the case of a female chronic myelocytic leukemia patient who received bone marrow from an HLA-identical male donor, specific stimulation of various subsets of cells enabled to discriminate between leukemic host cells and non-leukemic donor cells. Both the leukemia specific translocations and sex chromosomes were used as markers to analyze the flow karyograms obtained from the same sample.

5.2 Introduction

Flow karyotyping is a well-established method of analyzing chromosomes in suspension by means of a flow cytometer, resulting in either uni- or bivariate flow karyograms. In these karyograms virtually all human chromosomes can be discriminated on the basis of their size and base pair ratio (Langlois et al. 1982, Van den Engh et al. 1985, Arkesteijn et al. 1986, Harris et al. 1986, Arkesteijn et al. 1987, Harris et al. 1987, Arkesteijn et al. 1988, Van den Engh et al. 1988). The resulting chromosome clustering pattern is characteristic for the species from which the chromosomes are derived. Whenever a change in position of one of the chromosome clusters occurs due to an event through which the DNA content is altered (i.e. a translocation, deletion or amplification) this will show in the flow karyogram by a newly appearing peak or cluster and/or a quantitative reduction of the chromosome clusters that are involved in this event (Arkesteijn et al. 1988). Whether two chromosomes can be seen as separate clusters mainly depends on the quality of the flow karyogram. The coefficient of variation that is usually obtained in bivariate flow karyograms from leukemia patient material allows the detection of separate chromosomes that differ approximately 2% in DNA content.

Within each species, provided it is not inbred, the flow karyogram will display differences in the DNA content between the homologues of certain chromosomes. This phenomenon can be observed very well in uni- and bivariate flow karyograms. For man the largest variations occur in the chromosomes 1, 9, 16, and Y although in a bivariate flow karyogram almost every chromosome pair shows some degree of disparity.

When the difference between two homologues is large enough this will show in the bivariate flow karyogram as two separate clusters. Such homologue differences will contribute to the variation in the average standard position of the chromosome clusters in the bivariate flow karyogram (Gray et al. 1988). By means of family flow karyotyping it is possible to trace the origin of the homologue. Such investigations may shed light on inheritable diseases. (Harris et al. 1987, Van den Engh personal communications).

Whenever two clusters are observed for one chromosome in cases where altered cluster positions can be expected, like in the case of leukemia, additional analysis focusing on homologue differences is essential to identify small aberrations. The only feasible way to discriminate homologue differences from leukemia-specific chromosomal aberrations is by comparing this flow karyogram with the flow karyograms from first line relatives or, as is demonstrated in this study, with the flow karyograms of normal cells from the same patient.

Leukemia cells can be stimulated to proliferate in vitro by the addition of specific growth factors to the culture medium (Griffin et al. 1986, Delwel et al. 1987, Vellenga et al. 1987). We therefore made use of the possibility to specifically stimulate either the myelocytic leukemia cells with GM-CSF and IL3 or the normal T-cells with phytohemagglutinin (PHA). The flow karyogram from the PHA stimulated cells will indicate the normal homologue positions. Comparison of this with the flow karyogram from the GM-CSF and IL3 cultured cells will exclude homologue differences from leukemia associated chromosome aberrations.

Quantitative cluster analysis reveals which of the chromosomes are involved in a translocation, amplification or deletion. In this paper an example is given of a patient with acute myelocytic leukemia (AML) characterized by the loss of the Y chromosome as well as other rearrangements. Fluorescence in situ hybridization (FISH) with a Y specific DNA probe confirmed the loss of the Y chromosome in this particular patient. The procedure, which aims at specific stimulation of both normal and leukemic cells from blood or bone marrow also enables to determine the emergence or the persistence of relapsing leukemia and state of chimerism in (sex-mismatched) bone marrow transplant recipients. Here we report on a female patient with chronic myelocytic leukemia (CML) who received a bone marrow transplant from a male donor. At relapse, four years after transplantation, two cell populations could be distinguished by means of flow karyotyping; one of normal donor origin (indicated by the presence of the Y-chromosome) and one of leukemic origin (indicated by the presence of the Philadelphia chromosome and the absence of the Y-chromosome).

5.3 Materials and methods

Patients

A male patient (patient a) was diagnosed with acute myelocytic leukemia (AML; FAB classification M1). He was treated with daunorubicin and cytosine arabinoside. Blood samples were taken for flow karyotyping and FISH.

A female patient (patient b) who was diagnosed with chronic myelocytic leukemia (CML) was transplanted during the chronic phase with bone marrow from her HLA-identical brother. Four years after transplantation she showed clinical signs of an imminent relapse. At this time blood and bone marrow samples were taken for flow karyotyping and FISH.

Sample treatment

Either blood or bone marrow was collected in sterile heparinized flasks. After collection and during the whole process of cell separation the samples were kept at 4 C. Cells were isolated as described previously (Arkesteijn et al. 1988). In brief, nucleated cells were collected by layering either blood or bone marrow on lymphocyte separation medium (LSM; Organon, Durham, NC) and spun down in a table top centrifuge for 20 minutes at 700 G. Interphase cells were collected and washed twice in Hanks HEPES Buffered Balanced Salt Solution (HHBBSS). Only in the case of the male patient, in addition to this, the resulting cell suspension was mixed with 2-aminoethyl isothiouronium boride (AET)-treated sheep red blood cells (SRBC). This suspension was layered on LSM and spun down for a second time at 700 G for 20 minutes. The cell fraction originating from the AET-SRBC rosettes in the pellet was called the T-cell enriched fraction, the cell fraction originating from the interphase layer was called T-cell-depleted. Prior to culturing of the T-cell enriched fraction, the AET-SRBC were lysed by treating them for a short period with a buffer consisting of NH_4Cl 155 mM, EDTA 0.1 mM, and $NaHCO_3$ 11.9 mM and washed twice in HHBBSS.

Cell cultures

Cells were cultured in Alpha modified Eagle's medium. T-cells were cultured in the presence 10% fetal calf serum (in case of patient a, only the T-cell-enriched fraction; in case of patient b, a part of the total cell suspension). These cells were stimulated with 1% PHA.

Leukemic cells were stimulated with human recombinant GM-CSF (patient a and b,10 U/ml) and human recombinant IL3 (only patient a, 10 ng/ml). In the case of patient a, factors were added to only the T-cell-depleted fraction, and in patient b a part of the total cell suspension. IL3 was obtained from Gist-Brocades NV Delft NL, GM-CSF was obtained from Biogen SA, Willemstad NL-Antilles. After four days in culture at 37 C, 100% humidity and 5% CO_2, 250 ng/ml Tubulazole-C (Janssen Life

Science Products, Beerse, Belgium) was added during the last 4 hours of culture to accumulate cells in mitosis.

Chromosome isolation

Chromosomes were isolated as described previously (Arkesteijn et al. 1988) with some slight modifications. Cells were spun down on a discontinuous density gradient (Nycodenz, Nyegaard, Oslo, N.) containing 1% BSA in 4 ml plastic tubes at 4 C with a speed of 700 G The density of the upper layer was 1.062 g/cm^3 and the bottom layer was 1.080 g/cm^3. Mitotic T-cells were mainly found on the 1.080 layer whereas the mitotic leukemic cells layered mostly on top of 1.062. Dead cells and debris were found in the bottom fraction of the tube. The cells from the various densities were washed twice in cold HHBBSS. The washed cells were resuspended in prewarmed (37 C) swelling buffer (20 mM NaCl, 8mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5). After 2 - 5 minutes at 37 C the cells were lysed by the addition of an equal volume of Triton-X-100 (0.8% in water). The suspensions were then rapidly cooled in melting ice. The chromosomes were stained with Chromomycin A3 (CA3; 26 ng/ml) and DAPI (5.4 ng/ml, patient a) or CA3 and Hoechst 33258 (Ho; 5.4 ng/ml, patient b). Chromosome suspensions were either measured on the same day or stored at -20 C after the addition of dimethyl sulfoxide (DMSO; 10% final concentration). Frozen chromosome suspensions could be stored for weeks, defrosted, and refrozen again without any visible loss of resolution in the flow karyogram.

Flow karyotyping

Chromosomes were measured on the RELACS III, a dual-beam flow cytometer especially designed and built at the Radiobiological Institute TNO for the measurement of chromosomes in suspension (Van den Engh and Stokdijk 1989). CA 3 was excited with a laser tuned at 458 nm at 250 mW (Coherent Innova 90, Palo Alto, CA). Ho and DAPI were excited in the UV range (351 and 364 nm) at 350 mW laser power (Spectra Physics series 2000, Mountain View, CA). CA3 fluorescence was measured through a 550 nm longpass filter (LL550 Corion Corp., Holliston, MA). The fluorescence from Ho or DAPI was measured through two KV 408 filters (Schott Glaswerke, Mainz, Ger.). The CA3 fluorescence was used as a trigger signal. Data were stored in listmode on a computer (Hewlet Packard, series HP 9000). Further analysis was performed using the ELDAS software package (Jonker et al. 1987). Flow karyotyping as well as conventional cytogenetic analysis was done in a double blind fashion.

Fluorescence in situ hybridization (FISH)

In situ hybridization was performed on nuclei obtained from patient a and b after the first LSM treatments (i.e., after elimination of erythrocytes and granulocytes). Cells were fixed in methanol/acetic acid (3 : 1) and dropped on glass slides. The in situ hybridization and fluorescent detection protocol was described previously (Pinkel et al. 1986, Van Dekken et al. 1988). The human Y-specific repeat pY2.45 was obtained

commercially (Amersham Int., Amersham, UK). This satellite DNA probe hybridizes to the large block of heterochromatin found on the long arm of the Y chromosome (Cooke et al. 1982). Complete plasmid DNA was labeled with biotin-11-dUTP using the BRL-nick translation kit (BRL, Gaithersburg, MD) according to the manufacturers directions. The hybridized probe was detected using avidin-FITC. Nuclei were counterstained with propidium iodide (PI; Sigma, St. Louis, MO) to allow simultaneous observation of total DNA and hybridized probe. The DNA stain was used at a concentration of 1 µg/ml in an antifade solution containing p-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) to preserve the fluorescein fluorescence during microscopy (Johnson and Nogueira Araujo 1981).

5.4 Results

The flow karyograms from the AML patient (a) are shown in Figure 5.1. The differences between the karyogram derived from the GM-CSF/IL3- and the PHA-stimulated cells are indicated by solid arrows, the (homologue) clusters that appear in both karyograms are indicated by open arrows. The left panel shows the flow karyogram that was obtained from the T-cell-depleted fraction of nucleated peripheral blood cells from this male patient. The cells were stimulated in vitro with GM-CSF and IL-3.

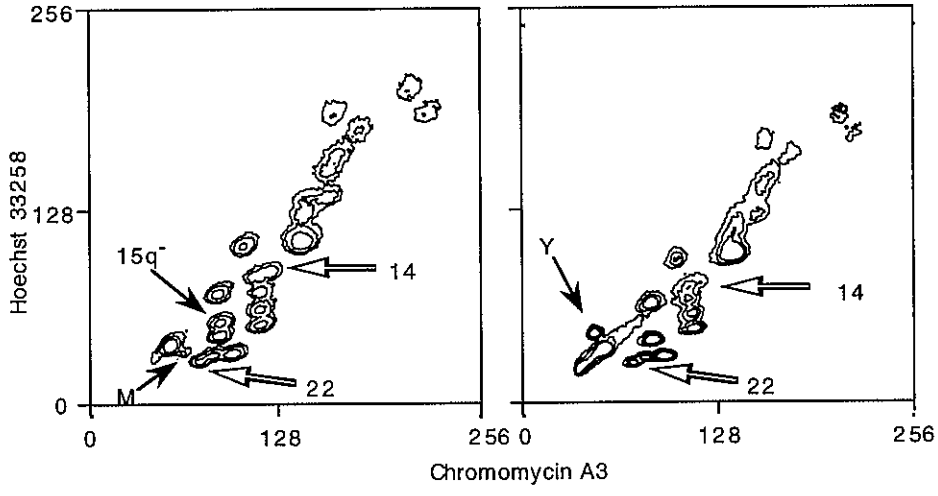

Figure 5.1
Bivariate flow karyograms from the same blood sample of an acute myelocytic leukemia patient (patient a).
Left panel: T-cell-depleted fraction stimulated with GM-CSF and IL3.
Right panel: T-cell enriched fraction (SRBC) stimulated with PHA.
Open arrows point to similarities in both flow karyograms, solid arrows point to differences between leukemia and normal cell types.
A limited amount of contour lines was drawn at levels that showed most optimal cluster separation.

A number of abnormalities where found in this flow karyogram i.e., the absence of the Y chromosome, an extra cluster closely above chromosome cluster 20 (indicated as 15q-) and a cluster close to chromosome 21 (indicated as M). Quantitative analysis of the chromosome clusters revealed the absence of one of the homologues in the cluster for chromosome 15. As can be seen in this karyogram a few clusters might be pointed out as being either chromosome abnormalities or homologue differences (arrows) i.e., two clusters on the position of chromosome 22 and the separation in two clusters for chromosome 14. The flow karyogram derived from the PHA-stimulated T-cell-enriched fraction in the right panel clearly shows the presence of the Y chromosome in the normal cells. Furthermore the clusters indicated by M and 15q- close to chromosome 20 and 21 are absent in the right panel, whereas the double clusters for chromosome 22 and 14 are present in both flow karyograms. Therefore, the latter two relate to homologue differences.

The (reciprocal) exchange of DNA, resulting in a linear shift in the number of channels to a new position in the flow karyogram, is inversely correlated to the channel shift for the second cluster in the translocation event. Therefore the aberrant cluster pattern cannot be explained by a translocation between chromosomes 15 an Y. In such a case a second new cluster would have appeared. No such cluster can be found in this karyogram. These observations strongly suggest that the Y chromosome is missing and that a part of one of the homologues of chromosome 15 is deleted. Calculations of the decrease in channel number of this new cluster compared to the original position of the #15 chromosomes indicates that 25% of the DNA is deleted. On the basis of quantitative analysis in the flow karyogram it was found at least 80% of the metaphases contained two of these partially deleted chromosomes while the remaining 20% contained one copy. The origin of the cluster indicated as M which was present in 20% of the metaphase cells analyzed by flow karyotyping remained unclear.

The loss of the Y chromosome was confirmed by the use of in situ hybridization with a Y specific probe to nuclei from unstimulated peripheral blood cells. Y chromosomes could be detected in only 5% of the nuclei of peripheral blood cells (Figure 5.2).

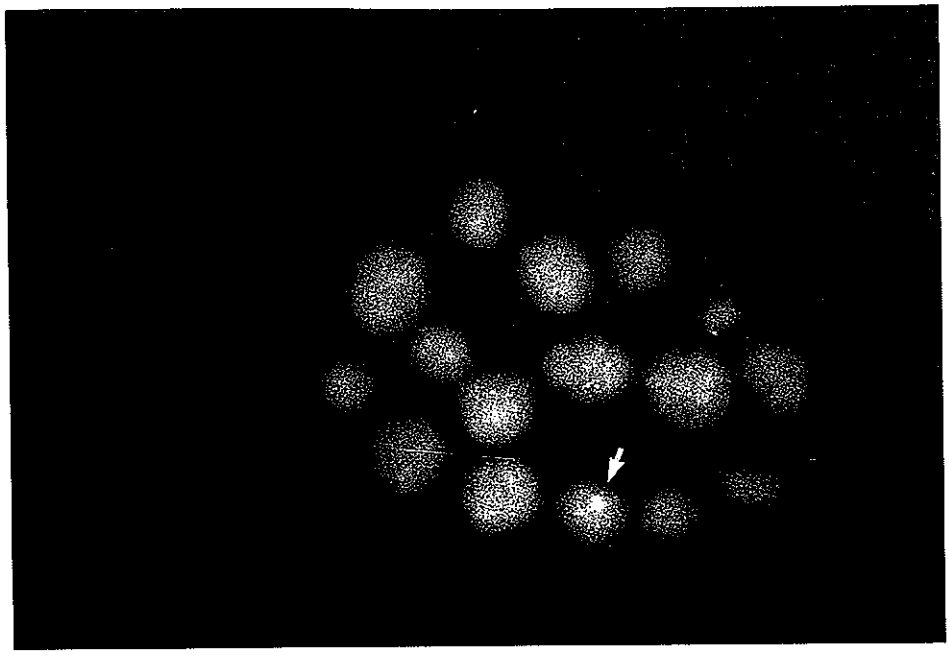

Figure 5.2
Photomicrograph, showing fluorescence in situ hybridization with a Y specific DNA probe on whole nuclei from patient a. Only a small fraction (5%) bears the Y chromosome as is visualized by a clear white spot (arrow). Nuclei are counterstained with propidium iodide (grey). Magnification 40 X objective

These results match the conventional cytogenetic analysis of this patient very well. In 83% of the metaphases the loss of the Y chromosome was noticed and a large deletion was found for the q arm of chromosome 15. In the conventional cytogenetic analysis which was derived from 31 metaphase spreads, no marker chromosome was found that could explain for the cluster that was indicated by M in the flow karyogram.

The flow karyogram from the sex-mismatched female bone marrow transplanted patient (b) is shown in Figure 5.3. In the right panel the normal flow karyogram from the male donor origin can be seen after stimulation of the cells with PHA, as can be recognized by the Y chromosome. In the left panel the Philadelphia chromosome can be seen as indicated. The Y chromosome is absent in this panel.

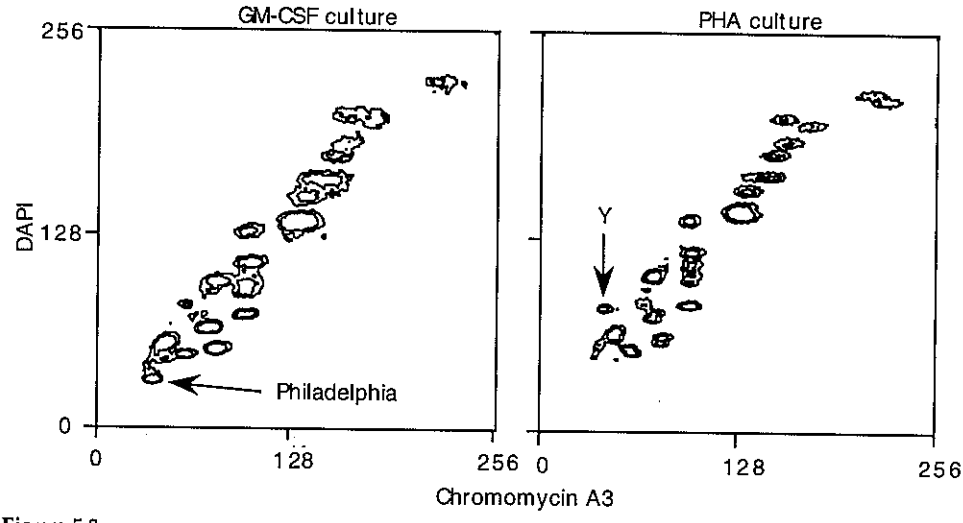

Figure 5.3
Bivariate flow karyograms from the same blood sample of a female CML patient four years after a sex-mismatched bone marrow transplantation.
Left panel: GM-CSF simulated cells.
Right panel: PHA stimulated cells.
The leukemia- and donor derived chromosomes are indicated by arrows.
A limited amount of contour lines was drawn at levels that showed most optimal cluster separation.

In situ hybridization to mononuclear peripheral blood cells of this patient revealed the presence of a Y chromosome in 68% of the cells (Figure 5.4)

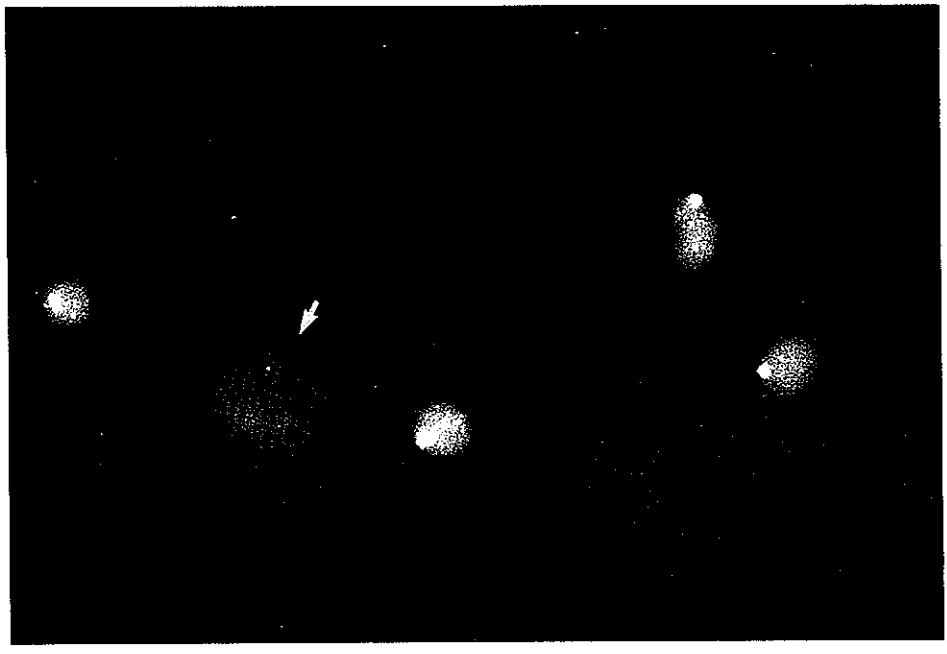

Figure 5.4
Fluorescence in situ hybridization with a Y specific DNA probe on whole nuclei from patient b. Among the Y-bearing cells of donor type, the presence of host derived leukemic cells (arrow) is evident.

5.5 Discussion

As is demonstrated by this study, leukemia-specific chromosomal aberrations can be detected by flow karyotyping very effectively.

For the analysis of flow karyograms in which small deviations of the expected chromosome position might occur as a result of amplifications, deletions and small translocations, it is very important to have a reference to exclude the possibility that variations in the positions of chromosome clusters in flow karyograms are caused by homologue differences. The flow karyogram from normal cells of a patient can serve as an internal reference for the discrimination between tumor-associated aberrations and homologue differences in this individual; in case of leukemia: the normal T-lymphocytes. The approach followed in this study was to collect the normal T-cells from the blood sample of leukemia patients. Separate culturing and specific stimulation of the T-cell-depleted and the T-cell-enriched fraction and subsequent flow karyotyping revealed whether differences appeared between the two flow karyograms (indicating the presence of leukemic cells). As can be seen in the case of patient a, the presence of leukemia associated aberrations (among which the loss of the Y chromosome) is only noticed in the T-cell-depleted and GM-CSF and IL3

stimulated fraction whereas the presence of the Y chromosome indicates that only normal cells were stimulated in the T-cell enriched fraction. In this way leukemia-associated chromosomal aberrations can be identified.

The possibility of stimulating different subsets of cells also provides a means of examining the chimeric state in (sex-mismatched) bone marrow transplanted patients. Again the difference in chromosomal constitution serves as an indicator for the type of cells that were stimulated and produced the flow karyogram. In the case of patient b, it is apparent that the leukemic cells of recipient origin start to take over while there still remains a fraction of normal donor derived T-lymphocytes. In this case the presence of the Philadelphia chromosome is evident; no homologue of chromosome 22 will appear in this region.

As demonstrated in this study the success of flow karyotyping of leukemia specimens mainly depends on the type of stimulating agent that is used for the outgrowth in vitro of a specific subpopulation in the sample. The specific outgrowth of a certain cell type does not allow to draw conclusions from the flow karyogram about the percentage of a certain type of aberration present in the specimen. It allows one to give a yes or no answer about the presence or absence of a certain subpopulation. Further, flow karyotyping gives insight in the type of aberration that is present. On the basis of this information in situ hybridization on nuclei with selected probes can confirm the aberrations and determine the real percentage of the leukemic fraction in the original sample without the bias of in vitro culturing.

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