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Cold Synchronization for the Study of Peripheral Blood and Bone Marrow Chromosomes in Leukemias and Other Hematologic Disease States

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Summary. A method of cold-shock synchronization of immature granulocytic cells from peripheral blood or bone marrow is described. It is shown that this method provides an increased yield of early metaphases and offers advantages over others currently employed.

The peaks of mitotic activity following the cold-shock treatment differ for patients with acute non-lymphoblastic leukemia (ANLL) and patients with chronic myeloid leukemia (CML) by an interval of 2 h.

This method is considered to be suitable for routine cytogenetic studies on hematological patients.

Introduction

Bone marrow aspirates for chromosomal studies in patients with hematologic disease frequently yield material that is unsuitable for conventional banding techniques. Chromosomes may have a 'fuzzy' character or chromatids appear widely separated. Too often studies are not possible because of 'dry taps' or extremely hypercellular marrow aspirates.

Synchronization of mitotic cells is not a new concept. Numerous physical and chemical methods of inducing synchronous mitoses in mammalian cell cultures have been reviewed (Nias and Fox, 1971; Newton, 1964a and b). Amethopterin synchronization is now used to obtain prometaphase chromosomes from human blood cell cultures (PHA-stimulated) (Yunis, 1976; Yunis et al., 1978).

Amethopterin synchronization of leukemic cells from bone marrow has recently been described by Hagemeijer et al. (1979); however, their mitotic index (MI) following synchronization is low for both acute nonlymphoblastic leukemia (ANLL) and chronic myeloid leukemia (CML), and unpublished observations on amethopterin synchronization performed in our laboratory suggest an optimal time for harvesting of 3.5 h for CML patients and 4.5 h for ANLL.

In this paper we describe a method of temperature-shock synchronization for immature granulocytic cells obtained from bone marrow or peripheral blood (PHA-unstimulated) to increase the yield of early metaphases suitable for solid stain analysis (Giemsa-stained) and banding, and this yield is compared with that obtained from cell cultures not subjected to temperature-shock synchronization. Advantages obtained by using this method of synchronization are a reduction in the fuzzy character of the chromosomes, particularly from some chronic myelogenous leukemia (CML) patients, and a general improvement in the quality of the trypsin-banded metaphases obtained, so that fewer metaphases are required for a complete analysis of all clones present. Serial studies can usually be performed on peripheral blood samples, thereby facilitating studies on cell lines without the necessity of a bone marrow aspiration when not otherwise indicated.

Methods

We collected 20 ml heparinized blood from each of seven patients, three of whom had ANLL (Table 1, Patients 1–3), three had CML (Table 2, Patients 1–3), and one had preleukemia (Table 1, Patient 4). Blood smears were made for each patient and a differential count (myelocytes, promyelocytes, and blasts) performed.

The blood was allowed to settle for 30–60 min at room temperature and the leukocyte-rich plasma (LRP) was transferred to alpha (MEM) medium supplemented with 20% fetal calf serum (Grand Island Biological), 1% antibiotic-antimycotic (Grand Island Biological), and 1% heparin. The cultures were placed in a CO_2 incubator at 37°C overnight before being transferred to 4°C for 8 h and returned to the 37°C incubator.

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Table 1. Mitotic index at peak times for ANLL patients studied

Patient no.[a]	% Immature granulocytes in PB	Mitotic index as a percentage		
		Amethopterin synchronization peak time	Cold-shock synchronization peak time	
			14.5 h	16.5 h
1	36	—	0	23.0 (17 h)
2	39	—	8.0 (14.0 h)	29.5 (17 h)
3	13	—	2.0 (14.0 h)	44.5 (17 h)
4[b]	Less than 1	—	0	50.0 (17 h)
5	27	3.0 (4.5 h)	1.0	8.0
6	35	10.0 (4.5 h)	2.0	5.0
7	67	6.0 (4.5 h)	1.0	8.0

[a] Patients 1—4, initial study, each sample treated with colcemid for a 1-h interval; Patients 5—7, follow-up study, each sample treated with colcemid for a 15-min interval
[b] Preleukemia

Table 2. Mitotic index at peak times for CML patients studied

Patient no.[a]	% Immature granulocytes in PB	Mitotic index as a percentage		
		Amethopterin synchronization peak time	Cold-shock synchronization peak time	
			14.5 h	16.5 h
1	7	—	38.0 (14.0 h)	7.0 (16.0 h)
2[b]	16	—	42.0 (14.0 h)	17.0 (16.0 h)
3[b]	42	—	14.5 (14.0 h)	9.5 (16.0 h)
4	12	—	4.0	1.0
5	26	—	6.0	1.0
6	31	—	15.0	0
7[b]	46	—	21.5	10.0
8	50	11.0 (3.5 h)	17.0	1.0
9	11	17.0 (3.5 h)	26.0	0
10	3	20.0 (3.5 h)	22.0	0
11	11	17.0 (3.5 h)	—	—
12	17	20.0 (3.5 h)	—	—

[a] Patients 1—3, initial study, each sample treated with colcemid for a 1-h interval; Patients 4—12, follow-up study, each sample treated with colcemid for a 15-min interval
[b] CML in blast crisis

An aliquot of each culture was removed every hour after the cold shock was initiated, treated with colchicine for 60 min, and centrifuged to collect the cell pellet. The resuspended pellet was smeared on microscope slides and stained with Wright's stain.

Only the granulocytic cells capable of division were examined and scored (i.e., myelocytes, promyelocytes, and blasts), to ascertain what percentage of these cells showed evidence of being in some stage of mitosis (for most patients studied, 100 dividing cells were analyzed for each aliquot; in patients with lower white blood cell counts or immature granulocyte counts, sometimes only 25 cells could be scored per aliquot).

The procedure described above was repeated, covering the broad 'peak periods,' thereby defining a more specific peak mitotic time (to within 15 min) for the leukemia patients studied (Table 1, Patients 5–7; Table 2, Patients 4–10).

Amethopterin synchronization was tested in parallel with the cold-shock cultures and aliquot samples evaluated in the manner previously described (see Tables 1 and 2 for comparative results).

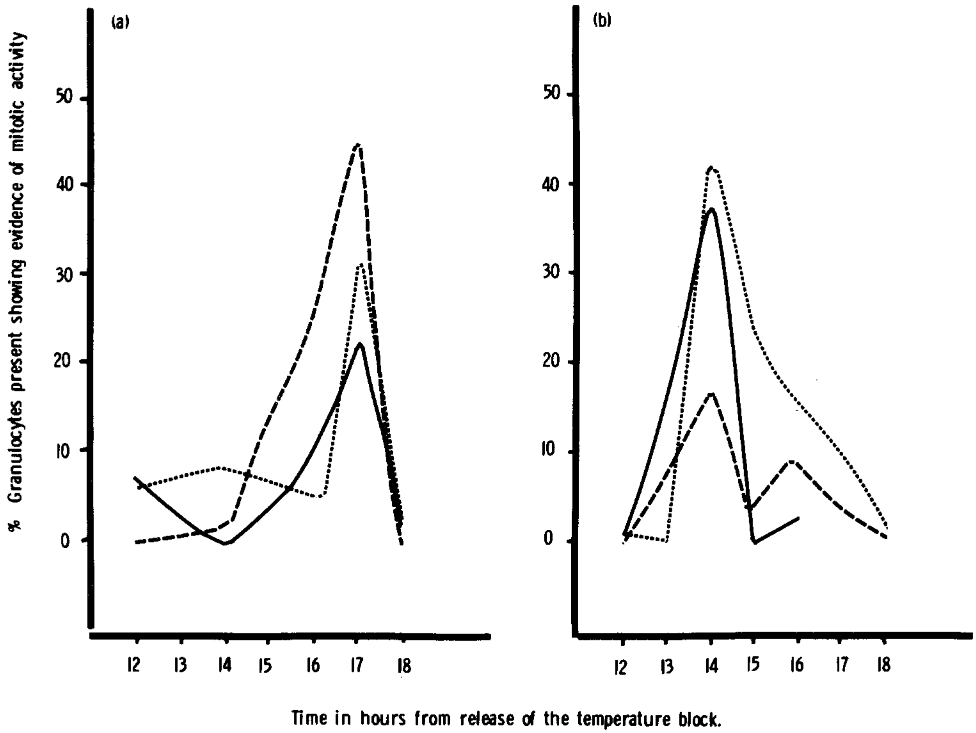

Fig. 1 a and b. Graph of MI after cold-shock synchronization of cells from ANLL patients (a: ——, Patient 1; ....., Patient 2; ---, Patient 3) and CML patients (b: ——, Patient 1; ....., Patient 2; ---, Patient 3)

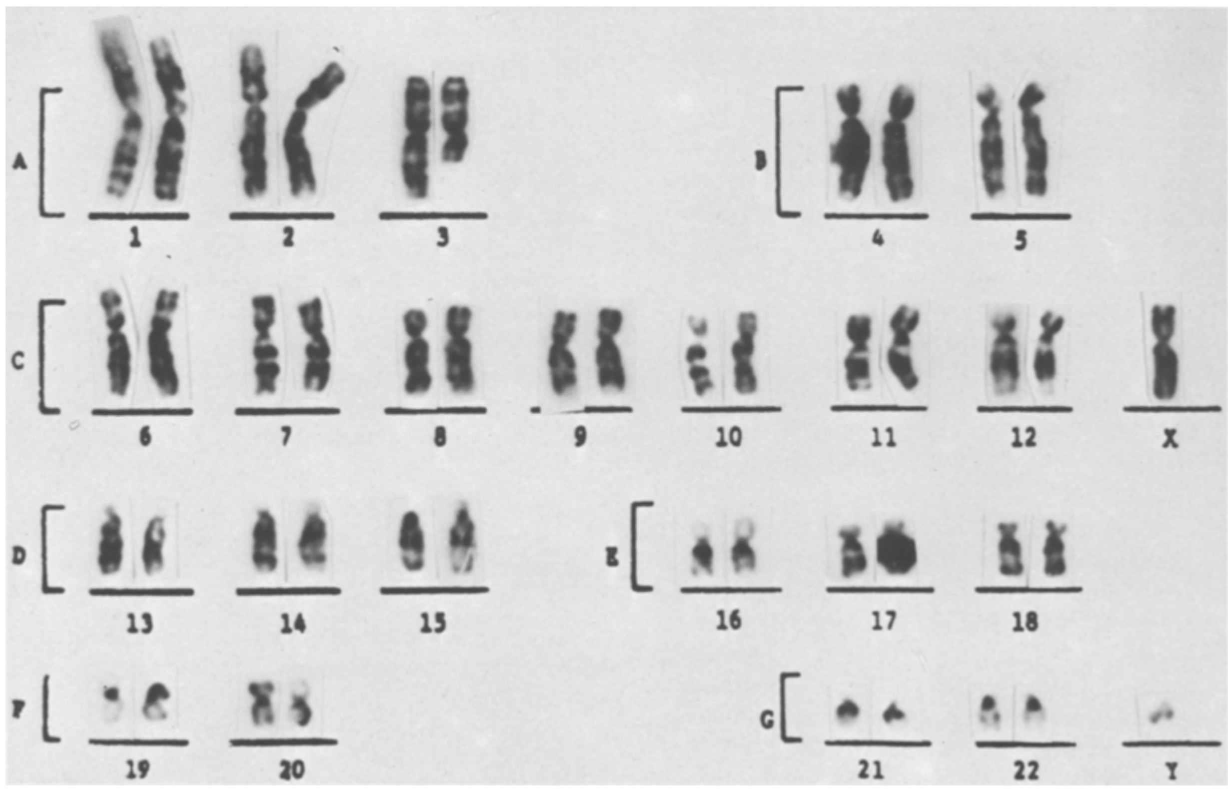

Fig. 2. Karyotype from a patient with ANLL: 46,XY,del(3)(q21),del(Y)(q12)

The critical timing in either synchronization method is the time from the release of the blocking agent to the harvest.

This cold-shock synchronization method has been adopted for routine examination in our studies of patients with hematologic disease. (See Appendix.)

Results

Initially a peak of mitotic activity was found for cells from ANLL patients 16–17 h after termination of the cold shock.

Further examinations over narrower time spans revealed peaks of mitotic activity, for CML cells at 14.5 h and for ANLL and preleukemia cells at 16.5 h after termination of the cold shock (Fig. 1a and b). A cell culture of normal PHA-stimulated lymphocytes (Ficoll-separated) also showed peak mitotic activity 16.5 h after termination of the cold shock.

In comparison, amethopterin synchronization showed peak mitotic activity for CML cells 3.5–4.0 h after release of the block, and for ANLL cells 4.5–5.0 h after release of the block with thymidine.

Discussion

It was noted (Rao and Engelberg, 1966) that immediately after a cell culture is transferred to a subnormal temperature, the MI increases as a consequence of prolongation of the M-phase of the cell cycle with respect to the G_1-, S-, and G_2-phases, and that following release of the temperature block little cell division is noted for a time; this interval approximates to the normal interphase period of the cell cycle. It is in this way that cold shock results in an accumulation of mitotic cells in the state of late telophase (Newton, 1964a).

Amethopterin synchronization, on the other hand, utilizes a thymidine-free state in the culture as a block to cell division in the G_1/S phase (Yunis et al., 1978) so that no nucleotides are available for incorporation into DNA. This blocking action is reversed on addition of thymidine, and a much shorter lag period (i.e., the interval to complete S and G_2) is seen before initiation of cell division.

We have found that a 5-ml sample of heparinized blood is usually sufficient when investigating CML patients, providing the white blood cell count is high enough, with sufficient immature granulocytes capable of division; we have obtained good results from as low as 4%—5% of immature granulocytic cells. When bone marrow aspirates are used 0.1—0.5 ml of the sample is cultured and synchronized.

We believe this method of cold-shock synchronization provides mitoses of at least as good a quality as those obtained with amethopterin synchronization, and a significantly increased MI over nonsynchronized cell cultures, where the average MI was found to be 2.5% (colcemid treatment for 1 h). This synchronization procedure is less costly and less time-consuming than amethopterin synchronization for routine use, and the morphology of the chromosomes is generally improved compared with bone marrow preparations that are not synchronized. Indeed, the quality of the cold-synchronized cultures we have obtained more closely resemble that of PHA-stimulated lymphocyte cultures than that of typical unsynchronized bone marrow cultures (i.e., chromatids generally remain together, metaphase spreading is improved, and the fuzzy character of the chromosomes of some CML patients is generally reduced).

A difficulty that may arise with this technique is a possible broadening of the peak of MI for CML patients in blast crisis (Fig. 1b; Table 1). In this instance, both peak periods should be covered. We have found it advisible in initial studies of all suspected CML patients to harvest at both peak periods, increasing the volume of the material harvested at 14.5 h. Treatment with actinomycin D (5 µg/ml) at the time of colcemid addition can produce more elongated chromosomes.

Figure 2 shows a karyotype from a cold-shock-synchronized culture of immature granulocytes from the peripheral blood (PHA-unstimulated) of a patient with ANLL. Initial studies on bone marrow from this patient were inconclusive—two clones of cells were present, the 3q− cell line was present but almost completely submerged (present in only 1/20 cells) by the 46XY cell line, which alone was banded at this time. Further bone marrow chromosome studies were impossible due to a packed marrow. Immature granulocytes from the peripheral blood were synchronized on two subsequent occasions: initially the 46,XY/46,XY, 3q− clones were revealed, and finally a 46,XY/46,XY, 3q−/46,XY,3q−,del,Yq clonal evolution.

We have been using this method of cold synchronization routinely and successfully and have found it superior to other methods previously tried.

Appendix

Cold-shock Synchronization for Preparation of Peripheral Blood and Bone Marrow Chromosomes

For CML patients 5 ml heparinized blood is usually sufficient, while for acute leukemias, preleukemias, and all other hematologic diseases 10 ml heparinized blood is obtained.

Leukocyte-rich plasma (LRP) is obtained by sedimentation for 30—60 min at room temperature (RT) and is incubated in

alpha (MEM) medium supplemented with 20% fetal calf serum (Grand Island Biological), 1% antibiotic-antimitotic (Grand Island Biological), and 1% heparin in a CO_2 incubator at 37°C overnight (or for up to 24 h).

For all non-CML patients the LRP is transferred to approximately 50 ml alpha (MEM) medium, whereas 0.2—0.25 ml LRP is cultured in 10 ml of alpha (MEM) medium for CML patients.

Samples are transferred to a 4°C refrigerator for a cooling period of 8 h and then returned to the 37°C incubator.

For acute leukemias, preleukemias, and other non-CML hematologic patients, colchicine (0.15 µg/ml) is added 16.25 h after termination of the temperature shock and allowed to act for 10 min before the sample is harvested in the same manner as a routine peripheral blood sample (hypotonic treatment: 0.075 *M* KCl at 37°C for 10 min) and fixed in acetic acid-methanol (1:3).

For CML patients, colchicine (0.15 µg/ml) is added 14.25 h after termination of the temperature shock for 10 min, and the hypotonic treatment is lengthened to 15 min before acetic acid-methanol fixation.

Slides are dried on a hot plate 72°C only until dry.

Trypsin banding is carried out with 0.11% (w/v) solution (DIFCO-Trypsin 1:250) for 10—15 s and stained with Leishman stain in phosphate buffer (pH 6.8, 17% solution GURR). The quality of the banding is also improved if slides are aged for 1 week at RT before banding studies are started.

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