Clonal Analysis of Basophil Differentiation in Bone Marrow Cultures From a Down's Syndrome Patient With Megakaryoblastic Leukemia

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We present the in vitro differentiation of marrow cells from a patient with Down's syndrome accompanied by megakaryoblastic leukemia into basophils in the presence of phytohemagglutinin-stimulated leukocyte conditioned medium, using a liquid culture and methylcellulose culture system. Identification of basophils was established by metachromatic staining with toluidine blue, transmission electron microscopy, and the presence of histamine. However, these basophils did not release histamine in response to calcium ionophore or chemotactic peptide. Samples from suspension cultures that contained 90% basophils showed chromosomal markers characteristic of leukemic cells (48, XY, +11, +21, t(1;15)) in all examined mitoses. The cellular composition of leukemic colonies grown in methylcellulose culture from single cells was studied using the micromanipulation technique. High plating efficiency and extreme predominance of basophil colonies were observed. In a total of 137 cultures, 79 revealed colony growth. Of 59 colonies that were analyzed by cytologic examination, 46 were pure basophil colonies. These basophil colonies showed disperse morphology, similar to that of a normal basophil colony. The clonality of the basophil colonies and skewing of lineage expression were documented from leukemic single-cell cultures. These data showed that leukemic cells have the capacity for differentiation into some lineages that are not expressed in vivo.

CASE REPORT

A 2-year-old boy with Down's syndrome was admitted to Saitama Children's Medical Center with anemia and ecchymoses in March 1984. Physical examination showed the presence of hepatosplenomegaly and heart murmur. His hemoglobin level was 9.9 g/dL and the white blood cell count was 3,700/µL with 6% blasts. Bone marrow examination revealed a normocellularity with an excess of myeloblasts (13%). Subsequent follow-up without chemotherapy showed no consistent change. In September, however, leukemic cells increased to 85% in the bone marrow. Diagnosis of megakaryoblastic leukemia was made at that time. In the peripheral blood, his white blood cell count was 6,500/µL with 8% blasts and 0% to 1% basophils. After administration of cytotoxic arabinoside and aclacinomycin, complete remission was achieved in December. The following studies were carried out, using the bone marrow cells obtained in September and December, with the informed consent from the parents of the patient. In February 1985, leukemic cells increased again and he died of a pneumonia.

MATERIALS AND METHODS

Cells. Bone marrow mononuclear cells were separated by centrifugation of Ficol-Hypaque (Lymphoprep, Nyegaard, Oslo); the interface cells were harvested and washed twice in Isco's medium (IMDM, GIBCO, Grand Island, NY). Cells from the mononuclear fraction were divided into several aliquots and kept in 10% dimethyl sulfoxide and 10% fetal calf serum (FCS, Flow Laboratories, North Ryde, N.S.W., Australia) at ~8 °C until use. These cells were thawed, washed, resuspended, and cultured under the appropriate conditions.

Cell culture. Methylcellulose culture was performed in 35-mm non-tissue culture dishes (Falcon, Oxnard, Calif) as previously described.

A 1-mL mixture of 10° to 10^2 bone marrow mononuclear cells, IMDM, 0.9% methylcellulose (Dow Chemical Co, Midland, Mich), 30% FCS, 1% bovine serum albumin (Sigma Chemical Co, St Louis), 2 units of erythropoietin (Toyobo, Osaka, Japan), and 5% (unless otherwise stated) medium conditioned by phytohemagglutinin-stimulated leukocytes (PHA-LCM) was incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. Using an inverted microscope, the culture plates were evaluated after 12 days for granulocyte-macrophage (GM) colonies, erythroid bursts, and mixed hematopoietic colonies.
DIFFERENTIATION OF PPO(+) LEUKEMIC CELLS

In vitro liquid culture was carried out by the following method: Cells were suspended in IMDM supplemented with 10% FCS and 5% PHA-LCM. Cells were incubated at 3.3 × 10⁷/mL in 25-cm² tissue culture flasks (Corning Glass, Corning, NY) in a humidified atmosphere of 5% CO₂ in air. Half of the culture medium was replaced once or twice a week with freshly prepared medium.

PHA-LCM was prepared as follows. Peripheral blood mononuclear cells from normal individuals were incubated at a concentration of 10⁶ cells per milliliter in IMDM, 10% FCS, and 0.5% PHA (Difco Laboratories, Detroit). After six days, the supernatant media were filtered through a 0.45-μm filter and stored frozen.

Cells were suspended in IMDM supplemented with 10% FCS and 5% PHA-LCM. Cells were incubated at 3.3 × 10⁵/mL in 25-cm² tissue culture flasks (Corning Glass, Corning, NY) in a humidified atmosphere of 5% CO₂ in air. Half of the culture medium was accomplished under direct microscopic visualization by using a fine Pasteur pipette (with a diameter of approximately 30 μm) attached to a micromanipulator (Narishige Scientific Instrument Lab, Tokyo). After confirming that a single cell was present in each culture dish, incubation was carried out at 37 °C in an atmosphere of 5% CO₂ in air. On days 6 through 9 of incubation, when the colonies appeared to have matured, individual colonies were lifted from the methylcellulose medium using a 3-μl Eppendorf pipette. The samples were spun in a Shandon Cytospin (Shandon Southern, Sewickley, Pa) and stained with May-Grunwald-Giemsa staining for differential counting.

Cytochemistry. Cells were stained with toluidine blue, peroxidase, Sudan black, alkaline phosphatase, or periodic acid Schiff staining. Nonspecific esterase and chloroacetate esterase staining were performed by the method described by Yam et al. Electron microscopy. Samples of fresh bone marrow or cultured cells were glutaraldehyde-fixed, processed, and stained with uranyl acetate for transmission electron microscopy using standard techniques. Platelet peroxidase (PPO) staining was done using the method described by Breton-Gorius et al. Histamine release. Histamine release with synthetic histamine releasers was performed. The calcium ionophore A23187, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), and compound 48/80 were used as synthetic histamine releaser. All releasers were purchased from Sigma Chemical Company. Cultured cells were washed twice with HEPES-buffered balanced solution and resuspended in the same buffer supplemented with 0.6 mmol/L CaCl₂ and 1 mmol/L MgCl₂. One-milliliter samples containing 1 to 2 × 10⁵ cultured cells per milliliter were challenged by incubation for 40 minutes at 37 °C with different doses of A23187 (0.1 to 1.0 μmol/L), compound 48/80 (1 to 100 μg/mL), or FMLP (10⁻⁴ to 10⁻⁶ mol/L).

After the challenge, samples were centrifuged at 200 g for ten minutes at 4 °C, and the supernatants were removed, acidified with perchloric acid, and stored at 4 °C until assay. Spontaneous histamine release was estimated without adding any releaser. Total histamine was measured by disintegrating the cells. Released histamine was expressed as a percentage of the total histamine corrected for spontaneous release.

Chromosome analysis. Chromosomes were studied on a sample of fresh bone marrow or cultured cells. The Giemsa banding technique was used. The chromosomes were classified according to the international nomenclature.

RESULTS

Morphology and methylcellulose culture. Mononuclear cells isolated by Ficoll-Metrizoate density centrifugation showed 92% leukemic cells. The remaining cells were lymphocytes and erythroblasts. Leukemic cells exhibited no granules and an undifferentiated appearance on light microscopic examination (Fig 1). Cytochemical studies on leukemic cells are summarized in Table 1. Approximately 74% of the leukemic cells were shown to possess PPO on electron microscopic study (Fig 2). The reaction was seen in the perinuclear space and endoplasmic reticulum, but not in the Golgi zone or granules.

We observed large numbers of basophil colonies in cultures of the patient's bone marrow mononuclear cells (in September 1984) in the presence of PHA-LCM. We carried out dose-response studies of the basophil colony formation at a concentration of 0% to 20%. Maximal colony formation was seen with 5%. The basophil colonies were seen as diffuse, small colonies consisting of less than 200 cells. May-

Fig 1. (A) May-Grunwald-Giemsa preparation of leukemic cells from bone marrow mononuclear cells. (B) Toluidine blue staining of leukemic cells. No granules were stained. (C) May-Grunwald-Giemsa preparation of cells from basophil colonies cultured for nine days in the presence of PHA-LCM. (D) Toluidine blue staining at pH 3.0 shows the metachromatic nature of the granules in the basophils.
The colony, we cultured single cells that were transferred colonies.

May-Grünwald-Giemsa staining of the colonies colonies, but they could not be analyzed by differential "whorls" were not seen.

normal basophils. The typical mast cell granules exhibiting granules, with features previously described as characteristic of human basophils. However, the electron-dense granules were smaller than the normal basophils. The typical mast cell granules exhibiting "whorls" were not seen.

Erythroid bursts and mixed colonies were not observed in cultures of the patient's bone marrow cells (in September 1984). In contrast to this, many GM colonies, erythroid bursts, and mixed colonies were noted in the cultures of bone marrow cells from the same patient in complete remission (in December 1984), as shown in Table 2. GM colonies consisted mainly of neutrophils and macrophages, with few basophil colonies.

**Table 1.** Cytotoxic Features of Freshly Isolated and Cultured Cells in a Case of Megakaryoblastic Leukemia

<table>
<thead>
<tr>
<th></th>
<th>Fresh Leukemic Cells</th>
<th>Cultured Basophils</th>
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<tbody>
<tr>
<td>Toluidine blue</td>
<td>–</td>
<td>+ + +</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sudan black</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Periodic acid Schiff</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Naphthyl butyrate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chloroacetate esterase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Platelet peroxidase*</td>
<td>+</td>
<td>–</td>
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*Electron microscopical observation.

An example of the in situ appearance of the pure basophil colony from a single cell is shown in Fig 3A. The colony showed diffuse morphology and small refractile cells. Some of these basophils had the spindle shape, as shown in the inset of Fig 3A. In contrast, the pure eosinophil colony from a single cell showed some aggregations and larger and darker cells (Fig 3B).

**Liquid culture.** Mononuclear cells from bone marrow were cultured in the presence of 5% PHA-LCM, and the recovered cells were examined by direct staining of the cell suspension with May-Grünwald-Giemsa and toluidine blue staining. Time course observations of cell proliferation are presented in Fig 4. The total number of nonadherent cells increased tenfold within one to two weeks. Differential counts of smears showed that basophils were less than 1% of the total cells before culture but increased to 90% within one to two weeks. Smears of the cells obtained in a 9-day-old culture were stained with May-Grünwald-Giemsa staining, and the differential counting is presented in Table 3. Simultaneous examination of the cell suspension by toluidine blue staining yielded concordant data. In the absence of PHA-LCM, the total number of cells was not increased. Bone marrow mononuclear cells from the patient in complete remission (in December 1984) were also cultured in the same manner. In the presence of PHA-LCM, basophils made up 4.8% of the total cells on day 9 of culture, as shown in Table 2.

**Table 2.** In Vitro Methylcellulose Culture of Marrow Cells From the Patient With Megakaryoblastic Leukemia

<table>
<thead>
<tr>
<th>Date</th>
<th>No. of Colonies per Dish</th>
<th>GM-Colonies</th>
<th>Erythroid Bursts</th>
<th>Mixed Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.20</td>
<td>600 ± 45</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12.19</td>
<td>45 ± 4</td>
<td>83 ± 5</td>
<td>2 ± 2</td>
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</table>

Bone marrow mononuclear cells were plated at a concentration of 1 x 10^4 per dish (9.20) or 1 x 10^5 per dish (12.19). Large number of basophil colonies (>90% of total colonies) were observed. Mainly neutrophil and/or macrophage colonies were observed.

**Fig 2.** (A) Ultrastructure of a megakaryoblastic leukemic cell identified by the presence of PPO. Original magnification x 8,700; current magnification x 6,625. The positive reaction is noted in the endoplasmic reticulum and nuclear envelope. (B) Ultrastructure of basophils in the colonies cultured for nine days in the presence of PHA-LCM. Many spherical and electron-dense granules were observed. Original magnification x 7,500; current magnification x 5,625. Detailed structure of the granules is highlighted in the inset. Original magnification x 37,000; current magnification x 27,750.
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Fig 3. (A) A dispersed pure basophil colony derived from a single cell. Spindle-shaped basophils are shown in the inset. Note that the cells are scattered widely. (B) A pure eosinophil colony derived from a single cell. Original magnification x25; current magnification x22. The cells appear larger and darker than in the pure basophil colony.

Histamine content and release. The estimated content was 0.02 pg per cell and 0.69 pg per cell on days 0 and 9 of culture, respectively. However, histamine release was 0% after challenge with a noncytotoxic dose of calcium ionophore A23187, compound 48/80, or FMLP.

Chromosome analysis. Samples from fresh bone marrow cells and cultured basophils showed abnormal karyotype 48, XY, +11, +21, t(1;15) in all examined 25 and 20 mitoses, respectively. Peripheral blood lymphocytes showed trisomy 21. The bone marrow cells from the patient in complete remission showed trisomy 21 in all examined ten mitoses.

DISCUSSION

Although persons with Down's syndrome have a markedly higher incidence of acute leukemia, the reports of megakaryoblastic leukemia are still uncommon. In this case, the diagnosis of megakaryoblastic leukemia was made on the basis of the presence of PPO in the majority of the leukemic cells. Transient abnormal myelopoiesis of Down's syndrome or CML were ruled out by the clinical course and chromosomal analysis.

We have demonstrated that the cells from the patient with megakaryoblastic leukemia can be differentiated into basophils in the presence of PHA-LCM. Studies with toluidine blue staining, transmission electron microscopy, and assay of histamine content suggested that the cultured cells were basophils. The histamine release was defective with the calcium ionophore A23187, FMLP, and compound 48/80.

It has been demonstrated that there are increased numbers of basophil precursors in the blood or bone marrow of patients with CML. However, pure basophil colonies were, at most, 10% of the total colonies. In this case, 78% of pure basophil colonies were formed in all colonies, although basophilia was not seen in vivo.

We could not obtain the metaphase on single colonies because the colonies consisted of a small number of cells, and mature basophils had limited proliferative capacity. The
data obtained from methylcellulose culture paralleled those from the suspension culture of leukemia cells in regard to the proliferation and differentiation of bone marrow cells. The colonies were considered to be leukemic in origin for the following reasons. Samples from suspension cultures that contained 90% basophils showed the abnormal karyotype characteristic of the leukemic clone in all examined 20 mitoses. Basophil differentiation in vitro is not associated with Down's syndrome because large numbers of basophils could not be grown in nine-day cultures of bone marrow cells from the same patient in complete remission. Moreover, basophils were defective in the function of histamine release. One of us (K.T.) observed that cultured basophils in human normal marrow cells showed histamine release after challenge with calcium ionophore but not with compound 48/80.3 Electron-dense granules in basophils were smaller than those of normal cultured basophils.

Recently, the cellular composition of murine or human normal hemopoietic colonies grown from single cells has been studied.12,13 Heterogeneity in the expression of differentiation programs is suggested from these experiments. To our knowledge, no report has yet established the growth or differentiation of cells in leukemic colonies derived from single cells. Of the 137 cultures of single cells from marrow mononuclear cells, 79 revealed colony growth. High plating efficiency (58%) may represent the proliferative pattern of the leukemic population. Of the 59 evaluable colonies, 46 pure basophil colonies were formed. Predominance of basophil colonies was not observed in the single-cell culture using human normal hemopoietic cells. Basophil colonies from leukemic single cells were disperse, similar to that of normal basophil colony described by Leary and Ogawa.15 The dish containing 10^4 mononuclear cells showed only 6% plating efficiency (Table 1). It is possible that some dispersed colonies, such as basophil colonies, would have been missed in situ unless a low cell concentration of cells was used for the culture.

Electron microscopy revealed that 74% of the leukemic cells were PPO positive. The single-cell experiment showed that 54% (74/137) of the mononuclear cells formed colonies or clusters consisting of mainly basophils. It is assumed that some of the PPO-positive cells could be differentiated into basophils. It remains to be solved whether the differentiation of basophil-like cells derived from PPO-positive precursors is an example of lineage infidelity.30

In summary, marrow cells from the Down's syndrome patient with megakaryoblastic leukemia could be differentiated to form colonies of cells with the morphological properties of basophils.

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REFERENCES

Clonal analysis of basophil differentiation in bone marrow cultures from a Down’s syndrome patient with megakaryoblastic leukemia

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