Human leukemia cell maturation induced by a T-cell lymphokine isolated from medium conditioned by normal lymphocytes

(hematopoiesis/leukemia differentiation/interferon/interleukin 2/colony-stimulating factor)

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ABSTRACT Human myelogenous leukemia cells in liquid culture can be induced to mature along the monocyte/macrophage pathway by a maturation inducer derived from the conditioned medium of activated human T lymphocytes. Serum-free conditioned medium was used for the isolation of the T-cell lymphokine. The maturation inducer was purified ≈ 6000 -fold by ammonium sulfate precipitation, low-salt elution from DEAE-Sephacrose CL-6B, gel filtration on Bio-Gel A-0.5m, and NaDodSO₄/PAGE under nonreducing conditions. The molecular weight of the maturation inducer was 36,000–58,000 on NaDodSO₄/PAGE. Terminal differentiation associated with inhibition of leukemia cell proliferation and expression of mature cell properties was observed with the isolated maturation inducer, identical to the activity observed with the unfractionated conditioned medium. Cell-cycle analysis revealed that the proportion of replicating S-phase cells was reduced from 40% to 7% after initial interaction of the maturation inducer with cells. The differentiating cells simultaneously acquired monocyte antigen, membrane complement receptors, phagocytic function, and monocyte/macrophage morphology. The maturation-inducing activity is dose-dependent, with more inducer causing the development of more mature cells in a shorter time period. The maturation inducer was shown to be stable after pH 2 treatment, independent of interleukin 2 and colony-stimulating factor, devoid of α -, β -, and γ -interferon, and not affected by antibody to interferon. The maturation inducer may play a role as a physiological regulator of monocytic and leukemia cell development.

Human and mouse myelogenous leukemia cells can be induced to differentiate and mature by many chemicals and physiological inducers. This maturation induction can be readily assayed in liquid culture, using leukemia cells. In recent years, a physiological inducer for the differentiation of human myeloid leukemia cells has been shown to be present in the culture media of mitogen-stimulated peripheral blood leukocytes (1) and lymphocytes (2, 3). Incubation of these conditioned media with leukemia cells from cell lines or from patients with acute myelogenous leukemia resulted in the maturation of the immature leukemia cells to monocytes and macrophages. The maturation inducer was shown to be a T-cell lymphokine, since it is derived from activated T cells (3–5) and is distinct from colony-stimulating factor (6). Analysis of the maturation events has revealed a complex process which includes a suppression of the growth of the leukemia cells, in parallel with the expressions of properties and functions of the maturing monocytic cells (3). By use of leukemia cells from the promyelocyte cell line HL-60 and myeloblasts from patients, the maturation inducer has been shown to mediate terminal differentiation of the leukemia cells, which then no longer proliferate (2–4).

The biological functions of the human maturation inducer, similar to those of the mouse differentiation factor for M1 leukemia cells (7), have suggested that this lymphokine is an important natural regulator for monocytic cells and leukemia development. However, the nature of the human maturation inducer, especially its relationship to other lymphokines, remained unclear. In this paper, we report the isolation of the maturation inducer from serum-free conditioned medium (CM) of normal lymphocytes and its effect on leukemia cell differentiation. The distinction of the maturation inducer from other known lymphokines is presented.

MATERIALS AND METHODS

Lymphocyte CM and Isolation of the Maturation Inducer. The maturation inducer for human leukemia cells has been identified in lymphocyte CM, which was used as the starting material for its isolation. Serum-free CM was prepared as described (8). Essentially, pooled normal peripheral blood lymphocytes were cultured at a density of 1×10^6 cells per ml in RPMI 1640 medium with 0.2% bovine serum albumin and 1% phytohemagglutinin. The medium from 3-day cultures was collected as CM.

CM (5.82 liters) was concentrated using a Millipore Pelli-con cassette system with a PTGC membrane (molecular weight cut-off, 10,000). It was precipitated with ammonium sulfate at 50% saturation and then at 70% saturation. The 70%-saturated ammonium sulfate precipitate was dissolved in and extensively dialyzed against 0.01 M Tris buffer, pH 8.0, and then was chromatographed on a DEAE-Sephacrose CL-6B column equilibrated with the same buffer. After the sample was loaded, the column was washed with two column volumes of buffer before a linear 0–0.5 M NaCl gradient was applied. Eluate fractions with inducing activity were concentrated by 70%-saturated ammonium sulfate precipitation. The precipitate was dissolved in and dialyzed against 10 mM sodium phosphate/0.15 M NaCl, pH 7.4. The sample was further purified by gel filtration on Bio-Gel A-0.5m in the same buffer.

Electrophoresis was performed in the buffer system described by Laemmli (9). Fifty microliters of sample was mixed with 50 μ l of electrophoresis sample buffer (4% NaDodSO₄/20% glycerol/160 mM Tris buffer, pH 6.8/4 mM phenylmethylsulfonyl fluoride) and applied to a 5–10% acrylamide gradient slab gel (16 $\times$ 19 cm). After electrophoresis, the gel was silver-stained (Bio-Rad).

Maturation Induction of HL-60 Leukemia Cells. The liquid culture assay with human leukemia cell line HL-60 promyelocytes (3) was used to analyze the maturation inducer isolated from lymphocyte CM. The HL-60 cell line was derived from a patient with acute promyelocytic leukemia (10). For each experiment, HL-60 cells were cultured at a density of 0.25×10^6 cells per ml of RPMI 1640 medium supplemented

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Abbreviation: CM, conditioned medium.

with 15% fetal calf serum and various concentrations of the maturation inducer. Control cultures were supplemented with an appropriate control medium for each analysis. Cellular proliferation and expressions of mature cell markers and functions were determined simultaneously during the culture period. The development of monocytes/macrophages, the acquisition of membrane complement receptors and monocyte antigen OKM1 phagocytic function, and morphological characteristics were assessed as described (3, 8). Cellular proliferation and cell-cycle-phase distribution were assayed using flow cytometry (3). Cells were stained for total DNA by suspending them in propidium iodide (0.05 mg/ml) sodium citrate (1 mg/ml)/0.1% Triton X-100 at 4°C for 30 min (11). A cytofluorograph system 50H (Ortho Diagnostics) equipped with a system 2150 computer was used for histogram production. The activity of the maturation inducer is defined as the minimal amount required in a given unit volume of liquid culture to induce a certain number of mature cells within a given time period. Thus, activity = $(1/t) \times (\% \text{ cells bearing mature-cell markers})$, where t is the day of the culture period on which the proportion of cells with mature cell markers is determined. One unit of activity is defined as 5% mature-cell-marker-bearing cells (the mean of cells bearing complement receptor, cells with macrophage/monocyte morphology, and phagocytic cells) obtained after 5 days of culture. The specific activity is equal to activity/mg of protein.

Activities of Lymphokines. To determine the presence of colony-stimulating activity for normal bone marrow cells, a soft-agar assay was carried out according to established procedures (12, 13). Fractions isolated from CM were introduced into the lower layer (0.5% agar) for stimulating bone marrow cells which were seeded in the upper layer (0.3% agar). The control lower layer contained 10^6 leukocytes and was used as the source of colony-stimulating factor. Approximately 10^5 bone marrow cells were used in the upper agar layer in each plate. The cultures were incubated for 7–14 days, and the average number of colonies having more than 40 cells is reported. Unit activities of α -, β -, and γ -interferons present in CM fractions were determined in M. Krim's laboratory (Sloan-Kettering Institute for Cancer Research, New York), by using assays with known human interferon standards and by using antisera against β and γ interferons. Purified human interleukin 2 and a mouse monoclonal anti-

FIG. 2. Gel filtration on Bio-Gel A-0.5m column. Fraction A (Fig. 1) was concentrated by a 70%-saturated ammonium sulfate precipitation. The precipitate was dialyzed against 10 mM phosphate buffer/0.15 M NaCl, pH 7.4, and chromatographed on the A-0.5m column (1.6×100 cm) equilibrated in the same buffer. The flow rate was 30 ml/hr. Elution positions of standard proteins used to calibrate the column are indicated, as is fraction I used for characterization of the inducer.

body against human interferon, with known antiviral neutralizing activity, were used in our experiments. These two reagents were gifts from C. Y. Wang (Sloan-Kettering Institute for Cancer Research, New York). Purified β -interferon was a gift from J. Wu (New York Medical College).

RESULTS

Isolation of the Maturation Inducer. The maturation inducer for human leukemia cells was isolated from lymphocyte CM. CM derived from pooled normal peripheral blood lymphocytes

FIG. 1. DEAE-Sepharose CL-6B chromatography. The 70%-saturated ammonium sulfate precipitate was dialyzed against 0.01 M Tris buffer, pH 8.0, and was applied to the column (2.6×40 cm). Flow rate was about 60 ml/hr. Beginning at fraction 31, a linear 0–0.5 M NaCl gradient was applied. Fraction A, used for further purification of the maturation inducer, is indicated by the bracketed line. CSF, colony-stimulating factor; IFN, interferon.

Table 1. Expressions of mature-cell characteristics in HL-60 cells induced by maturation inducer

Inducer	% cells						
	Characteristics				Morphology		
	Monocyte antigen OKM1	Complement receptors	Phagocytosis	S-Phase cells	Promyelocyte	Monocyte	Macrophage
None	0	1	2	40	96*	0	0
Fraction I	33	46	82	14	25	15	60
Unfractionated CM	65	42	80	12	20	10	70

Proportion of OKM₁-positive cells was determined on day 3 in culture. All other data represent 5 days after culturing.

*Differential cell counts of HL-60 cells reveal 96% promyelocytes, 3% myeloblast-like cells, and 1% myelocyte-like cells.

phocytes depleted of monocytes and B lymphocytes yielded identical activities of the maturation inducer. The serum-free CM was concentrated and subsequently subjected to ammonium sulfate precipitations at various concentrations. The precipitate at 70% saturation was found to be most active and was chromatographed on a DEAE-Sephacrose CL-6B column. The profile of elution with a NaCl gradient is depicted in Fig. 1. Material eluted at 0.04–0.08 M NaCl (fraction A) contained the maturation inducer. Colony-stimulating factor and γ -interferon were eluted with 0.12–0.15 M and 0.19–0.22 M NaCl, respectively. Fraction A subsequently was further purified by gel filtration on Bio-Gel A-0.5m. As shown in Fig. 2, fraction I contained the major maturation-inducing activity, which is described in the following section, and all fractions were devoid of colony-stimulating factor and γ -interferon.

Maturation-Inducing Activity of Isolated Fractions. Supplementation with either the active fractions or unfractionated CM produced gradual changes in the characteristics of promyelocytes, indicative of differentiation to monocytes and macrophages. The active fractions induced the development of cell aggregates and adherent cells during day 1 in culture. Table 1 summarizes a representative set of marker analyses during cell maturation induced by fraction I (see Fig. 2 and previous section). Final concentrations of 5–10 μ g/ml and 20–50 μ g/ml were the minimal and optimal ranges, respectively, of fraction I for inducing the terminal differentiation of HL-60 cells. The inductive activity of fraction I was dose-dependent. When a higher dose, such as 50 μ g/ml, was used, a larger number of mature cells was observed after 3–5 days in culture in comparison to that seen at 20 μ g/ml. Concomitantly, proliferation of these cells was drastically reduced, leading to an earlier termination of these differentiated cultures.

When fraction I was used at 20 μ g/ml, the proportion of cells bearing the normal monocyte antigen OKM1 increased. A significant increase in complement receptor-bearing cells occurred 3 days after culturing; it increased from 1–2% to >32% after 4 days and remained at this level for the balance of the culture period. The capacity to phagocytize increased after treatment with the maturation inducer. More than 80% of the cells were phagocytic by days 6–8. The earliest significant morphological changes were observed after 3 days in culture, with $\approx$ 25% of the cells being monocytes and macrophages and promyelocyte populations decreasing concomitantly. Macrophages became the predominant cell type in the later culture period. Proliferation of the cells also was measured during the differentiation period by using flow cytometry (Table 1). There was a steady decrease of cell proliferation, as measured by the proportion of S-phase cells. A reduction in S-phase cells was observed in cultures incubated with fraction I, from $\approx$ 40% S-phase cells on day 1 to $\approx$ 14% on day 5. This was in contrast to control cells, which maintained the same percentage of proliferating cells. The activity of fraction I thus was shown to be the same as that of the unfractionated CM in inducing a terminal differentiation of leukemia cells.

Comparison of the Maturation Inducer to Other Lymphokines. To determine the nature of the maturation inducer, we compared the activity to interleukin 2, interferons, and colony-stimulating factor. Addition of purified human interleukin 2 (0.5–40 units/ml) or purified human β -interferon (5–800 units/ml) to the HL-60 cell differentiation assay had no effect on inducing the development of mature cells and the suppression of proliferation of HL-60 cells (Table 2). It was determined that fraction I is devoid of α -, β -, and γ -interferon and interleukin 2, while approximately 1200 units of γ -interferon was found per ml of unfractionated CM. It should

Table 2. Activity of maturation inducer that is distinct from interferons, colony-stimulating factor, and interleukin 2

Inducer	Target cells	% cells				Colonies per 10 ⁵ cells
		Monocyte antigen OKM1 (day 2)	Complement receptor (day 4)	Monocyte/macrophage (day 7)	S-phase (day 5)	
None	HL-60	0	1	0	42	
Purified IL-2, 0.5–40 units/ml	HL-60	0	2	0	40	
Purified IFN- β , 5–800 units/ml	HL-60	0	2	0	45	
Fraction I, 20 μ g/ml	HL-60	29	35	82	12	
Fraction I + anti-IFN- γ	HL-60	25	40	85	8	
Fraction I, treated at pH 2	HL-60	22	29	75	15	
Human IgG, 0.5–3 mg/ml	HL-60	0	2	0	40	
Fraction I as feeder, 0.01–200 μ g/ml	Normal bone marrow					2–5
None	Normal bone marrow					1–6
Leukocytes as feeders	Normal bone marrow					38–65

IL-2, interleukin 2; IFN, interferon.

be pointed out that the maturation inducer was clearly separated from colony-stimulating factor and γ -interferon on the DEAE-Sepharose CL-6B column (Fig. 1). When fraction I was preincubated with an equal volume of a mouse monoclonal anti-human γ -interferon antibody that has antiviral neutralizing activity and then used in the HL-60 differentiation assay, it maintained its maturation-inducing activity (Table 2), indicating that the maturation inducer is not γ -interferon. Fraction I also was incubated at pH 2.0, dialyzed against culture medium, and tested in the differentiation assay. Compared to the untreated fraction I, the acid-treated fraction, which was used at the same protein concentration, induced a similar number of mature cells and a similar effect on the reduction of proliferation. Human IgG (0.5–3 mg/ml) added to the liquid culture had no effect on the parameters examined in Table 2. Pretreatment of fraction I with Pronase resulted in 70% reduction of the maturation-inducing activity of the lymphokine.

To test for the presence of colony-stimulating activity, we added fraction I at 0.01–200 μ g/ml to the feeder layers of the soft-agar cultures for colony formation of normal bone marrow cells. Only 2–5 colonies were observed with four different marrow preparations. In control experiments using leukocytes as a source of colony-stimulating factor, a large number of bone marrow colonies were formed, indicating that the maturation-inducing activity of fraction I is distinct from colony-stimulating factor.

NaDodSO₄/PAGE. To estimate the molecular weight of the maturation inducer, fraction I was electrophoresed, without treatment with a reducing agent, in a NaDodSO₄/polyacrylamide gel. After electrophoresis, the gel was cut into four pieces (Fig. 3): IA (from the top of the gel to M_r 130,000), IB (M_r 130,000–58,000), IC (M_r 58,000–36,000), and ID (M_r 36,000–tracking dye front). Each gel piece was placed in a tube with 5 mg of bovine serum albumin as carrier, ground into small fragments with a stainless steel rod, and immersed in 5 ml of phosphate-buffered saline. Protein was eluted at room temperature for 12 hr on a rocker platform. Eluted proteins were dialyzed against phosphate-buffered saline and tested in the HL-60 cell differentiation assay. The specific activity of the maturation inducer eluted from IC was >6000-fold that of CM (Table 3). The molecular weight of the inducer was calculated to be 36,000–58,000. Two major bands corresponding to molecular weights of 37,000 and 50,000 were revealed by silver staining.

DISCUSSION

In this paper, we have presented evidence that activated normal T lymphocytes produce a lymphokine that induces the maturation of human leukemia cells. Biochemical fractionation has shown that this isolated lymphokine retains the biological function of the activity present in unfractionated lymphocyte CM. The lymphokine mediates the maturation of leukemia cells to monocytes and macrophages through complex events involving a mechanism that induces cessation of cell proliferation and development of mature cell markers, characteristic of terminal differentiation. Since this mediator is derived from normal T cells (3, 4), this T-cell

FIG. 3. NaDodSO₄/PAGE (5–10% acrylamide gradient) in the absence of reducing agent. The gel was silver-stained. Lane 1: marker proteins (β -galactosidase, catalase, fumarase, and glycerol-3-phosphate dehydrogenase; M_r values at left). Lane 2: 53 μ g of CM protein. Lane 3: 5 μ g of fraction A protein (see Fig. 1). Lane 4: 1 μ g of fraction I protein (see Fig. 2); regions tested for inducer activity (IA–D) are delineated.

lymphokine may play a role as a physiological regulator of monocytic cell development.

We also present evidence that the maturation inducer is distinct from some of the known lymphokines. The maturation inducer was separable from (Fig. 1) and shown to lack the activity of colony-stimulating factor, supporting the reports of others (6, 7) who have used column chromatography to separate colony-stimulating factor of mouse and man from the maturation-inducing activity. Our data suggest that interferon(s) is not the maturation inducer mediating the terminal differentiation of HL-60 leukemia cells. This distinction is indicated by the separation of the maturation inducer and γ -interferon by DEAE-Sepharose CL-6B chromatography, the absence of α -, β -, and γ -interferon in the isolated fractions, the failure of anti- γ -interferon antibody to block the terminal maturation process, and the acid-stability of the maturation inducer. The possibility that interferon has a role in the total inductive activity of the lymphocyte CM or is a mediator for partial differentiation cannot be completely ruled out. γ -Interferon may mediate a partial differentiation of leukemia cells by inducing certain states to the differentiated state but not by inducing the terminal differentiation of the cell lineage, as indicated by the report of the induction of monocytic cell maturation from cells of chronic myelogenous leukemia (14).

The proteinaceous nature of the maturation inducer is indicated by its sensitivity to Pronase. Since the lymphokine

Table 3. Purification of maturation inducer

Fraction	Protein, mg	Total activity	Yield, %	Specific activity	Fold purification
CM	6175	38,840	100	6.2	1
70% (NH ₄) ₂ SO ₄ precipitate	895	12,967	33.3	14.4	2.3
DEAE-Sepharose CL-6B	34.52	3,320	8.5	96.1	15.5
Bio-Gel A-0.5m	1.39	393	1.0	282.7	45.5
NaDodSO ₄ /PAGE fraction IC	0.008	333	0.8	41,625	6713

has no detectable interleukin 2 and interleukin 2 does not induce maturation of leukemia cells, the maturation inducer is distinct from interleukin 2. The activity of the maturation inducer cannot be attributed to interleukin 1, because interleukin 1 and the lymphokine are products of non-T lymphocytes and T lymphocytes, respectively. The activity of the maturation inducer is not due to human IgG, because human IgG has no inducing effect on leukemia cells, and IgG and the lymphokine originate from different cell types.

Physiological mediators that have different capabilities for inducing leukemia cell differentiation may exist. Some may interact with cells at earlier stages than others. They also may differ by mediating either partial or terminal differentiation of the cell lineage. These different mediators may be products of different cell types. Recently, we have reported the constitutive production of a maturation-inducing activity by T-cell line HUT-102, similar to that from normal lymphocytes (15); this activity has since been isolated (16). A comparison of these mediators may reveal the importance and specificity of each as a regulator in monocytic and leukemia cell development.

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