

Characterization of Factors Stimulating Differentiation of Mouse Myeloid Leukemia Cells from a Yoshida Sarcoma Cell Line Cultured in Serum-free Medium¹

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ABSTRACT

A clone, YS-T22, of cells from Yoshida sarcoma cell line, YSSF-212T, grown in "serum-free" culture medium produced factors stimulating differentiation of mouse myeloid leukemia cells (M1) to macrophages and granulocytes. The formation of macrophages and granulocytes was accompanied by induction of phagocytosis, locomotive activity, and lysosomal enzyme activities. The rates of induction of these differentiated phenotypes were proportional to the concentration of the factor added and the period of treatment.

The factor stimulating differentiation of M1 cells was a heat-labile, nondialyzable proteinaceous substance that was inactivated by trypsin but not by ribonuclease or glycosidases.

On diethylaminoethyl cellulose chromatography, the factor stimulating differentiation of M1 cells from conditioned medium of YS-T22 cells was eluted in various fractions with or without activity of the colony-stimulating factor.

INTRODUCTION

Myeloid leukemia cell line M1, derived from an SL strain mouse with spontaneous leukemia, can be induced to differentiate *in vitro* into macrophages and granulocytes by treatment with various proteinaceous D-factors³ (1, 3, 8-11, 13-16, 18, 19-21) and other chemicals (4-8, 12, 18-23).

D-Factors like those originally found in CM of mouse embryo cells (11) were also detected in CM of embryo cells of various other mammals (14). The D-factors in mouse embryo cells were shown to be glycoproteins with molecular weights of 40,000 to 50,000 and to differ in nature from the CSF (13).

Although D-factors were found in the CM of primary and secondary cultures of embryo cells of various mammals, they were scarcely detectable in CM of established cell lines, including tumor cells (14). One exceptional established cell line, E1, derived from mouse embryo cells, was found to produce D-factor with a molecular weight of 68,000 (3); but since this factor was a single protein with CSF activity (3), it was thought to be different from the factors produced by primary and secondary cultures of mouse embryo cells (11, 13, 14).

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³ The abbreviations used are: D-factor, factor stimulating differentiation of mouse myeloid leukemia M1 cells; CM, conditioned medium; CSF, colony-stimulating factor; PBS, phosphate-buffered saline (138 mM sodium chloride-2.7 mM potassium chloride-8 mM dibasic sodium phosphate-1.5 mM monobasic potassium phosphate, pH 7.4).

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There are no previous reports of studies on production of D-factor by an established cell line in serum-free medium. However, recently, Ohno *et al.* (17) observed production of CSF with a molecular weight of 22,000 by a Yoshida sarcoma cell line, YSSF-212T, maintained in serum-free culture medium. This medium avoids the complication of contaminating substances from serum.

Therefore, in this study, we used YSSF-212T cells in serum-free medium to investigate the mechanisms regulating the production of D-factors and CSF by cultured cells. We first examined whether YSSF-212T cells could actually produce D-factor for M1 cells as well as CSF and found that this was the case. Although uncloned YSSF-212T cells produced only a small amount of D-factor, one clone, the YS-T22 clone, was found to produce a large amount. We then examined the properties of D-factor. Results showed that D-factor from YS-T22 cells was nondialyzable, heat labile, and heterogeneous, some fractions from DEAE-cellulose being associated with CSF.

MATERIALS AND METHODS

Cells and Cell Culture. Yoshida rat sarcoma cells (YSSF-212T, a line maintained in culture without serum for more than 212 transfers) were cultured in serum-free medium (24), supplemented with 1% bovine serum albumin Fraction V (Armour Co., Kankakee, Ill.) and 1- μ g/ml amounts each of oleic acid, linoleic acid, and poly-L-arginine.

A myeloid leukemia cell line, M1, from an SL mouse with spontaneous myeloid leukemia was cultured in Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins and supplemented with 10% calf serum inactivated by heating at 56° for 30 min (11).

Cloning of Cells. Twenty-two clones (YS-T1-YS-T22) of Yoshida sarcoma YSSF-212T cells were isolated in 0.5% agar containing serum-free medium. The cloned cells were cultured in serum-free medium as described under "Cells and Cell Culture."

Preparation of CM. Cells of each clone (YS-T1-YS-T22) of YSSF-212T cells were seeded at a concentration of 2×10^5 cells/ml and cultured for 3 days at 37° in a humidified atmosphere of 5% CO₂ in air. The CM was obtained by centrifugation of the cultures at $800 \times g$ for 10 min.

Test for Lysozyme Activity. Lysozyme activity was determined by a modification of the lysoplate method of Osseman and Lawlor with lysoplates containing 1% agar, m/15 phosphate buffer (pH 6.6), 0.05 M NaCl, and heat-killed *Micrococcus lysodkiticus* (0.5 mg/ml) (12). After incubation for 24 hr

at 27°, the diameters of the clear zones were measured.

Test for Phagocytic Activity. M1 cells were incubated at a concentration of 5×10^5 cells/ml with dexamethasone or CM for 2 days. Then, a sample of the cells was tested for phagocytic activity as described previously (7, 9).

Examination of Morphologically Differentiated Cells. M1 cells that had been incubated with or without CM were smeared on a glass slide and stained with May-Grünwald-Giemsa solution for examination of morphology. Morphologically differentiated cells were classified as mature macrophages (Fig. 1b) or granulocytes (Fig. 1, e and f), and intermediate cells (Fig. 1, c and d) were classified as cells that were morphologically intermediate between typical unchanged myeloblastic cells (Fig. 1a) and mature macrophages (Fig. 1b) or granulocytes (Fig. 1, e and f). The percentage of morphologically differentiated cells was estimated by examination of at least 250 cells.

Test for Migrating Activity. The M1 cells were seeded at a cell density of 1000 cells/6-cm diameter plastic Petri dish in soft agar (0.3%) on a basal agar layer (0.5%) by the method of Ichikawa (11). One ml of CM from YS-T22 cells was poured onto 10-day-old colonies. The number and type of colonies were examined 7 days later and classified as "compact type," without migrating cells, or "dispersed type," with migrating cells, as described by Ichikawa (11).

Enzymatic Digestion of D-Factor in CM from YS-T22 Cells. CM of YS-T22 cells was dialyzed against PBS for 2 days at 4°. Then, a sample of the CM was incubated with pancreatic RNase (0.5 mg/ml), trypsin (1 or 2.5 mg/ml), or mixed glycosidases (1 mg/ml) at 37° for 2 hr. After incubation with trypsin, the same concentration of trypsin inhibitor (1 or 2.5 mg/ml) as trypsin was added to terminate the enzyme action, and incubation was continued for 1 hr. No inhibitor was added to mixtures with RNase or mixed glycosidases. As controls, these enzymes were incubated with PBS under the same conditions. At the concentrations used, these enzymes had no apparent effect on the cells. RNase, trypsin, and trypsin inhibitor (soybean trypsin inhibitor) were obtained from Sigma Chemical Co., Saint Louis, Mo., and mixed glycosidases were obtained from Seikagaku Kogyo Co., Tokyo, Japan.

Test for CSF Activity. The activity of CSF was assayed in 35-mm plastic Petri dishes with 10^5 mouse bone marrow nucleated cells, obtained from male C3H mouse femur, 0.3% (w/v) agar, 60% (v/v) McCoy's Medium 5A, 20% horse serum, and 20% (v/v) CSF solution in PBS, in a total volume of 1 ml. This mixture was incubated in a humidified CO₂ incubator for 7 days, and then the numbers of colonies containing more than 50 cells were counted as described previously (17).

DEAE-Cellulose Column Chromatography. CM of YS-T22 cells was treated with 90% saturated ammonium sulfate (6.2 g protein), and the resulting precipitate was dialyzed against 2 mM sodium phosphate buffer, pH 7.2, and loaded on a DEAE-cellulose column (6 x 24 cm) equilibrated with the same buffer. Proteins were eluted with an increasing concentration of NaCl in 10 mM sodium phosphate buffer, pH 6.5, and the absorption of the eluate was monitored at 280 nm. Fractions of 17.5 ml were collected, and the 538 fractions collected were combined into 19 fractions. These 19 fractions were concentrated to one-fifth of their original volume by ultrafiltration with a membrane filter (PM-10, Amicon Corp., Cambridge, Mass.), and samples were used for assay of CSF and D-factor activities.

RESULTS

Production of D-Factor from YSSF-212T Cell Clones

Scarcely any activity of D-factor was detectable in CM from uncloned YSSF-212T cells (Table 1). However, CM from clone YS-T22 had high D-factor activity, like that of dexamethasone described previously (Table 1). As shown in Chart 1, production of D-factor by the YS-T22 cells increased progressively for about 2 to 3 days after inoculation of 2×10^5 YS-T22 cells/ml of medium and then reached a plateau, whereas the cell number increased for 3 days and reached a plateau on Day 4 (Chart 1).

D-Factor activity in the CM was enhanced by dialysis, and the difference in the D-factor activities of dialyzed and undialyzed CM was greater after longer times after cultivation of the cells (Chart 1). The result suggests that the CM contains some dialyzable factors inhibiting the D-factor.

Induction of Differentiation of M1 Cells by CM from YS-T22 Clone Cells

Since CM from YS-T22 clone cells produced high D-factor activity for induction of phagocytic activity of M1 cells, the effects of the CM on induction of several markers of differentiation of M1 cells were examined in detail.

Table 1
Induction of differentiation of M1 cells with CM of various clones of YSSF-212T cells

Clone	Concentration of CM	Differentiation of M1 cells	
		Lysozyme ^a activity (units/mg protein)	Phagocytosis ^b (%)
Untreated		0.1	0.3
YSSF-212T	50%	2	2
YS-T1-YS-T21	50%	1.5-6	2-8
YS-T22	50%	27	48
Dexamethasone ^c	10^{-5} M	38	50

^a See "Materials and Methods." Values are means of duplicate determinations.

^b The percentage of phagocytic cells among at least 300 viable cells was calculated as described in "Materials and Methods." Values are means of duplicate determinations.

^c A known inducer of differentiation of M1 cells used as a positive control.

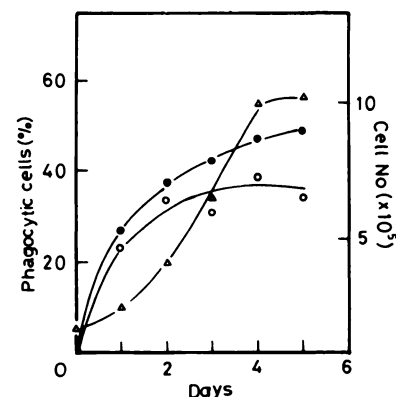


Chart 1. Time course of production of D-factor in CM of YS-T22 cells. YS-T22 cells were inoculated at 2×10^5 cells/ml into serum-free culture medium as described in "Materials and Methods." On the days indicated after inoculation of the cells, the cell number (Δ) was determined, and CM of the cells was harvested for examination of its activity for induction of phagocytosis in M1 cells. Percentage of phagocytic M1 cells induced by treatment with CM with (●) or without (○) dialysis against PBS.

Induction of Phagocytic Activity. Chart 2 shows the dose-dependent induction of phagocytosis of M1 cells by incubation of the cells with culture media containing various amounts of CM from YS-T22 clone cells. The percentage of phagocytic cells increased linearly up to about 50% with increase in the amount of CM in the medium to 50% (v/v) of the medium.

Morphological Changes. Although untreated M1 cells stained with Giemsa had a large round nucleus and a small amount of strongly basophilic cytoplasm (Fig. 1a), many of the cells treated with CM from YS-T22 clone cells were either macrophage-like cells with a smaller nucleus and more abundant, weakly basophilic cytoplasm (Fig. 1b) or granulocyte-like cells with polymorphic nuclei (Fig. 1, e and f). Cells in morphologically intermediate stages of differentiation between typical myeloblastic cells and mature granulocytes or macrophages were also seen on treatment of M1 cells with CM from YS-T22 clone cells (Fig. 1, c and d).

The number of morphologically differentiated cells increased with time of cultivation with CM from YS-T22 clone cells and with the concentration of CM in the culture medium. After treatment of M1 cells with 50% CM from T22 clone cells for 3 days, 25 and 17% of the cells were morphologically differentiated into macrophage- and granulocyte-like cells, respectively, and 43% were differentiated into morphologically intermediate stages of maturation, as shown in Table 2. Under similar conditions without CM, no mature macrophage- or granulocyte-like cells were observed.

Induction of Migrating Activity. When M1 cells were incubated in soft-agar plates, they produced compact colonies in which the cells adhered together. However, when they were incubated with CM from T22 clone cells, they formed dispersed colonies. As shown in Chart 3, the percentage of dispersed colonies composed of cells with migrating activity such as macrophage- or granulocyte-like cells increased linearly to about 70% with increase in the concentration of the CM from T22 clone cells to 50% of the medium.

Properties of D-Factor in CM from YS-T22 Clone Cells

The ability of CM from YS-T22 clone cells to induce phagocytic activity of M1 cells was almost completely inactivated by heating the CM at 70° for 10 min, but it was stable for at least 1 month at 4° and it was not lost on dialysis, as described above.

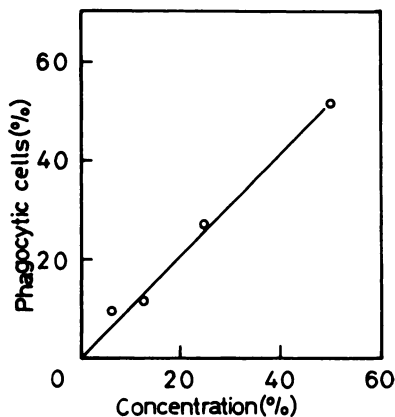


Chart 2. Effects of various concentrations of CM of YS-T22 cells on induction of phagocytic cells. M1 cells were treated with CM of YS-T22 cells for 2 days.

Table 2
Induction of morphological changes in M1 cells by CM of YS-T22 cells

CM (50%)	Morphological changes (%) ^a			
	Myeloblasts	Intermediate	Macrophages	Granulocytes
Untreated	90	10	0	0
Treated	15	43	25	17

^a See "Materials and Methods." Values are means of duplicate determinations.

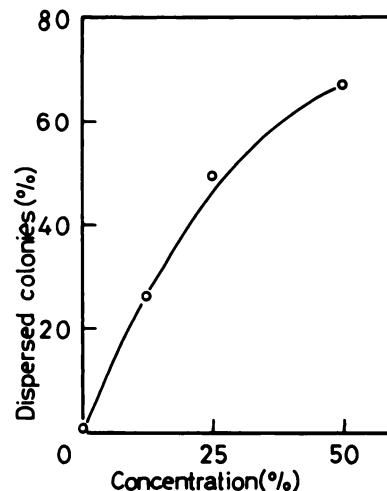


Chart 3. Induction of locomotive activity of M1 cells by CM of YS-T22 cells. The percentage of dispersed-type colonies and the total colonies were determined 7 days after pouring 1-ml volumes of various concentrations of CM from YS-T22 cells onto 10-day-old M1 cell colonies.

The phagocytosis-inducing activity of the CM decreased markedly on treatment of the CM from YS-T22 clone cells with trypsin but not with RNase or glycosidases (Table 3). These results indicate that D-factor in the CM from YS-T22 clone cells is a heat-labile, nondialyzable, proteinaceous substance.

Relationship between D-factor and CSF in CM from YS-T22 Clone Cells

Since production of CSF from YSSF-212T cells was reported previously, we examined the relation of CSF to D-factor. The material precipitated from the CM with 90% saturated ammonium sulfate was fractionated by DEAE-cellulose column chromatography, and the activities of CSF and D-factor in each fraction were measured. As shown in Chart 4, although both activities were clearly detected in various fractions, their distributions were heterogeneous. Fraction 1 contained high D-factor activity, but no CSF activity, whereas Fractions 2 and 4 had CSF but no D-factor activity and, although both activities were detected in Fractions 3 and 5, the activity of CSF was higher than that of D-factor in Fraction 3 while the reverse was true in Fraction 5.

DISCUSSION

Scarcely any D-factor activity was found in the conditioned medium from YSSF-212T cells, but fairly high activity was found in CM from clone YS-T22 cells derived from YSSF-212T cells. As described above, Maeda and Ichikawa (14) examined various tissue culture cells for production of D-factor and found

that primary and secondary cultures of embryo cells of various mammals usually produced appreciable D-factor, whereas cultures of established cell lines including some tumor cells did not. As an exceptional established cell line, E1 cells derived from mouse embryo cells produced high D-factor activity (3).

The mechanism regulating production of D-factor by the cells is unknown, but these results suggest that production or activity of D-factor may be suppressed during culture of primary cultured cells for establishment of cell lines. Among the cell population of established cell lines, however, a small proportion of the cells, such as YS-T22 cells from YSSF-212T, seem to produce D-factor, and these may be responsible for the production of the slight amount of the D-factor in CM from whole populations of cells of established cell lines. The possibility that production of D-factor by the whole population of cells from established cell lines is suppressed also cannot be neglected.

The D-factor from YS-T22 cells was a heat-labile, nondialyzable, proteinaceous substance with a heterogeneous elution profile on DEAE-cellulose column chromatography. Not all fractions of the D-factor of YS-T22 cells eluted from the column were associated with CSF (Chart 4). Some fractions had only D-factor or CSF activity. However, both activities were found in some fractions, and further purification of these fractions is required to determine definitely whether there is any association of the 2 activities.

On gel filtration on Ultrogel AcA44, Fraction 1 with only D-factor activity separated into 2 fractions with molecular sizes of about 45,000 and 88,000 (data not shown).

The present results and those of Maeda *et al.* on D-factor in

CM of mouse embryo cells show that D-factor is not always associated with the activity of CSF. Furthermore, Gallagher *et al.* (2) reported that CM of whole human embryo cells prompted growth and differentiation of culture of leukocytes from patient with acute myelogenous leukemia but had no stimulatory effect on peripheral blood or bone marrow cells from normal donors, suggesting that the activity in the CM was also distinct from CSF. Further purification and characterization of the D-factor and CSF are under way to elucidate the detailed molecular properties of the 2 factors.

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Table 3

Effects of various enzyme treatments on activity of CM of YS-T22 cells for induction of phagocytosis of M1 cells

Enzyme	Concentration (mg/ml)	Phagocytic cells ^a (%)
Untreated		39
Trypsin	1	19
	2.5	13
Mixed glycosidases ^b	1	26
RNase	0.5	39

^a The percentage of phagocytic cells among at least 300 viable cells was calculated as described in "Materials and Methods." Values are means of duplicate determinations.

^b "Mixed glycosidases" was a mixture of α - and β -N-mannosidases, α - and β -glucosidases, α - and β -galactosidases, α - and β -N-acetylglucosaminidases, α -L-fucosidase, β -N-acetylgalactosaminidase, and β -xylosidase.

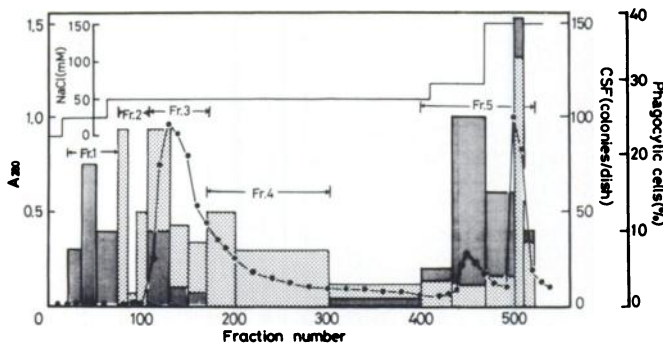


Chart 4. DEAE-cellulose column chromatography of D-factor and CSF from CM of YS-T22 cells. ●, A_{280 nm}; stippled bars, CSF (colonies/dish); solid bars, percentage of phagocytic cells.

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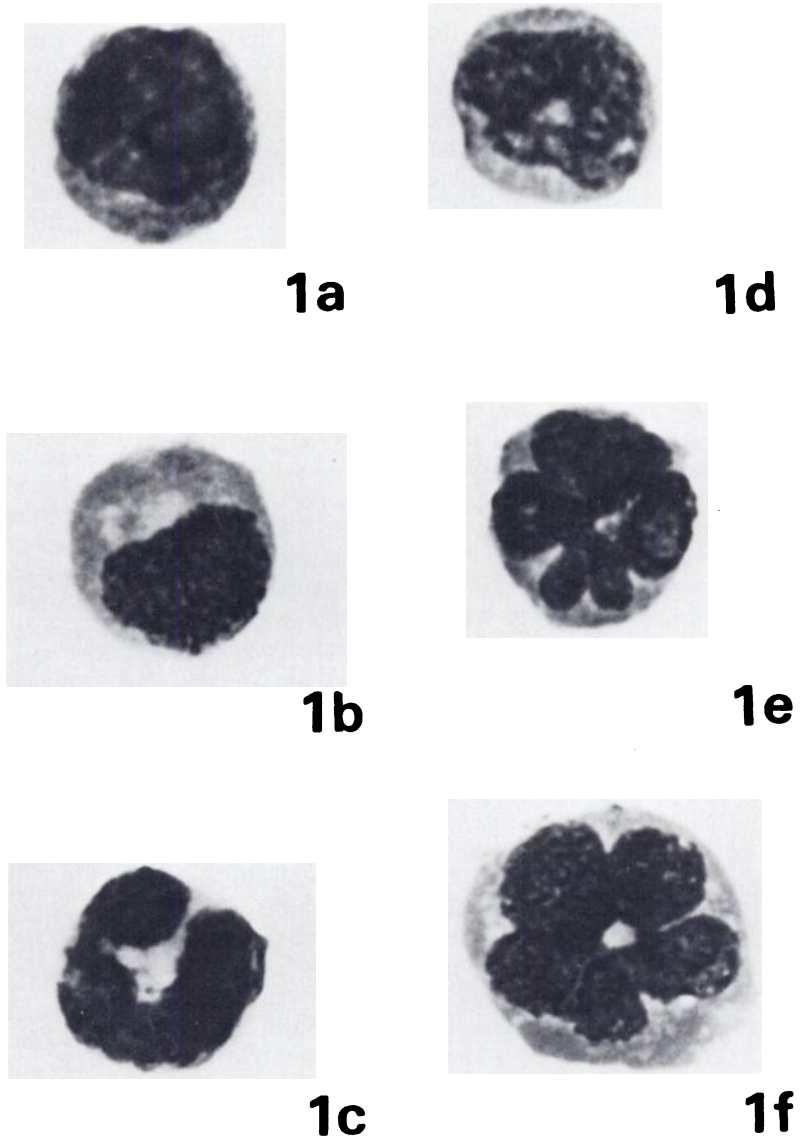


Fig. 1. Morphological changes of M1 cells treated with CM of YS-T22 cells. *a*, untreated myeloblastic M1 cell; *b*, macrophage; *c* and *d*, cells in intermediate stages of differentiation; *e* and *f*, granulocyte. May-Grünwald-Giemsa, $\times 1200$.