Combined Effects of Differentiation-inducing Factor and Other Cytokines on Induction of Differentiation of Mouse Myeloid Leukemic Cells

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Mouse myeloid leukemic M1 cells are induced to differentiate into macrophage-like cells by differentiation-inducing factors (D-factors) and granulocyte colony-stimulating factor. We examined the effects of recombinant human tumor necrosis factor (rTNF), lymphotoxin (rLT) and interleukin 1 (rIL-1) on the induction of differentiation of M1 cells, compared with the effects of D-factor purified from the conditioned medium of mouse Ehrlich ascites tumor cells and recombinant human granulocyte colony-stimulating factor (rG-CSF). rIL-1 induced phagocytic activity, a typical marker of cell differentiation, in at most 30% of M1 cells at concentrations ranging from $10^{-10}M$ to $10^{-7}M$. The differentiation-inducing activity of rIL-1 was similar to that of rG-CSF and less than that of D-factor. rTNF induced phagocytic activity in 14% of M1 cells only at a high concentration ($10^{-7}M$). rLT did not induce differentiation of the cells even at $10^{-7}M$. rTNF stimulated induction of differentiation of M1 cells by D-factor, rG-CSF or rIL-1 by two or three fold. The combination of any two of the cytokines D-factor, rG-CSF and rIL-1 induced differentiation of M1 cells more efficiently than any of these cytokines alone. Moreover, the combination of three cytokines rG-CSF, rIL-1 and rTNF, all of which are known to be produced by macrophages, was more effective than the combination of any two of these cytokines in induction of differentiation of M1 cells.

Key words: Myeloid leukemic cells — Cytokines — Cell differentiation

Mouse myeloid leukemic M1 cells can be induced to differentiate into macrophages by protein inducers (D-factors)[4] and various chemicals.[1, 2]

We purified D-factor with a molecular weight of 62,000 from the conditioned medium of mouse fibroblast L929 cells[3] and D-factor with a molecular weight of 45,000 from the conditioned medium of mouse Ehrlich ascites tumor cells.[4] The half-maximally active concentration of the D-factor was $4 \times 10^{-11}M$.[3, 4] The antiserum to D-factor from L929 cells inhibited the D-factor activity in conditioned medium of Ehrlich cells, embryo cells, lung tissue and lymphocytes stimulated with concanavalin A, and serum from mice injected with endotoxin.[5] However, it did not affect the D-factor activity with a molecular weight of 20,000–25,000 in conditioned medium of macrophages.[5, 6]

Recently, we determined the partial amino acid sequence of the D-factor from Ehrlich cells (unpublished data), finding that D-factor differed from the colony-

stimulating factors (growth factors for normal precursor cells for granulocytes and macrophages), interferons, TNF, LT and interleukins. Gearing *et al.* cloned a cDNA for a murine leukemia-inhibitory factor (LIF) that induced differentiation of M1 cells.[7] Our partial amino acid sequence of D-factor showed homology with that of LIF, suggesting that D-factor and LIF are the same molecule.

On the other hand, human myeloid leukemic cells have been reported to be induced to differentiate into macrophages by the conditioned medium of activated human lymphocytes or T cell lines.[2, 8, 9] Although human TNF, LT and interferon-γ induced differentiation of human myeloid leukemic cells,[10–15] the presence of a differentiation-inducing factor distinct from TNF, LT and interferon-γ on the basis of its biochemical characteristics[8–10] was reported in the conditioned medium of lymphocytes, but it has not yet been purified.

G-CSF was reported to induce differentiation of mouse myelomonocytic leukemic WEHI-3B cells and human leukemic cells[16, 17] and we also found that human rG-CSF could induce differentiation of M1 cells.[18] Recently, Tamatani *et al.*[19] reported that IL-1 induced the differentiation of M1 cells in the presence of TNF. In this study, we investigated the effects of the purified D-factor, human rG-CSF, rTNF, rLT and rIL-1 on differentiation of M1 cells. Human G-CSF, TNF, LT and IL-1 show

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[4] Abbreviations used are: D-factor, differentiation-inducing factor; G-CSF, granulocyte colony-stimulating factor; rG-CSF, recombinant granulocyte colony-stimulating factor; TNF, tumor necrosis factor; rTNF, recombinant tumor necrosis factor; IL-1, interleukin 1; rIL-1, recombinant interleukin 1; LT, lymphotoxin; rLT, recombinant lymphotoxin.

species-crossreactivity for murine cells. We found that rIL-1 by itself induced differentiation of M1 cells when added at more than $10^{-10}M$ and that the combination of any two of the purified D-factor, rG-CSF, rTNF, rLT and rIL-1 induces differentiation of M1 cells more efficiently than any of these cytokines alone.

MATERIALS AND METHODS

Cells and cell culture Myeloid leukemic M1 cells originated from a spontaneous myeloid leukemia in an SL mouse.[1] Clone T22-3, a subclone of M1 cells, was used in this study. The cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo), supplemented with double the usual concentrations of amino acids and vitamins and 10% (v/v) heat-inactivated calf serum at 37°C under 5% CO_2 in air.

D-factor D-factor (molecular weight 45,000) was purified to homogeneity from conditioned medium of Ehrlich ascites tumor cells as described.[4] Its specific activity was 6.4×10^7 U/mg protein; 50 U of D-factor was defined as the activity inducing 50% phagocytic cells in 1 ml of M1 cell culture.

Other cytokines Purified human recombinant TNF (molecular weight 17,700) and human recombinant IL-1 (molecular weight 18,000) were kindly provided by Dainippon Pharmaceutical Co., Ltd., Osaka. Purified human recombinant LT (molecular weight 20,000) was kindly provided by Boehringer-Ingelheim, Vienna; it was *E. coli*-derived and had a specific activity of 1.2×10^8 U/mg on L-M mouse cells. Human recombinant G-CSF (molecular weight 18,700) was obtained as an *E. coli* product and purified to homogeneity as previously described.[18]

Phagocytosis M1 cells (3×10^5 cells/ml) were incubated with cytokines for 2 days and then their phagocytic activity was determined as previously described.[3] Cells were incubated in serum-free medium containing 0.2% of a suspension of polystyrene latex particles (average diameter, 1.099 μm; Dow Chemical Co., Indianapolis, IN) for 4 h at 37°C. Then the cells were thoroughly washed 3 times with phosphate-buffered saline, and the percentage of phagocytic cells in more than 200 cells was determined. Cells containing more than 10 latex particles were scored as phagocytic cells.

Cell morphology The percentage of cells that were morphologically similar to macrophages was determined in smear preparations stained with May-Grünwald-Giemsa solution.

RESULTS

Induction of differentiation of M1 cells by cytokines We examined the effects of rTNF, rLT and rIL-1 on differen-

tiation of M1 cells (Fig. 1). rIL-1 induced phagocytic activity, a typical marker of differentiation of M1 cells. The percentage of phagocytic cells increased gradually with increasing concentration of rIL-1 from $10^{-10}M$ to $10^{-7}M$, and the maximum percentage of phagocytic cells induced by rIL-1 (10^{-7} M) was 30%. Under the same conditions, the maximum percentages of phagocytic cells
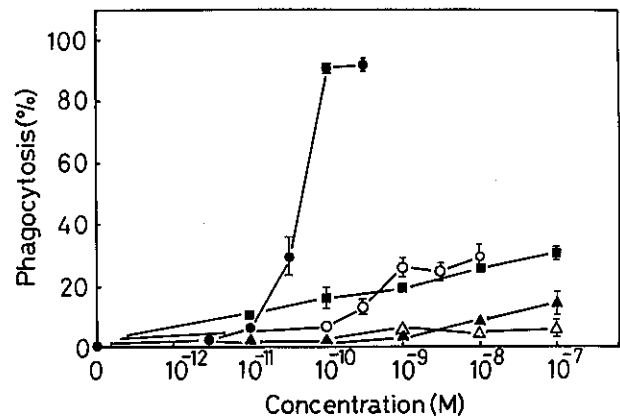

Fig. 1. Induction of differentiation of M1 cells by cytokines. M1 cells were incubated with various concentrations of cytokines for 2 days and then their phagocytic activity for polystyrene latex particles was assayed. ●, Purified D-factor; ○, rG-CSF; ▲, rTNF; △, rLT; ■, rIL-1. Values are means of duplicate determinations; bars, SE. The second experiment gave similar results.
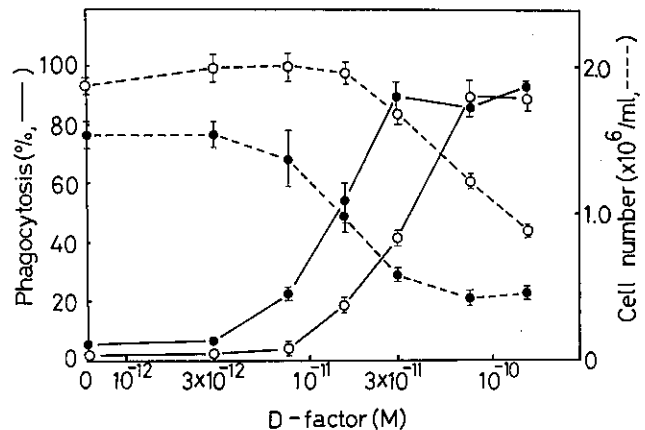

Fig. 2. Effects of rTNF on growth and differentiation of M1 cells induced by D-factor. M1 cells were incubated with various concentrations of D-factor in the presence (●) or absence (○) of rTNF ($10^{-8}M$) for 2 days. Then, their phagocytic activity (——) was assayed and cell numbers (----) were counted in a Coulter counter. Values are means of duplicate determinations; bars, SE. Three other experiments gave similar results.

Table I. Effect of rTNF on Induction of Morphological Differentiation of M1 Cells by D-factor[a]

| Treatment | | Cell type (%)[b] | | |
Cytokine	Concentration (M)	Blasts	Intermediates	Macrophages
None		97.3±1.6[c]	2.7±1.5	0
D-factor	10^{-10}	4.0±1.7	14.0±4.6	82.0±6.0
D-factor	10^{-11}	93.0±3.4	6.6±3.4	0.4±0.5
rTNF	10^{-8}	88.3±1.7	11.0±2.4	0.8±1.0
D-factor	10^{-11}			
+ rTNF	10^{-8}	15.6±4.8	70.4±4.5	14.0±2.9

a) M1 cells (5×10^4 cells/ml) were incubated with D-factor and/or rTNF for 4 days.
b) Morphology of the cells was examined by staining the cells with May-Grünwald-Giemsa solution.
c) Mean ±SE of duplicate determinations.

induced by D-factor ($10^{-10} M$) and rG-CSF ($10^{-9} M$) were 92% and 28%, respectively. Defferentiation-inducing activity of rIL-1 was similar to that of rG-CSF, yet considerable less than that of D-factor. rIL-1 induced not only phagocytic activity but also lysozyme activity and morphological differentiation in M1 cells (data not shown). The effect of IL-1 was not due to contamination by endotoxin because addition of polymyxin B (20 μg/ml) did not inhibit the differentiation-inducing activity of IL-1 (data not shown).

rTNF induced phagocytic activity in a small portion of M1 cells only at a high concentration ($10^{-7}M$). rLT did not induce differentiation of the cells even at $10^{-7}M$. Neither rTNF nor rLT was toxic to M1 cells at the concentrations tested.

Stimulation of differentiation of M1 cells by rTNF We next examined the effects of the combination of rTNF and other cytokines on differentiation of M1 cells. rTNF at $10^{-8}M$ stimulated D-factor-induced phagocytic activity of M1 cells and shifted the D-factor dose-response curve to the left by approximately two fold (Fig. 2). Stimulation by rTNF was observed at the concentrations above $10^{-10}M$ (data not shown). rLT also stimulated D-factor-induced differentiation (data not shown). rTNF at $10^{-8}M$ was not toxic but decreased the growth of M1 cells to 80% of the control (Fig. 2). Differentiation induced by D-factor is associated with a decrease in cell growth.[2] The combination treatment with rTNF and D-factor was more effective for inhibition of cell growth than either cytokine alone (Fig. 2).
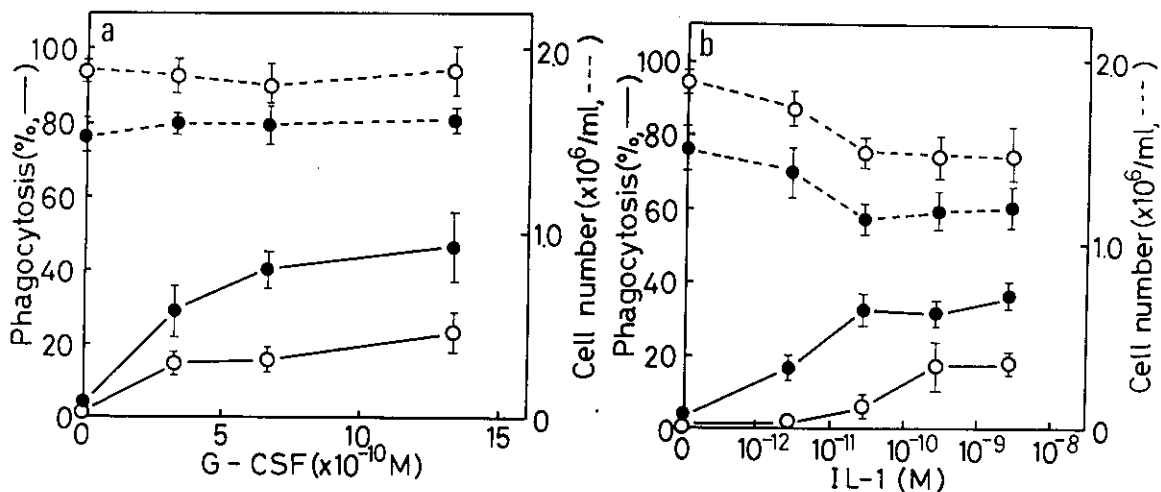

Fig. 3. Effects of rTNF on growth and differentiation of M1 cells induced by rG-CSF or rIL-1. M1 cells were incubated with various concentrations of rG-CSF (a) or rIL-1 (b) in the presence (●) or absence (○) of rTNF ($10^{-8}M$) for 2 days. Then, their phagocytic activity (———) was assayed and cell numbers (----) were counted in a Coulter counter. Values are means of duplicate determinations; bars, SE. Three other experiments gave similar results.

rTNF also stimulated induction of the morphological differentiation of M1 cells by a suboptimal concentration of D-factor (Table I). Untreated M1 cells were myeloblastic cells with a large round nucleus and little cytoplasm. On treatment of M1 cells with 10^{-10} M D-factor for 4 days, 82% of the cells differentiated morphologically into macrophage-like cells. When M1 cells were treated with $10^{-11}M$ D-factor or $10^{-8}M$ of rTNF alone, a

small portion of the cells differentiated into macrophages or intermediate forms between myeloblastic cells and macrophages. However, on simultaneous treatment with rTNF (10^{-8} M) and D-factor (10^{-11} M), 70% and 14% of the cells differentiated into the intermediates and macrophages, respectively.

rTNF also enhanced the differentiation-inducing activity of rG-CSF (Fig. 3a) and rIL-1 (Fig. 3b) by two or
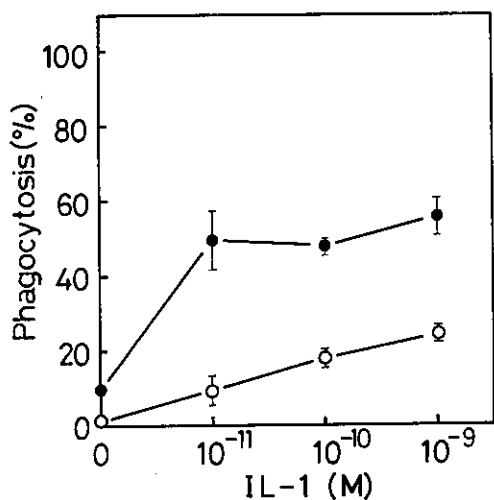

Fig. 4. Effect of rG-CSF on differentiation of M1 cells induced by rIL-1. M1 cells were incubated with various concentrations of rIL-1 in the presence (●) or absence (○) of rG-CSF ($10^{-9}M$) for 2 days and then their phagocytic activity was assayed. Values are means of duplicate determinations; bars, SE. The second experiment gave similar results.
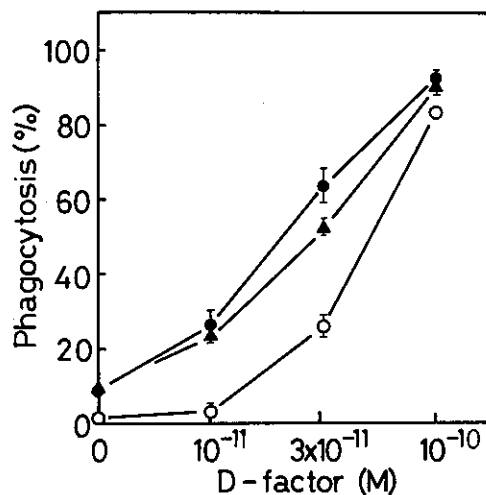
Fig. 5. Effects of rG-CSF and rIL-1 on differentiation of M1 cells induced by D-factor. M1 cells were incubated with various concentrations of D-factor with rG-CSF ($10^{-9}M$, ●) or rIL-1 (10^{-11} M, ▲) or without (○) for 2 days and then their phagocytic activity was assayed. Values are means of duplicate determinations; bars, SE. Three other experiments gave similar results.
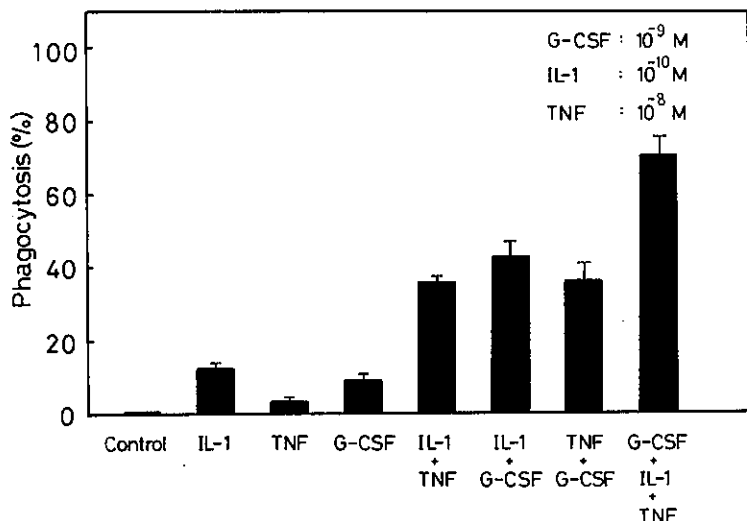

Fig. 6. Effects of combinations of rG-CSF, rIL-1 and rTNF on differentiation of M1 cells. M1 cells were incubated with rG-CSF, rIL-1 and/or rTNF for 2 days and then their phagocytic activity was assayed. Values are means of triplicate determinations; bars, SE. Three other experiments gave similar results.

three fold but the maximum percentage phagocytic cells did not exceed 50%. After incubation of M1 cells for two days, rIL-1 at more than $10^{-10}M$ decreased the cell growth to 70% of the control but rG-CSF had no effect. The combination of rTNF and rG-CSF or rIL-1 decreased the cell growth more efficiently than any of these cytokines alone.

IL-1 and TNF were reported to stimulate the production of prostaglandins in many cells, including monocytes and macrophages.[20, 21] Honma et al. reported that prostaglandin E did not directly induce but enhanced induction of differentiation of M1 cells by dexamethasone.[22] However, the action of rIL-1 and rTNF might not be mediated by the production of prostaglandin E, because indomethacin did not block the effects of rIL-1 and rTNF (data not shown).

Combined effects of other cytokines Finally, we examined the effect of the combination of other cytokines on differentiation of M1 cells. The combination of rG-CSF and rIL-1 was more effective induction of phagocytic activity in M1 cells than either cytokine alone, but the maximum percentage of phagocytic cells was 60% (Fig. 4). Moreover, rG-CSF or rIL-1 enhanced induction of differentiation of M1 cells by a suboptimal concentration of D-factor (Fig. 5).

Macrophages are known to produce TNF, IL-1 and G-CSF.[23, 24] D-factor activity was also found in the conditioned medium of macrophages[2] but has not yet been purified. We examined the effect of the combination of these three cytokines on M1 cells (Fig. 6). When M1 cells were incubated simultaneously with rTNF, rIL-1 and rG-CSF, a high percentage (70%) of M1 cells became phagocytic cells. The combination of three cytokines was more effective than the combination of any two of these cytokines.

DISCUSSION

We examined the effects of the cytokines rTNF, rLT and rIL-1 on differentiation of M1 cells, compared with those of D-factor and rG-CSF. TNF and LT were reported to induce differentiation of human myeloid leukemic cell lines at low concentrations from $10^{-11}M$ to $10^{-10}M$.[10-13] However, TNF and LT were ineffective for M1 cells at these concentrations (Fig. 1). A slight increase in phagocytic activity was observed when M1 cells were treated with a high concentration $(10^{-7}M)$ of rTNF.

We found that rIL-1 induced differentiation of M1 cells. The differentiation-inducing activity of rIL-1 was similar to that of rG-CSF but less than that of D-factor (Fig. 1). One possibility to account for the different sensitivity of M1 cells to cytokines is that there are at least two populations of cells in M1 cells. However, this

is unlikely because the low sensitivity of M1 cells to rG-CSF was observed even when a newly isolated subclone was used.[18]

Synergistic effects between TNF and other inducers, such as interferon-γ, retinoic acid and vitamin D_3 were reported in studies on the induction of differentiation of human myeloid leukemic cells.[10, 25, 26] In this study, we found that rTNF enhanced induction of differentiation of M1 cells by D-factor, rG-CSF or rIL-1(Figs. 2 and 3). Moreover, we found that the combination of any two of the cytokines, D-factor, rG-CSF and rIL-1, induced differentiation of M1 cells more efficiently than any of these cytokines alone (Figs. 4 and 5).

Our data on the combined effect of IL-1 and TNF are consistent with the earlier report by Tamatani et al.[19] that IL-1 induced differentiation of M1 cells in the presence of TNF. They also reported that IL-1 induced differentiation of M1 cells in the presence of a low dose of lipopolysaccharide.[27] However, in their experiments, IL-1 did not induce the differentiation of M1 cells by itself. The discrepancy between their results and ours on the differentiation-inducing activity of IL-1 might be due to the concentration of IL-1 used. They examined the effect of IL-1 at maximally $3 \times 10^{-11}M$ but we found the differentiation-inducing activity of IL-1 at more than $10^{-10}M$.

We previously reported that there are two types of D-factor for M1 cells which differ from each other in antigenicity and molecular size.[5, 6] One is the D-factor (molecular weight 40,000–70,000) produced by mouse Ehrlich cells, L929 cells and activated lymphocytes, and the other is the D-factor (molecular weight 20,000–25,000) produced by macrophages. The latter has not yet been purified. Macrophages are known to produce TNF, IL-1 and G-CSF[23, 24] and the combination of rTNF, rIL-1 and rG-CSF induced differentiation of M1 cells more efficiently than the combination of any two of these cytokines (Fig. 6). Recently, interleukin-6, also produced by macrophages, was found to induce differentiation of M1 cells.[28] These results suggest that at least a part of the D-factor activity found in the conditioned medium of macrophages may be accounted for by the combined effect of these cytokines.

In conclusion, our results suggest that the combination of two or three cytokines described here is of potential value for differentiation therapy of myeloid leukemia.

ACKNOWLEDGMENTS

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