Preparation and neutralization characteristics of an antibody to the factor inducing differentiation of mouse myeloid leukemic cells

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Mouse myeloid leukemic M1 cells can be induced to differentiate into macrophages and granulocytes in vitro by a protein inducer (D-factor) in body fluids and conditioned media from various cells and tissues. Antiserum to D-factor was prepared by immunizing a rabbit with D-factor partially purified from conditioned medium of mouse L929 cells. At 1:20 dilution, the antiserum nearly completely suppressed the activities of various D-factor preparations from mice and partially suppressed the activities of preparations from rats and hamsters. It did not cross-react with factors stimulating colony formation of macrophages and granulocytes from normal bone marrow cells.

Myeloid leukemic cell *Differentiation factor* *Antibody* *Bone marrow cell* *Colony-stimulating factor*

1. INTRODUCTION

Mouse myeloid leukemic M1 cells can be induced to differentiate either in vitro or in vivo by a protein inducer (D-factor) and various chemicals [1–3]. D-factor has also been referred to as MGI-2 [4] and has been found in culture fluids of various cells and tissues, including mouse embryo cells [1,5], hamster embryo cells [6], mouse spleen lymphocytes [7], mouse fibroblast L cells [8], rat YS cells [9] and mouse lung [10], and in body fluids such as ascitic fluids [11] and serum of mice injected with bacterial endotoxins [12]. D-factors are heterogenous in molecular size and charge [2] and the relation of D-factors from different sources is still unknown.

Preparations of D-factor all contain CSF activity essential for the in vitro growth of granulocyte and macrophage colonies from normal bone marrow cells. The relation between D-factor and CSF is not well understood. We [7] previously showed that D-factor and CSF in conditioned medium of mouse spleen cells stimulated with Con A or lipopolysaccharide were distinct substances. Furthermore, we [8] recently found that mouse L cells produced D-factor which was a glycoprotein with a M_r of 50000–70000 and that it could be separated from CSF by gel filtration. However, in [4] it was reported that D-factor from conditioned medium of Krebs ascites tumor cells was separated from the bulk of CSF activity but not from some residual CSF activity.

To elucidate the relation of D-factors from different sources and the role of D-factor in growth and differentiation of normal bone marrow cells, we prepared antiserum to D-factor from L cells. This paper reports studies on the neutralization of D-factors and CSF's from various sources by the antiserum.

Abbreviations: D-factor, differentiation-stimulating factor; MGI, macrophage and granulocyte inducer; CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; CM, conditioned medium

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2. MATERIALS AND METHODS

2.1. *Assay of D-factor*

Activity of D-factor was assayed by measuring induction of phagocytic activities in M1 cells (clone T-22) as in [13]. M1 cells (5×10^5 cells) were incubated for 2 days in 1 ml of Eagle's minimal essential medium containing D-factor, and then incubated further for 4 h with a suspension of polystyrene latex particles. Cells containing more than 10 latex particles were scored as phagocytic cells. Induction of phagocytic cells was proportional to the concentration of D-factor. Data in the present experiments were obtained in the linear dose–response range. Fifty units of D-factor was defined as the activity inducing 50% phagocytic cells under these conditions.

2.2. *Purification of D-factor from L cells*

Serum-free conditioned medium of L929 cells was prepared as in [15]. D-factor was precipitated from the medium with 90% saturation of ammonium sulfate, applied to a Sephadex G-200 column as in [8], and then chromatographed on a Phenyl-Sepharose column as in [16].

2.3. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was performed essentially as in [17]. Gels (5×110 mm) containing 7.5% polyacrylamide were used. D-factor (2500 units, 55 μg of protein) was allowed to stand for 30 min at room temperature in the presence of 1% SDS before the run, and then subjected to electrophoresis at 7 mA/gel for 5.5 h. Sections (2.6 mm) of the gel were eluted overnight with 0.25 ml of phosphate-buffered saline containing 0.1% bovine serum albumin at room temperature, and 0.1-ml samples of the eluates were assayed for D-factor activity. Bovine serum albumin (M_r 68000), ovalbumin (M_r 45000) and trypsin inhibitor (M_r 21500) were used as marker proteins.

2.4. *Preparation of anti-D-factor antiserum*

A rabbit was immunized by intradermal injection of partially purified L cell D-factor in 50% Freund's complete adjuvant at intervals of 2 weeks; it was injected 5-times with 1000 units of D-factor (22 μg of protein) and then 4-times with 5000 units of D-factor. The resulting serum was heated at 56°C for 30 min. IgG was separated on Protein A Sepharose CL-4B (Pharmacia Fine Chemicals) as in [18].

2.5. *Sources of D-factor and CSF*

Conditioned medium of mouse lung tissue [10], L cells [8], mouse embryo cells [19], hamster embryo cells [6], mouse macrophage-like Mm-1 cells [20], mouse spleen cells stimulated with Con A [7] and rat Yoshida sarcoma cell line YS-T22 cells [9], mouse endotoxin serum [12] and ascitic fluid of rats bearing AH130 hepatoma cells [11] were prepared as described before.

2.6. *Assay of CSF*

CSF was assayed as in [7]. Bone marrow cells (10^5) of adult ICR mice were seeded into a 35 mm petri dish containing 1 ml of 0.3% agar medium and CSF preparation. After incubation for 7 days, the number of colonies was counted. Data were obtained in the concentration range giving a linear dose–response relationship. The morphology of cells was determined in whole-mount preparations [14] stained with Giemsa solution.

3. RESULTS

3.1. *Partial purification of D-factor from conditioned medium of L cells*

D-factor was concentrated from serum-free conditioned medium of L cells by ammonium sulfate precipitation. Although CSF from L cells was eluted in several fractions on gel filtration, most of the D-factor activity was separated from CSF activity by gel filtration on Sephadex G-200, as in [8]. The resulting preparation of D-factor was then purified further by Phenyl-Sepharose chromatography. The purified D-factor preparation had a specific activity of 60-times that of the original conditioned medium. When this preparation was subjected to electrophoresis on polyacrylamide gel in the presence of SDS, a protein band associated with D-factor activity was observed. This protein constituted 3.5% of the total protein in the gel, judging from the densitometer tracing (fig.1). On treatment of M1 cells (clone T-22) with 100 units of the D-factor preparation, nearly all the cells differentiated into macrophage-like cells by day 4 and no granulocytic cells were observed (unpublished).

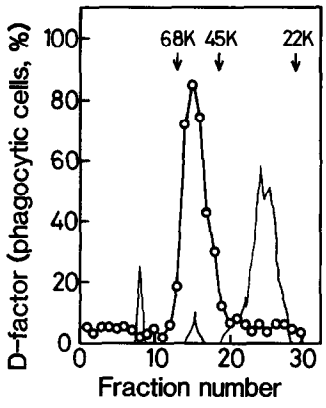

Fig.1. SDS–polyacrylamide gel electrophoresis of partially purified D-factor of L cells. Fractions were assayed for ability to induce phagocytic activity in M1 cells (○—○). Another gel run in parallel was stained for protein with Coomassie Brilliant Blue and scanned with a microdensitometer (———).

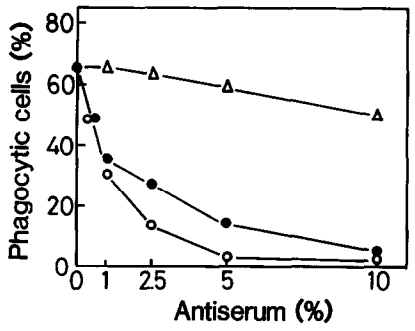

Fig.2. Neutralization of D-factor of L cells by its antibody. Conditioned medium of L cells (0.5 ml) was treated with various concentrations of antiserum (○); IgG purified from the antiserum (●); or control serum (△) at room temperature for 30 min. Then it was adjusted to a total volume of 1 ml with culture medium and assayed for D-factor activity. The percentage of antiserum was expressed as the final concentration in 1 ml of medium.

3.2. *Production of antiserum against L cell D-factor*

Initially, 6 rabbits were immunized with 1000 units of D-factor preparation, purified as described above. Even after 5 injections of the D-factor, the titers of sera from 6 rabbits for 50% inhibition of D-factor were below 1:20. When one of the rabbits was then injected 4-times more with 5000 units of the D-factor, the titer of this serum for 50% inhibition was 1:100. This serum at 1:20 dilution completely inhibited induction of phagocytic activity in M1 cells by L cell D-factor (fig.2). However, it did not inhibit induction of phagocytic cells when added 24 h after treatment of M1 cells with D-factor. M1 cells can also be induced to differentiate by dexamethasone, lipopolysaccharide or poly(I) [2]. The antiserum did not inhibit induction of phagocytic cells by these inducers (not shown). Untreated control rabbit serum did not significantly inhibit D-factor activity. IgG purified from the antiserum on Protein A-Sepharose inhibited D-factor activity. These results indicate that inhibition of D-factor activity was the direct result of an anti-D-factor antibody rather than of non-specific factors.

3.3. *Neutralization of D-factor from various sources*

Cross-reactivity against various D-factor preparations was examined with antiserum to D-factor of L cells. The antiserum at 1:20 dilution almost completely neutralized the D-factor activity of various mouse preparations including endotoxin serum and conditioned media from L cells, embryo cells, spleen cells stimulated with Con A and lung tissue (fig.3a). It also inhibited D-factor activity in rat ascitic fluid and conditioned media of rat

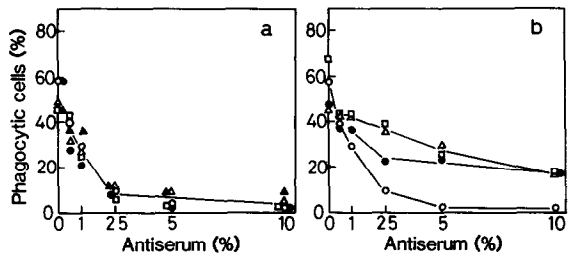

Fig.3. Neutralization of D-factor from various sources: (a) Murine D-factor from various sources was treated with various concentrations of antiserum to D-factor of L cells as described in the legend to fig.2 and then assayed for D-factor activity. Sources of D-factor: 0.5 ml of L cell CM (○); 0.5 ml of mouse embryo CM (△); 0.1 ml of mouse spleen cell CM (□); 25 μl of mouse lung CM (●); 38 μl of mouse endotoxin serum (▲); (b) Sources of D-factor: 0.5 ml of L cell CM (○); 0.4 ml of hamster embryo CM (△); 0.4 ml of rat YS cell CM (□); 0.5 ml of rat ascitic fluid (●).

Yoshida sarcoma cells and hamster embryo cells, although its extents of inhibition were less than with mouse D-factor (fig.3b).

3.4. *Effect of anti-D-factor antibody on CSF activity*

For examination of the relation between D-factor and CSF, several CSF preparations were treated with the antibody to D-factor of L cells. Since the untreated antiserum and control rabbit serum slightly enhanced colony formation from normal bone marrow cells by L cell CSF, purified anti-D-factor IgG was used in the experiments. This IgG did not inhibit the CSF activities of conditioned media from L cells, lung tissue, mouse Mm-1 cells or rat Yoshida sarcoma cells. Furthermore, it did not change the proportions of granulocyte and macrophage colonies significantly (table 1).

4. DISCUSSION

D-factors for myeloid leukemic M1 cells have shown to be heterogeneous in molecular size and charge [2]. Here, we showed that the antigenic properties of D-factor from various preparations from mice were similar to those of D-factor from mouse L cells. D-factors from rats and hamsters were also neutralized by the antibody to D-factor of L cells, but the titers of inhibition were somewhat less than those observed with mouse D-factor. The activities of L cell CSF (M-CSF) and lung CSF (GM-CSF) were not inhibited by the antibody.

Besides M1 cells, a mouse myelomonocytic leukemic cell line, WEHI-3B cells, can also be induced to differentiate into macrophages and granulocytes by a differentiation factor (DF) obtained from similar sources to those of the D-factor for M1 cells [16,21,22]. A similar substance(s) is probably responsible for the inductions of differentiation of M1 cells and WEHI-3B cells. The DF activities in conditioned media of mouse lung and endotoxin serum could be separated from the bulk of GM-CSF and M-CSF activities, but not from some G-CSF activity [16]. However, in [22] G-CSF from WEHI-3B-D$^-$ cells (a differentiation-noninducible cell line) did not induce differentiation of WEHI-3B-D$^+$ cells (a differentiation-inducible cell line). Since DF preparations for WEHI cells and D-factor preparations for M1 cells with CSF activity have not yet been purified to homogeneity [4,16], there is no evidence at present that D-factor or DF is a subset of CSF.

Although D-factor or DF seems to be important for suppression of development of myeloid leukemia [2,3,21], the roles of these factors in normal hematopoiesis are unknown. In [23] it was claimed that CSF induced growth of normal precursor cells of granulocytes and macrophages and induced production of D-factor by the precursor cells, that caused their own differentiation. We are now purifying D-factor further to elucidate its true target cells in vivo.

Table 1

Effect of anti-D-factor antibody on CSF activity[a]

CSF	Antibody	No. of colonies	Colony type (%)		
			G	M	Mix
L cell CM	–	109 ± 20	0	95	5
(50 μl)	+	108 ± 11	0	97	3
Lung CM	–	70 ± 5	53	20	27
(50 μl)	+	77 ± 10	47	22	31
Mm-1 cell CM[b]	–	29 ± 4	34	31	35
(150 μl)	+	28 ± 4	46	32	22
YS cell CM	–	54 ± 2	41	38	21
(150 μl)	+	46 ± 3	42	36	22

[a] Various CSF preparations were treated with purified IgG equivalent to 0.1 ml of original antiserum at room temperature for 30 min and assayed for CSF activity. Colony type: granulocytes (G), macrophages (M) and a mixture of granulocytes and macrophages (Mix)

[b] Ten-times concentrated

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