# Characterization of a factor inducing differentiation of mouse myeloid leukemic cells purified from conditioned medium of mouse Ehrlich ascites tumor cells

Mikio Tomida, Yuri Yamamoto-Yamaguchi and Motoo Hozumi

*Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama-Ken 362, Japan*

Received 24 September

A factor inducing differentiation of mouse myeloid leukemic cells (M1) into macrophages was purified to apparent homogeneity from 168 l of CM of Ehrlich ascites tumor cells. The purified factor was half-maximally active at $2 \times 10^{-11}$ M. The factor was analyzed by radioiodination, SDS-polyacrylamide gel electrophoresis and autoradiography. Its $M_r$ was 40000–50000. On reduction, the factor lost activity, but showed no subunit structure. Treatment of the factor with endo-β-*N*-acetylglucosaminidase F, but not endo-β-*N*-acetylglucosaminidase H, gave rise to a molecule of $M_r$ 20000–28000. The activity of the factor from Ehrlich cells was completely neutralized by antiserum to the factor of $M_r$ 50000–70000 from mouse fibroblast L929 cells.

*Myeloid leukemic cell* *Macrophage* *Differentiation factor* *Structure* *Glycoprotein* *Antigenicity*

## 1. INTRODUCTION

Mouse myeloid leukemic M1 cells can be induced to differentiate into macrophages by protein inducers (D-factors) and various chemicals [1–3]. On differentiation, M1 cells express a wide variety of morphological, biochemical and functional characteristics of normal macrophages, such as phagocytic and locomotive activities, lysosomal enzyme activities, Fc and C3 receptors and prostaglandin production. Untreated M1 cells are leukemogenic in syngeneic mice, but on differentiation M1 cells lose their leukemogenicity. Injection of D-factor, also named MGI-2, into mice inoculated with M1 cells inhibited the proliferation of the leukemic cells in the bone marrow and prolonged the survival of the mice [4]. Therefore, D-factors are possibly important for suppressing development of myeloid leukemia and may be useful for therapy of leukemia [2,3].

Although D-factor is thought to be produced in vivo mainly by activated macrophages and lymphocytes [5], it is also produced spontaneously in vitro by some established cells. We [6] reported a procedure for purification of a D-factor of $M_r$ 62000 from CM of mouse fibroblast L929 cells and suggested that the D-factor is distinct from CSF, the growth factor for normal precursors of macrophages and/or granulocytes. However, further studies on this factor were difficult because L cells produced very little D-factor and the yield of purified D-factor was low.

Here, we report the purification of a D-factor of $M_r$ 40000–50000 from a more readily available source, CM of Ehrlich ascites tumor cells, and discuss the relation between the two D-factors of different sizes.

*Abbreviations:* D-factor, differentiation-inducing factor; MGI, macrophage and granulocyte inducer; M-, G- and GM-CSF, macrophage, granulocyte and granulocyte-macrophage colony-stimulating factors, respectively; HPLC, high-performance liquid chromatography; CM, conditioned medium

*Published by Elsevier Science Publishers B.V.*
00145793/84/$3.00 

## 2. MATERIALS AND METHODS

### 2.1. *Assay of D-factor*

D-Factor was assayed by measuring induction of phagocytic activity in M1 cells (clone T-22) as in [7], since induction of phagocytic activity in the cells was associated with the inductions of other phenotypic markers of cell differentiation, such as morphological differentiation and lysozyme activity. M1 cells ($5 \times 10^5$ cells) were incubated for 2 days in 1 ml of Eagle's minimal essential medium containing D-factor, and then for 4 h with a suspension of polystyrene latex particles. Induction of phagocytic cells was proportional to the concentration of D-factor. Fifty units of D-factor was defined as the activity inducing phagocytic activity in 50% of the cells under these conditions.

### 2.2. *Preparation of CM of Ehrlich ascites tumor cells*

Ehrlich ascites tumor cells were passaged in ICR mice by intraperitoneal inoculation of about $10^7$ cells. The cells were harvested from the peritoneal cavity after 9 days, washed 3 times with phosphate-buffered saline and seeded at $10^6$ cells/ml in spinner flasks containing 8 l of serum-free Eagle's medium. After incubation for 2 days at 37°C, the culture fluid was collected and centrifuged (10000 $\times$ g, 20 min) to remove cells and cell debris.

### 2.3. *Purification procedures*

Volumes of 24 or 32 l of CM of Ehrlich ascites tumor cells were concentrated to 2 l with an Amicon H10P10 hollow fiber and then to 200 ml with an H1P10 hollow fiber. Thereafter, D-factor was purified essentially as in [6], but the Sephadex G-200 column was replaced by an Ultrogel AcA 44 column (LKB-Produkter AB, Stockholm).

### 2.4. *Protein determination*

Protein in fractions before the second HPLC step was determined by the dye fixation method (Bio-Rad Laboratories) with bovine serum albumin as a standard. Since the protein content of fractions after the second HPLC step was low, it was determined from the absorbance profile assuming that 1 mg protein/ml had an average absorbance of 1 at 280 nm.

### 2.5. *Iodination of D-factor*

Purified D-factor was iodinated as in [8,9]. Aliquots of D-factor from the TSK G3000SW column were dried under vacuum in a Speed Vac unit. Then the preparation was mixed first with 10 $\mu$l of 0.2 M sodium phosphate buffer, pH 6.5, containing 0.04% (w/v) polyethylene glycol 6000 and 10 $\mu$l of 50% (v/v) dimethyl sulfoxide then with 10 $\mu$l of carrier-free $^{125}$I (1 mCi, Amersham Japan, Tokyo) and finally with 10 $\mu$l of a solution of chloramine T (200 $\mu$g/ml). The mixture was stood at 0°C for 30 min and then 10 $\mu$l of sodium metabisulfite (200 $\mu$g/ml) and 10 $\mu$l of 0.1 M KI were added. The iodinated D-factor was separated from unbound $^{125}$I by chromatography on an Ultrogel AcA 44 column in phosphate-buffered saline.

### 2.6. *SDS-polyacrylamide gel electrophoresis*

The $^{125}$I-labeled D-factor was subjected to electrophoresis on 12% acrylamide slab gels (100 mm long, 140 mm wide, 1.5 mm thick) as in [10], at a constant current of 30 mA for 3 h. Autoradiography was performed at $-70$°C for 1 day with X-Omat RP film (Kodak) and intensifying screens (DuPont Cronex HI Plus). The distribution of D-factor activity on the gel was examined in the absence of 2-mercaptoethanol. The activity was eluted from sections of the gel as in [11].

### 2.7. *Endoglycosidase treatment*

Endo-$\beta$-*N*-acetylglucosaminidase F (EC 3.2.1) from *F. meningosepticum* and endo-$\beta$-*N*-acetylglucosaminidase H (EC 3.2.1.96) from *S. plicatus* were purchased from New England Nuclear, Boston, MA. D-Factor labeled with $^{125}$I was incubated with 2 units of endoglycosidase F for 18 h at 37°C in reaction mixtures consisting of 0.1 M sodium phosphate buffer, pH 6.1, 50 mM EDTA, 1% Nonidet P-40, 0.5% SDS and 0.5% 2-mercaptoethanol. The $^{125}$I-labeled D-factor was treated with 2.5 $\mu$g of endoglycosidase H for 18 h at 37°C in a reaction mixture consisting of 500 mM sodium citrate buffer, pH 6.0, 0.5% SDS and 0.5% 2-mercaptoethanol.

### 2.8. *Anti-D-factor antiserum*

Antiserum was prepared by immunizing a rabbit with D-factor partially purified from the CM of L cells as in [11].

## 3. RESULTS

### 3.1. *Purification of D-factor*

D-Factor was purified from CM of Ehrlich ascites tumor cells by a similar method to that used for purification of D-factor from CM of L cells. A summary of the purification is shown in table 1. D-Factor from Ehrlich cells and D-factor from L cells were eluted in similar positions from a phenyl-Sepharose column and a μBondapak $C_{18}$ column on reverse-phase HPLC. On gel filtration of these preparations, the main D-factor activity from Ehrlich cells was eluted in fractions corresponding to $M_r$ 40000–50000 (fig.1), whereas the D-factor from L cells was eluted in fractions corresponding to $M_r$ 50000–70000 [6,7]. A minor fraction of activity of D-factor of $M_r$ 25000 was found in a crude preparation from Ehrlich cells, but this activity was lost during subsequent purification. Aliquots of fractions from the high performance gel filtration column of TSK G3000SW (fig.2) were radioiodinated and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Fraction 64 gave two bands of contaminating material, but fractions 65 and 66 each gave a single band of material of $M_r$ 40000–50000 that coincided with the biological activity (not shown). Results on the induction of phagocytic activity of M1 cells by purified D-factor are shown in fig.3. Assuming that the D-factor has an $M_r$ of 45000, it was half-maximally active at $2 \times 10^{-11}$ M. Even at $5 \times 10^{-9}$ M (11200 U/ml) it did not induce colony formation of macrophages or granulocytes from normal bone marrow.

### 3.2. *Effect of glycosidase treatment of D-factor*

CM of Ehrlich cells was applied to a column of Con A-Sepharose (Pharmacia) as in [6,13]. Half the D-factor activity was adsorbed to the column and could be eluted with methyl-α-D-glucopyranoside. Therefore, D-factor from Ehrlich cells, like that from L cells, contains carbohydrate. To determine the nature of the carbohydrate and $M_r$ of the deglycosylated D-factor, we treated the purified radiolabeled D-factor with endoglycosidases in the presence of SDS and 2-mercaptoethanol. Treatment with endo-β-*N*-acetylglucosaminidase F, but not endo-β-*N*-acetylglucosaminidase H, reduced the $M_r$ of the D-factor from 45000 to 24000 (fig.4). Treatment of the D-factor with 2-mercaptoethanol resulted in loss of biological activity, but no change in its $M_r$ occurred. Therefore D-factor is a glycoprotein that contains internal disulfide bonds but not subunits.

### 3.3. *Antigenicity of D-factors*

We prepared antiserum against partially purified D-factor from L cells. The antiserum almost completely neutralized the D-factor activity in mouse endotoxin serum and CM from L cells, embryo cells, lung tissue and spleen cells stimulated with

Table 1

Purification of D-factor from Ehrlich cell-conditioned medium

| Step | D-factor[a] ($U \times 10^{-3}$) | Protein | Specific activity (U/mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| CM (168 liters) | 16488 | 5.2 g | $3.2 \times 10^3$ | 1 | 100 |
| Hollow fiber | 7304 | 2.3 g | $3.2 \times 10^3$ | 1 | 44.3 |
| DEAE-cellulose | 6438 | 916 mg | $7.0 \times 10^3$ | 2 | 39.0 |
| Ultrogel AcA 44 | 3715 | 326 mg | $1.1 \times 10^4$ | 4 | 22.5 |
| Phenyl-Sepharose | 2055 | 28 mg | $7.3 \times 10^4$ | 23 | 12.5 |
| μBondapak $C_{18}$ (1st) | 1848 | 4 mg | $4.8 \times 10^5$ | 149 | 11.0 |
| μBondapak $C_{18}$ (2nd) | 1135 | 422 μg | $2.7 \times 10^6$ | 843 | 6.9 |
| TSK G3000SW | 1104 | 24 μg | $4.5 \times 10^7$ | 14200 | 6.7 |

[a] 50 units were defined as the activity inducing 50% of phagocytic cells in 1 ml of M1 cell culture

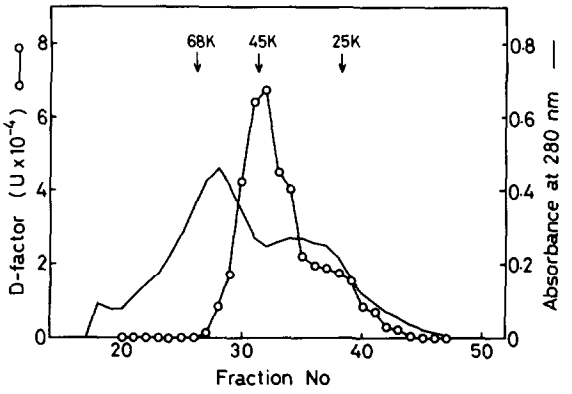


Fig.1. Gel filtration of D-factor from Ehrlich cells. Run-through fractions from the DEAE-cellulose column were concentrated and applied to an Ultrogel AcA 44 column. Fractions were assayed for D-factor activity (○). Absorbance at 280 nm (——). Bovine serum albumin (68 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) were used as marker proteins. K, kDa.

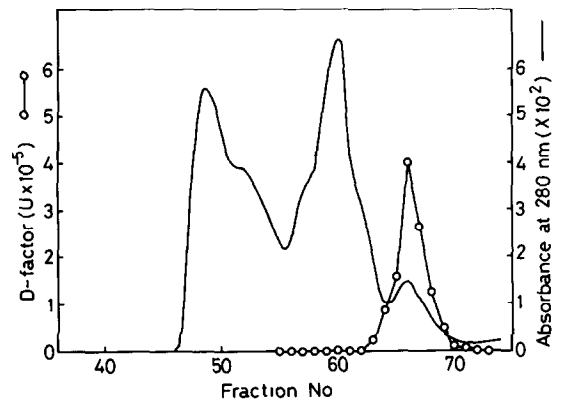


Fig.2. High-performance gel filtration chromatography of D-factor on TSK G3000SW. Active fractions from the reverse-phase HPLC step were applied to a TSK G3000SW column (Toyo Soda, Tokyo) and eluted with 45% acetonitrile containing 0.1% trifluoroacetic acid as in [6]. (○—○) D-Factor activity, (——) absorbance at 280 nm.

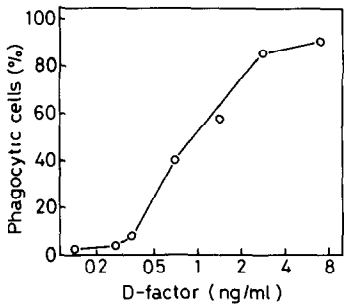


Fig.3. Induction of phagocytic activity in M1 cells by purified D-factor. M1 cells were incubated for 2 days with various concentrations of D-factor from the TSK G3000SW column and then their phagocytic activity for polystyrene latex particles was assayed.

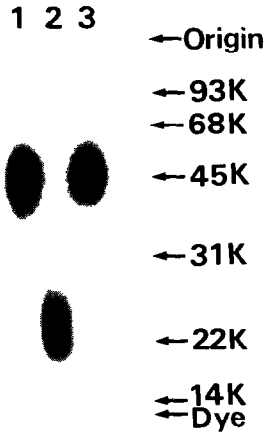


Fig.4. SDS-polyacrylamide gel electrophoresis of $^{125}$I-labeled D-factor treated with glycosidase. (1) Untreated and without 2-mercaptoethanol, (2) treated with endo-β-*N*-acetylglucosaminidase F, (3) treated with endo-β-*N*-acetylglucosaminidase H. The molecular mass markers used were: phosphorylase B (93 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (22 kDa) and lysozyme (14 kDa).

Con A [11]. However, it did not affect the D-factor activity of $M_r$ 20000–25000 in CM of peritoneal macrophages or differentiated M1 cells [12]. As shown in fig.5, D-factor activity in CM of Ehrlich cells was completely suppressed by the antiserum. Results with CM of L cells and macrophages are also shown. At 1:20 dilution, the antiserum completely suppressed D-factor activity in a fraction corresponding to $M_r$ 25000 obtained by gel filtration of CM of Ehrlich cells (see fig.1).

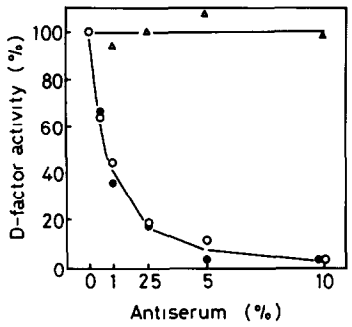


Fig.5. Antigenicity of D-factors. CM of Ehrlich cells (○), L cells (●) and peritoneal macrophages (△) were treated with various concentrations of antiserum to D-factor from L cells at room temperature for 30 min. Then D-factor activity was assayed by measuring induction of phagocytic activity of M1 cells. Values are percentages of the activity of untreated controls.

## 4. DISCUSSION

A factor inducing differentiation of mouse myeloid leukemic M1 cells has been purified to homogeneity from conditioned medium of mouse Ehrlich ascites tumor cells. The D-factor was half-maximally active at $2 \times 10^{-11}$ M. Even at $5 \times 10^{-9}$ M (11200 U/ml) it did not induce colony formation of macrophages or granulocytes from normal bone marrow. Since other purified factors stimulating growth and differentiation of hematopoietic progenitor cells, such as M-CSF [8], GM-CSF [13], G-CSF [14] and interleukin 3 [9], induced colony formation of macrophages and/or granulocytes at $10^{-11}$–$10^{-12}$ M, the D-factor is distinct from the CSFs so far purified. It is also distinct from a factor ($M_r$ 24000–25000) inducing differentiation of the mouse myelomonocytic leukemia cell line WEHI-3B, since the latter was identified as a G-CSF [14].

We [6] previously purified a D-factor ($M_r$ 62000) that is also half-maximally active at $1.7 \times 10^{-11}$ M from CM of L cells. On treatment of L cells with tunicamycin, the $M_r$ of the D-factor decreased from 67000 to 25000, indicating that the D-factor contained Asn-linked oligosaccharides [7]. Here, we showed that the $M_r$ of the D-factor from Ehrlich cells was decreased from 45000 to 24000 by treatment with endo-$\beta$-*N*-acetylglucosaminidase F, but not endo-$\beta$-*N*-acetylglycosaminidase H. The endoglycosidase F cleaves glycans of both 'high mannose' and 'complex' types linked through asparagine to a protein backbone [15], whereas the endoglycosidase H cleaves only glycans of the 'high mannose' type [16]. Therefore, the carbohydrate moieties of the D-factor are of the Asn-linked 'complex' type. However, the carbohydrate moieties of D-factor are not essential for its biological activity, since D-factor produced in the presence of tunicamycin is fully active [7]. The activities of the D-factors from Ehrlich cells and L cells were both completely inhibited by treatment with antiserum to D-factor from L cells. These results suggest that the difference between the sizes of the D-factors from Ehrlich cells and L cells is due to a difference in the degrees of glycosylation of the same or a similar polypeptide moiety.

Although we demonstrate carbohydrate in the D-factor from Ehrlich ascites tumor cells and L cells, authors in [17] reported that the D-factors (MGI-2) from Krebs ascites tumor cells may not contain carbohydrate, because none of them bound to concanavalin A, soybean agglutinin or wheat germ agglutin agarose columns. Nearly all the activity of the D-factor (95%) from L cells [6] and 53% of the activity of D-factor from Ehrlich cells bound to Con A-Sepharose. Therefore, the degree of glycosylation of the D-factors from Krebs ascites tumor cells may be lower than that of our preparations.

At present, the physiological role of D-factor without CSF activity is unknown. Radiolabeled D-factor will be useful for elucidating its normal target cells in vivo.

## ACKNOWLEDGEMENT

This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

[1] Ichikawa, Y. (1969) J. Cell. Physiol. 74, 223–234.
[2] Sachs, L. (1978) Nature 274, 535–539.
[3] Hozumi, M. (1983) Adv. Cancer Res. 38, 121–169.
[4] Lotem, J. and Sachs, L. (1984) Int. J. Cancer 33, 147–154.
[5] Yamamoto, Y., Tomida, M. and Hozumi, M. (1980) Cancer Res. 40, 4804–4809.

[6] Tomida, M., Yamamoto-Yamaguchi, Y. and Hozumi, M. (1984) J. Biol. Chem. 259, 10978–10982.
[7] Yamamoto, Y., Tomida, M., Hozumi, M., Ayusawa, D., Seno, T. and Tamura, G. (1981) Cancer Res. 41, 2534–2539.
[8] Stanley, E.R. and Guilbert, L.J. (1981) J. Immunol. Methods 42, 253–284.
[9] Ihle, J.N., Keller, J., Henderson, L., Klein, F. and Palaszynski, E. (1982) J. Immunol. 129, 2431–2436.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Tomida, M., Yamamoto-Yamaguchi, Y. and Hozumi, M. (1983) FEBS Lett. 151, 281–285.
[12] Tomida, M. (1984) in: Mononuclear Phagocytes, Physiology and Pathology (Dean, R.T. and Jessup, W. eds) Elsevier, Amsterdam, New York, in press.
[13] Burgess, A.W., Camakaris, J. and Metcalf, D. (1977) J. Biol. Chem. 252, 1998–2003.
[14] Nicola, N.A., Metcalf, D., Matsumoto, M. and Johnson, G.R. (1983) J. Biol. Chem. 258, 9017–9023.
[15] Tarentino, A.L. and Maley, F. (1974) J. Biol. Chem. 249, 811–817.
[16] Elder, J.H. and Alexander, S. (1982) Proc. Natl. Acad. Sci. USA 79, 4540–4544.
[17] Lipton, J.H. and Sachs, L. (1981) Biochim. Biophys. Acta 673, 552–569.