Induction of Differentiation of Mouse Myeloid Leukemia M1 Cells by Serum of Patients with Chronic Myeloid Leukemia

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We examined the capacities of sera from patients with myeloid leukemia to induce differentiation in mouse myeloid leukemic M1 cells. Higher differentiation-inducing activity (D-activity) was detected in sera of patients with chronic myelomonocytic leukemia or chronic myeloid leukemia (CML) than in sera of patients with acute myeloid leukemia and normal volunteers. The D-activity in the sera was lost on heating the sera at 56° for 30 min. The major peak of D-activity on Sephadex G-200 gel filtration had an apparent molecular weight of 160,000. The origin of the D-activity in sera of patients with CML was studied by culturing fractions of peripheral blood cells of patients with D-activity for 3 days and then measuring the ability of the conditioned medium (CM) to induce differentiation of M1 cells. The cells in the myeloblast and promyelocyte fraction differentiated spontaneously into macrophage-like cells during culture for 3 days and the cells in the late granulopoietic cell fraction differentiated into neutrophil-like cells. Higher D-activity was present in CM of cells in the myeloblast and promyelocyte fraction than in CMs of late granulopoietic cell fractions. These results suggest that human leukemic cells produce D-activity for M1 cells during their differentiation into macrophage-like cells.

Key words: Leukemia — Serum — Differentiation inducer

Mouse myeloid leukemia cells (WEHI-3B D[+], R453 and M1) and human promyelocytic leukemia cells (HL-60) differentiate *in vitro* and *in vivo* into forms that are functionally and morphologically similar to macrophages and granulocytes on treatment with various inducers.[1-5] On differentiation, these leukemic cells lose proliferating capacity *in vitro* and leukemogenicity to syngeneic mice or athymic nude mice.[1-5]

Factors regulating differentiation of leukemic cells can be detected by studies with these differentiation-inducible leukemic cells. Non-differentiating M1 cells produce a factor(s) that inhibits induction of differentiation of M1 cells, whereas differentiated M1 cells produce a factor that induces differentiation of M1 cells.[6-10] Human leukemia HL-60 cells and M1 cells produce leukemia-associated inhibitors that suppress normal granulopoiesis *in vitro*.[7, 9, 11-13] These results suggest that leukemia cells produce factors that regulate growth and differentiation of the leukemic cells and normal granulopoietic cells.

Recently, it has become possible to detect the activities regulating differentiation of leukemic cells in patients by using various differentiation-inducible leukemic line cells. Mouse myeloid leukemic line cells, such as M1, WEHI-3B D[+], 7-M12 and NFS-60, can be induced to differentiate *in vitro* by various inducers. The differentiation inducers differ in their target specificities for different clones of myeloid leukemic cells.[14-18] For example, i) M1 cells are responsive to differentiation-inducing factor (D-factor, LIF or MGI-2), which differs from that of WEHI-3B D[+] or 7-M12 cells; ii) WEHI-3B D^{+} cells are responsive to G-CSF,[*4] which also induces differ-

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[*4] Abbreviations: CML, chronic myeloid leukemia; CMMOL, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; D-activity, differentiation-inducing activity; CM, conditioned medium; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin 3.

entiation of M1 cells but not 7-M12 cells; iii) 7-M12 and NFS-60 cells are responsive to GM-CSF or IL-3, which can not induce differentiation of M1 cells.

Metcalf[19, 20] reported that the sera of some patients with AML or acute infections had high capacities to induce differentiation in colonies of WEHI-3B D$^+$ cells and that the sera from some patients with acute infections also had high capacities to induce differentiation in HL-60 colonies. In this work, we analyzed the mechanisms of regulation of growth and differentiation of leukemic cells by examining the capacities of sera and CM of cells from patients with myeloid leukemia to induce differentiation of M1 cells, because M1 cells were responsive to a specific inducer (D-factor, LIF or MGI-2) which differs from that of WEHI-3B D$^+$ cells.

MATERIALS AND METHODS

Cells and Cell Culture Mouse myeloid leukemic M1 cells originated from a spontaneous myeloid leukemia in an SL mouse.[1] The cells were cultured in Eagle's minimal essential medium supplemented with double the usual concentrations of amino acids and vitamins and 10% (v/v) heat-inactivated (56° for 30 min) calf serum at 37° under 5% CO$_2$ in air. The cells were transferred every 2–3 days.

Assay of D-activity D-activity was assayed by measuring induction of phagocytic activity of M1 cells as described previously[6] and cell morphology was observed in smears treated with May-Gruenwald-Giemsa solution. Relative D-activity was calculated as a percentage of that with control CMMOL serum (No. 45). CMMOL serum (5%) induced 35.0–60.3% phagocytic M1 cells.

Human Serum Human sera were obtained from patients with CML in the chronic phase (14 samples), CML in the blastic crisis (6 samples), CMMOL (3 samples), AML (18 samples) and normal healthy volunteers (8 samples). The sera were dialyzed against phosphate-buffered saline (PBS) for 2 days. The FAB classification of the AML was M2 (7), M3 (4), M4 (6) and M5 (1). The dialyzed sera were filtered through an HA filter of 0.45 μm pore size (Millipore Corp., USA), divided into portions and stored at −40° until use.

Cell Separation by Ficoll-Hypaque and Percoll Peripheral blood cells were obtained from patients with CML in the chronic phase (Nos. 47 and 56), CMMOL (No. 59) and a normal healthy volunteer (No. 1). The cells were diluted with RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and then overlaid at room temperature on a cushion of 10 ml Ficoll-Hypaque

(Pharmacia Fine Chemical Co., Sweden) and centrifuged at 800g for 40 min. Leukemic cells, lymphocytes and mononuclear cells were concentrated in the middle layer (Fr. A), while erythrocytes and polymorphonuclear leukocytes were obtained in the bottom layer (Fr. B). The cells in each fraction were washed twice and suspended in RPMI-1640 medium.

The CML (No. 56) cells in Fr. A isolated by Ficoll-Hypaque centrifugation were suspended in PBS at a concentration of $2 \times 10^7/3$ ml. Density gradient centrifugation was performed on Percoll (Pharmacia Fine Chemicals) by the method of Pember et al.[21] Percoll was made isotonic with 10-fold concentrated PBS. Three milliliters of the cell suspension was thoroughly mixed with 6 ml of isotonic Percoll and the mixture (9 ml) was then placed in a glass tube (15 ml, Corex, USA) and centrifuged in a Sorvall RC 5 centrifuge in an SS-34 fixed angle rotor for 25 min at 18,000 rpm. The self-generated gradients were fractionated into Fr. 1 (upper 3 ml), Fr. 2 (medium 3 ml) and Fr. 3 (bottom 3 ml) with an automated system consisting of a peristaltic pump and fraction collector. Cell fractions were diluted with 5 vol of PBS and washed twice by centrifugation for 10 min at 400g. The recovery of total cells from the gradient was 54%.

Conditioned Medium (CM) Fr. A cells (10^6 nucleated cells/ml), Fr. B cells (10^6/ml), Fr. 1 cells (5×10^5/ml), Fr. 2 cells (5×10^5/ml) and Fr. 3 cells (5×10^5/ml) were cultured for 3 days in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. The cells were removed by centrifugation and the CM was harvested. CM of PHA-stimulated Fr. A cells of normal volunteers was prepared in the presence of 1% PHA-M (Difco, USA).

RESULTS

Effect of Human Serum on Growth and Differentiation of M1 Cells M1 cells (5×10^5/ml) were cultured for 2 days with or without 2.5–10% (v/v) human serum, and then the number of cells (Fig. 1) and the percentage of phagocytic cells were determined (Fig. 2). As shown in Fig. 1, sera from both normal and leukemic donors at concentrations of 5–10% inhibited the growth of M1 cells, but the inhibitory activities of some sera of patients with CML were lower than those of other patients. The sera of many patients with CML had higher D-activity than sera of normal volunteers or patients with AML (Fig. 2). To determine the relation between the D-activity in sera and the type of leukemia, the D-
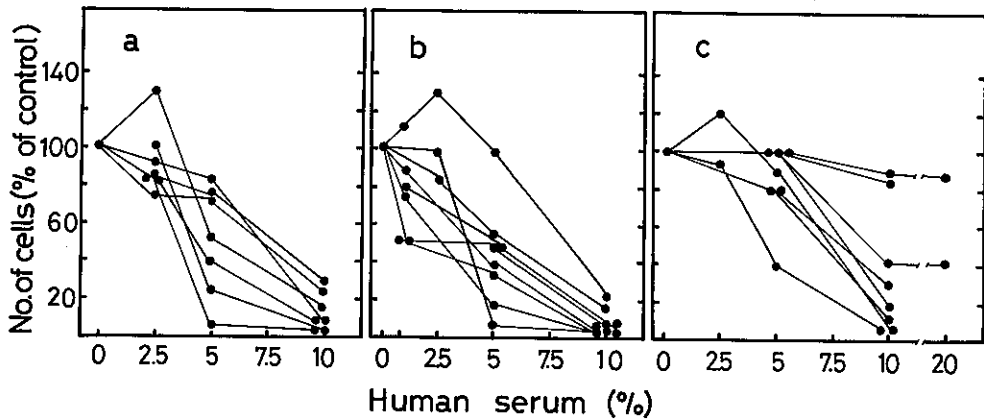
Fig. 1.   Effects of human sera on growth of M1 cells. M1 cells (5×10^5/ml) were cultured in the presence of human sera and after 2 days, numbers of viable cells were counted in a hemocytometer. The number of control cells in the absence of human serum was 1.0–2.0×10^6 cells/ml. Values are averages for 2 independent samples. a, normal sera; b, AML sera; c, CML sera.
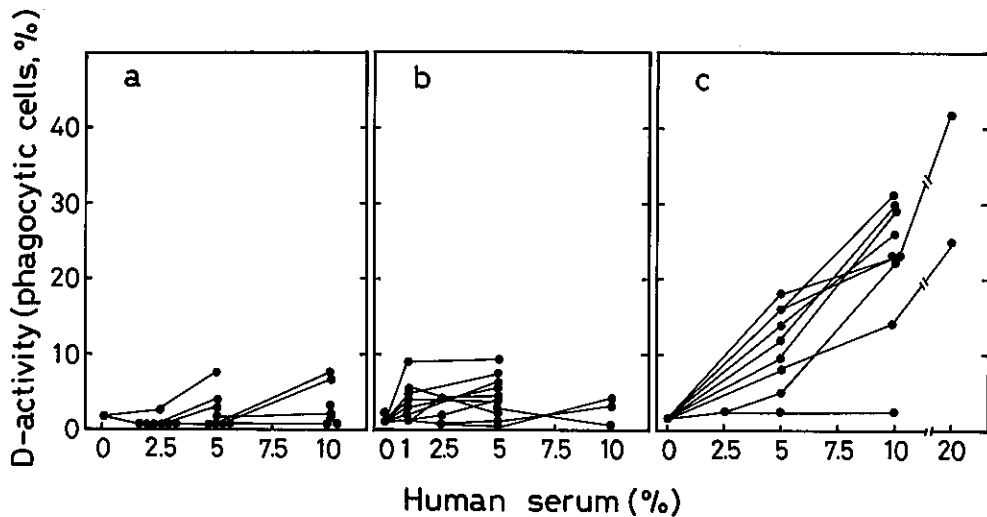

Fig. 2.   D-Activities of human sera. M1 cells (5×10^5/ml) were cultured in the presence of human sera and after 2 days, the phagocytic activity of M1 cells, a marker of differentiation, was examined. Values are averages for 2 independent samples. a, normal sera; b, AML sera; c, CML sera.

activities in 5% sera (v/v) of patients with CML (14 samples), CMMOL (3), CML-BC (6), AML (18) and normal volunteers (8) were assayed. Figure 3 shows the relative D-activities in these sera. The average relative D-activities in sera of patients with CMMOL, CML, CML-BC, AML and normal volunteers were 73%, 46%, 38%, 21% and 17%, respectively.   Morphological differentiation was also observed in May Gruenwald-Giemsa stained smears (data not shown). The percentages of mature macrophages in control and CMMOL-serum(5%)-treated cells were 0% and 17.5%, respectively.

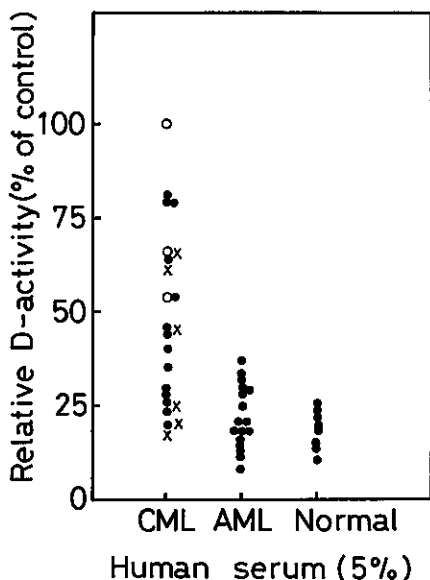**Properties of D-activity in CML and CMMOL Sera**   The D-activity of CML

Fig. 3. Relative D-activities of human sera. M1 cells $(5 \times 10^5/\text{ml})$ were cultured in the presence of 5% human sera and after 2 days the phagocytic activities of the M1 cells were determined. Serum of a patient with CMMOL (No. 45) was used as a control. Control serum at 5% induced 45.0–60.3% phagocytic activity. Open circles (○) and crosses (×) show values with sera of patients with CMMOL and CML in blastic crisis, respectively.
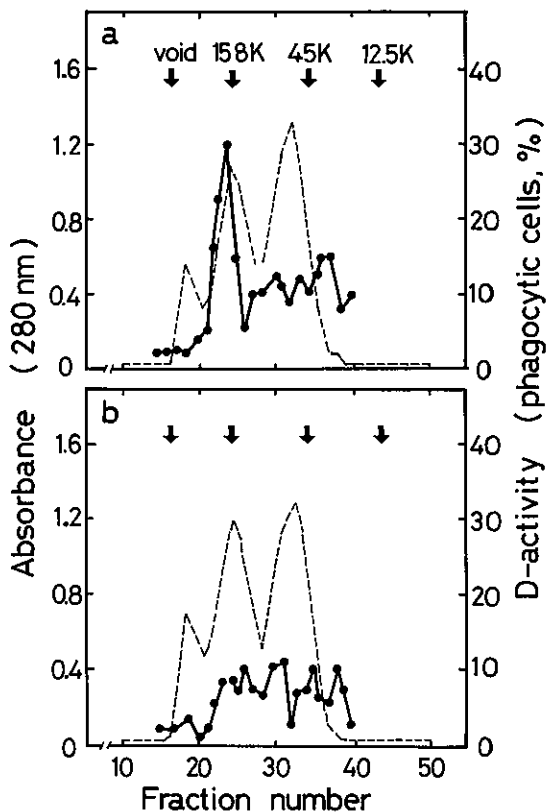

Fig. 4. Sephadex G-200 gel filtration profile of CML serum (a) and normal serum (b). The serum (0.8 ml) was applied to a Sephadex G-200 column (1.9×86 cm) equilibrated with PBS and fractionated. Each fraction (0.5 ml) was assayed for D-activity. Molecular weight standards were as follows: aldolase (158K), ovalbumin (45K) and cytochrome c (12.5K). (----), absorbance at 280 nm; (●—●), D-activity.

serum (Nos. 22, 23 and 47) was nondialyzable and was inactivated by heating at 56° for 30 min. To exclude the effect of contamination in serum of lipopolysaccharide, which is a differentiation inducer of M1 cells, we examined whether the D-activity was blocked by incubation with polymyxin B. Polymyxin B is known to bind lipopolysaccharide and to block several actions of lipopolysaccharide.[22] The D-activity of CML serum (Nos. 22, 23 and 47) was stable on treatment with polymyxin B (10 μg/ml). CML-serum (No. 47) with D-activity was chromatographed on Sephadex G-200. The D-activity was eluted as a single peak with a molecular weight of 160,000 (Fig. 4a). The peak fraction with the same molecular weight obtained from normal serum (No. 1) did not exhibit D-activity (Fig. 4b).

To examine whether the inhibitory activity that suppresses the D-activity observed in CML or CMMOL sera is detected in normal or AML sera, the D-activity in CML serum was assayed in the presence of normal or AML sera. The inhibitory activity was not detected in normal (Nos. 1, 2 and 3) or AML (No. 11, FAB-M2; No. 24, FAB-M4; No. 33, FAB-M3) sera (data not shown).

The D-activity in CML (Nos. 22, 23 and 47) and CMMOL (No. 59) sera (5–20%) did not induce differentiation of HL-60 cells (data not shown).

D-activity in CM of CMMOL and CML Cells Heparinized peripheral blood samples of patients with CMMOL (No. 59), CML (No. 47), and a normal volunteer (No. 1)

Table I. Data on Peripheral Blood Cells and D-activity of Various Subjects

	Subject (diagnosis)		
	No. 47 (CML)	No. 59 (CMMOL)	No. 1 (Healthy)
Peripheral blood			
WBC $(10^3/\mu l)$	452.0	49.5	6.8
Myeloblasts (%)	5.0	9	0
Promyelocytes (%)	6.5	35	0
Myelocytes (%)	10.5		0
Metamyelocytes (%)	9.0		0
Mature granulocytes (%)	58.5	18	55
Eosinophils (%)	3.0		4
Basophils (%)	5.5		0.5
Monocytes (%)	1.5	18	5.5
Lymphocytes (%)	0.5	20	35
D-activity[a]			
CM of Fr. A cells	35.0 ± 3.4	88.3 ± 1.3	12.8 ± 5.0
CM of Fr. B cells	5.0 ± 0.5	ND	ND
CM of PHA-Fr. A cells[b]	ND	ND	69.3 ± 3.2

a) Peripheral blood cells were fractionated into Fr. A and Fr. B on Ficoll-Hypaque, and the cells in each fraction (10^6 nucleated cells/ml) were cultured for 3 days. The CM (20%) was assayed for D-activity.
b) CM of PHA-stimulated Fr. A cells of a normal volunteer.
ND, not determined.

Table II. Morphological Changes in Fr. 1, Fr. 2 and Fr. 3 Cells of a Patient with CML during Culture

Cells[a]	Myeloblasts and promyelocytes (%)	Cells in intermediate stages (%)	Late granulopoietic cells (%)	Macrophages (%)
Fr. 1	72.3 ± 8.5	25.5 ± 5.8	2.2 ± 2.9	0
Fr. 1 culture[b]	5.1 ± 2.4	29.2 ± 7.6	7.1 ± 2.2	58.7 ± 7.6
Fr. 2	23.7 ± 8.1	68.5 ± 8.7	7.8 ± 2.8	0
Fr. 2 culture[b]	2.2 ± 2.8	20.6 ± 3.1	44.5 ± 6.7	32.8 ± 6.6
Fr. 3	4.0 ± 2.0	40.0 ± 4.3	56.0 ± 2.8	0
Fr. 3 culture[b]	0.3 ± 0.6	12.7 ± 1.8	78.8 ± 2.3	8.2 ± 3.2

a) See legend to Fig. 5.
b) The cells (5×10^5/ml) in each fraction were cultured for 3 days.

were obtained and the cells were fractionated into Fr. A (leukemic immature cells and mononuclear cells) and Fr. B (erythrocytes and mature granulocytes) by Ficoll-Hypaque density gradient centrifugation. Lymphocytes in Fr. A of normal blood were concentrated by using Ficoll-Hypaque. Then 10^6 nucleated cells/ml were cultured. Table I shows data on peripheral blood and the D-activity of the CM of Fr. A cells. Higher D-activity than in the normal Fr. A cells was detected in those of the patients with CML and CMMOL. The CM of Fr. A cells also induced morphological dif-

ferentiation of M1 cells. The percentages of mature macrophages in control and CM-treated cells were 0% and $55.7 \pm 1.1\%$ (SE), respectively. The D-activity in the CM was inactivated by treatment with protease or by heating at $56°$ for 30 min (data not shown). No D-activity was detected in the CM of either normal or leukemic Fr. B cells.

When the normal Fr. A cells, which were mainly lymphocytes, were treated with PHA for 3 days, their CM showed high D-activity (Table I). PHA-treated lymphocyte-CM was reported to contain inducers of differentiation
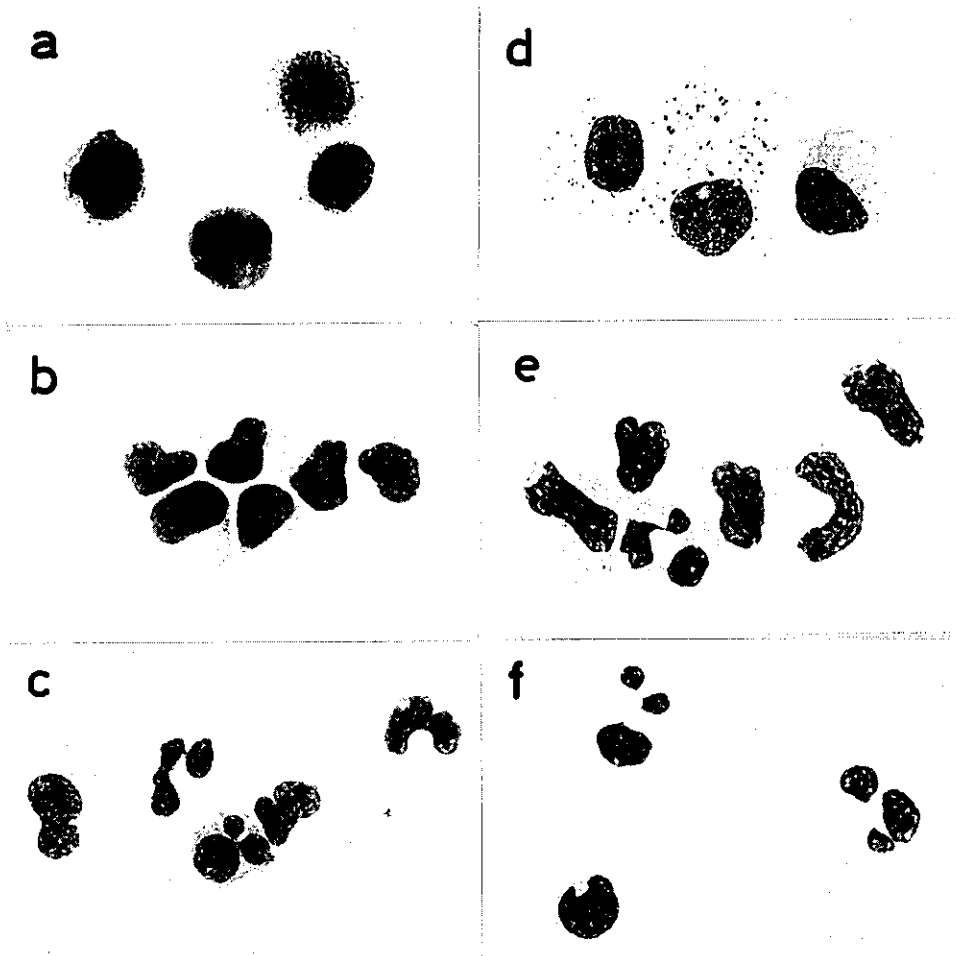
Fig. 5. Spontaneous differentiation of CML cells fractionated by Ficoll-Hypaque and Percoll density gradient centrifugation. Peripheral blood cells of a patient with CML (No. 56) were fractionated into Fr. A cells by Ficoll-Hypaque density gradient centrifugation. The Fr. A cells, which were prominently leukemic cells, were further fractionated into Fr. 1 (a), Fr. 2 (b) and Fr. 3 (c) by Percoll density gradient centrifugation (Table II). The cells (5×10^5/ml) in each fraction were cultured for 3 days (d–f), and the D-activity in the CM was determined. The highest D-activity was found in the CM of Fr. 1 cells. During 3 days of culture, the cells in each fraction differentiated spontaneously into macrophage-like cells (d, e) and polymorphonuclear leukocytes (f).

of HL-60 cells.[23] The CM of CMMOL Fr. A cells (without treatment with PHA) exhibited higher D-activity than that of PHA-treated lymphocytes, but did not induce differentiation of HL-60 cells (data not shown).

We fractionated the Fr. A cells of CML (No. 56) further into Fr. 1, Fr. 2 and Fr. 3 by using Percoll. The percentages of cells in Fr. 1, Fr. 2 and Fr. 3 were 2.6%, 83.5% and 13.9%, respectively; Fr. 1 (Fig. 5a) contained mainly myeloblasts and promyelocytes, Fr. 3 (Fig. 5c) contained mainly late granulopoietic cells, and Fr. 2 (Fig. 5b) contained cells in intermediate stages between those in Fr. 1 and Fr. 3 (Table II). The cells in each fraction (5×10^5/ml) were cultured for 3 days (Fig. 5d–f). The D-activities in the CM from cells of Fr. 1, Fr. 2 and Fr. 3 are shown in Fig. 6. The highest D-activity was found in CM of cells in Fr. 1; the CM of cells in Fr. 3 did not exhibit
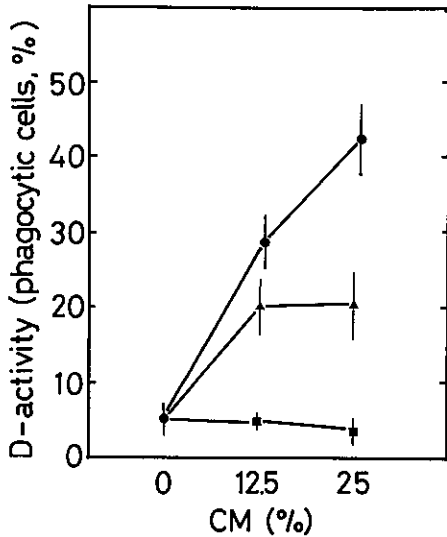
Fig. 6. D-activity in CM from CML (No. 56) cells fractionated by Percoll density gradient centrifugation. CML Fr. A cells were further fractionated into Fr. 1, Fr. 2 and Fr. 3 on Percoll (Table II and Fig. 5). The cells (5×10^5/ml) were cultured for 3 days and the D-activity in their CM was assayed. (●), CM of Fr. 1 cells isolated by Percoll density gradient centrifugation of Fr. A cells; (▲) CM of Fr. 2 cells; (■), CM of Fr. 3 cells.

D-activity. During culture for 3 days, the cells in Fr. 1 differentiated spontaneously into mainly macrophage-like cells (Fig. 5d), while those in Fr. 3 differentiated into neutrophil-like cells (Fig. 5f). These results suggest that CML and CMMOL cells produce D-activity during their maturation into macrophages.

Discussion

In this work we examined the capacities of sera from patients with myeloid leukemia to induce differentiation of M1 cells. We found that sera of some patients with CML and CMMOL had higher differentiation-inducing activity than sera of patients with AML and normal volunteers.

Metcalf[19, 20] reported that sera from some patients with AML or acute infections had high capacities to induce differentiation in colonies of WEHI-3B D$^+$ cells, and that the sera from some patients with acute infections also had high capacities to induce differentia-

tion in HL-60 colonies. In our present study none of the patients had infections, and the D-activity for M1 cells in the sera was not associated with a capacity to induce differentiation of HL-60 cells. The differentiation inducers differ in their target specificities for different clones of mouse myeloid leukemic line cells.[14-18] These target specificities of inducers are probably exerted in different clones of human myeloid leukemic line cells. Therefore, the D-activity for M1 cells in CML-serum should have some activity on certain human myeloid leukemic cells but not HL-60 cells.

The differentiation-inducing factors for M1 cells have been purified to homogeneity from medium conditioned by mouse L-cells (D-factor)[14] and Krebs II ascites tumor cells (LIF or MGI-2).[15, 16] However, these factors can not induce differentiation of WEHI-3B D$^+$ and HL-60 cells. On the other hand, unlike the CSFs, the differentiation-inducing factors have no proliferative stimulus for normal and leukemic progenitor cells. Therefore, they may have potential therapeutic value in the management of myeloid leukemia. Recently, a cDNA for murine LIF was isolated and recombinant LIF was produced in a yeast expression system.[24] A human homologue of the cloned murine LIF gene was then isolated from a genomic library by using the murine cDNA as a hybridization probe and the factor encoded by this gene was expressed in yeast cells.[25] The recombinant human LIF induced the differentiation of murine M1 leukemic cells in a manner analogous to murine LIF. Since murine LIF has target specificites for different clones of mouse myeloid leukemic line cells, human LIF may also have them.

The molecular size of the component responsible for the D-activity in CML serum (Fig. 4a) seems to be larger than those of the purified murine D-factor, LIF and MGI-2. The larger molecular size of the D-activity may be a result of complex formation in the CML serum. The human LIF had 78% amino acid sequence homology with murine LIF.[25] The relationship to human LIF of the D-activity in CML-serum remains unknown. The possible identity to human LIF can only be proven by direct amino acid sequencing of the purified proteins.

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The role of CML serum D-activity for mouse M1 cells in human hematopoietic regulation is unknown. D-activities for M1 cells were found in human amniotic fluid,[26] urine and saliva.[27] The presence of D-activity in these body fluids is of special interest in connection with *in vivo* regulation of proliferation and differentiation of leukemia cells. These D-activities in human body fluids were postulated to be due to macromolecules of proteinous nature since they were nondialysable, heat-labile, and sensitive to trypsin. The properties of the D-activity were similar to those in serum of the patient with CML. However, the origin of the D-activity in human body fluids is unknown. The D-activity in CML serum may be derived from leukemic cells, since this activity was found in the CM of leukemic cells of patients with CML and CMMOL (Fig. 6). The immature leukemic cells differentiated spontaneously into macrophage-like cells during culture (Fig. 5). These results suggest that the leukemic cells produce D-activity during the process of differentiation into macrophage-like cells. It is of interest to examine whether the human LIF gene is highly expressed in the CML cells.

The leukemic cell fraction contains a few lymphocytes. The CM of the PHA-stimulated normal lymphocytes showed an increased capacity to induce differentiation of M1 cells (Table I). Therefore, we could not exclude the possibility that CML lymphocytes contaminated in leukemic cell fraction produced the D-activity without PHA-stimulation. Although the CM of PHA-stimulated normal human lymphocytes also induced differentiation of HL-60 cells, the CMs of Fr. A cells from the patients with CML or CMMOL did not. These results suggest that the D-activity in CM of leukemic cells is not due to the contaminating lymphocytes.

HL-60 cells can be induced to differentiate into macrophages by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or into granulocytes by retinoic acids.[5] It is of interest to examine whether HL-60 cells produce D-activity for M1 cells during their differentiation into macrophages or granulocytes. In a preliminary experiment, higher D-activity was detected in the CM of TPA-treated HL-60 cells than in the CMs of retinoic acid-treated HL-60 cells or untreated cells. These results

also suggest that the cells produce D-activity in the process of differentiation into macrophages.

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