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TUMOR NECROSIS FACTOR AS AN INTERLEUKIN 1-DEPENDENT DIFFERENTIATION INDUCING FACTOR (D-FACTOR) FOR MOUSE MYELOID LEUKEMIC CELLS

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SUMMARY: Experiments were conducted to purify the differentiation-inducing factor (D-factor), which induces differentiation of mouse myeloid leukemic cell line, Ml, into macrophage-like cells, in a conditioned medium of guinea pig peritoneal macrophages stimulated with lipopolysaccharide. On gel filtration under high performance liquid column chromatography (HPLC), D-factor eluted at the position of 45-15 KD. By the subsequent separation on DEAE HPLC the D-factor activity disappeared. However, in the presence of recombinant human IL 1α the D-factor activity appeared at a position where tumor necrosis factor (TNF) eluted. Even after fractionation on hydroxyapatite HPLC the IL 1-dependent D-factor was co-chromatographed with TNF. Recombinant human TNF as well as the partially purified guinea pig TNF induced differentiation of Ml cells in conjunction with either the partially purified guinea pig IL 1 or recombinant human IL 1α, although these factors by themselves did not induce differentiation. These findings suggest that a part of D-factor activity in the conditioned medium resulted from the cooperative effects between TNF and IL 1.

Myeloid leukemic cell lines have been widely used as a model to study the mechanism of differentiation of monocytes/macrophages or granulocytes. Mouse myeloid leukemic cell line, Ml, can be induced to differentiate into macrophages or granulocytes in vitro by several stimuli, such as lipopolysaccharide (LPS) (1), glucocorticoid hormones (2) and a protein factor, differentiation-inducing factor (D-factor). D-factor(s) has been found in conditioned media of various tissues and cells (3). Activated- or stimulated-macrophages are reported to produce D-factor(s) (4). However, the characteristics of D-factor produced by macrophages are largely unknown. Macrophages are also known to produce other monokines, interleukin 1 (IL 1) and tumor necrosis factor (TNF)

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Abbreviations used: Ml, a mouse myeloid leukemic cell line; D-factor, differentiation-inducing factor; TNF, tumor necrosis factor; IL 1, interleukin 1; HPLC, high performance liquid column chromatography.

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(5). Recently, TNF was reported to induce differentiation of human myeloid leukemic cell lines into macrophage-like cells (6,7). On the other hand, we observed that purified human IL 1 was cytostatic or cytocidal for several types of tumor cells as well as for M1 cells (8). All the IL 1s, human recombinant IL 1α, natural IL 1β and mouse recombinant IL 1α, appeared to augment the differentiation of M1 cells into macrophage-like cells induced by small amount of LPS, although these IL 1s by themselves did not induce differentiation (9). Thus, we speculated that IL 1 may relate to D-factor(s). In the present study, we attempted to purify D-factor(s) elaborated by guinea pig macrophages, and demonstrate that D-factor can not be separated from TNF, and the highly purified guinea pig TNF as well as recombinant human TNF induced differentiation of M1 cells into macrophage-like cells only in the presence of IL 1.

MATERIALS AND METHODS

Reagents: Purified human recombinant IL 1α and human recombinant TNF were kindly provided by Dainippon Pharmaceutical Co. Ltd. Guinea pig IL 1 was partially purified by HPLC gel filtration of a conditioned medium of guinea pig macrophages. The fractions of 20-10 KD were pooled and used as guinea pig IL 1.

Assay of D-factor: D-factor was assayed by measuring the induction of Fc receptors (FcR) for IgG or phagocytosis activity in clone, M1/436-7, of M1 cells. This clone is known to differentiate into macrophages but not into neutrophils or granulocytes (10). M1 cells (2×10^4 cells) were cultured in 100 µl of RPMI 1640 (M.A. Bioproducts, MD) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Utah) for 3 days under 5% CO_2 in air. Usually polymyxin-B (10 µg/ml) was added to neglect the effect of endotoxin which may contaminate the culture. The assay for FcR was performed by the modified method of Bianco et al (11). Briefly, cells were cultured with 4% EA (ox erythrocytes sensitized with IgG of rabbit anti-ox erythrocyte antiserum) at 37°C for 3 hr. After culture, the percentage of M1 cells with more than 3 EA attached was determined by counting at least 200 cells in hemocytometer. To test phagocytosis activity, cells were cultured with 4% polystylene latex particles for 8 hr, then the percentage of cells ingesting more than 10 latex particles was determined in a hemocytometer by counting at least 200 cells.

Preparation of a conditioned medium of guinea pig peritoneal macrophages: Hartley guinea pigs, weighing 300-400 g, were injected intraperitoneally with 15 ml liquid paraffin. After 4 days the peritoneal exudate cells were collected. More than 80% were macrophages by morphological criteria. The cells (1×10^6/ml) were treated with 30 µg/ml of LPS (E.coli 0127:B8, Difco Laboratories, Detroit) for 5 hr in RPMI 1640 supplemented with 1% FCS. Then the cells were washed 3 times with the medium, and then cultured for 20 hr in RPMI 1640, 1% FCS. The culture supernatants were collected, and concentrated by ultrafiltration through Amicon Diaflow membrane YM2 (Average molecular weight cut, 2000).

HPLC column chromatography: Every HPLC manuplation was performed using Beckman Model 344 HPLC system at room temperature. TSK gel G3000 SW, TSK gel DEAE-5PW (Toyo Soda, Japan) and hydroxyapatite column (BIO-RAD, Richmond, CA) were used. After column chromatography each fractions were dialysed at 4°C against PBS and then RPMI 1640, and were used for D-factor or TNF assay.

Assay for IL 1 activity: The IL 1 activity was determined by measuring the incorporation of [^{3}H]Thymidine (2 Ci/mmol, New England Nuclear, Boston, MA) by C3H/HeJ mouse thymocytes cultured for 3 days in the presence of 0.5 μg/ml concanavalin A and serially diluted IL 1 as described (8). One unit per milliliter was defined as the reciprocal of the dilution at which 50% of the maximum thymocyte proliferation response was obtained.

Assay for TNF activity: The activity of TNF was defined by a L929 fibroblast cell lytic assay. Briefly, 100 μl of a cell suspension of TNF-sensitive mouse L929 fibroblast cells (3 x 10^5 cells/ml) were cultured with serially diluted test samples in wells of flat-bottomed microtiter plate at 37°C for 18 hr in 5% CO_2 in air in the presence of actinomycin D (1 μg/ml). After culture, the plates were washed, and cell lysis was determined by staining the plates with crystal violet (0.5%) in methanol/water (1:4). After solubilizing the dye-staining cells with 0.1 ml of 0.1% SDS, the dye uptake was calculated by an automatic micro ELISA autoreader (Immuno Reader NJ-2000, Inter-Med). One unit of TNF activity was defined as the reciprocal of the dilution of samples that lysed 50% of the test cells.

RESULTS AND DISCUSSION

The purification of D-factor in a conditioned medium of LPS-stimulated guinea pig peritoneal macrophages was attempted. The medium was concentrated and applied to HPLC TSK G3000 column. As shown in Figure 1, D-factor activity assessed by the effect on FcR expression eluted in the position of 45-35 KD with a shoulder of 30-15 KD. Another monokine, IL 1 detectable by thymocyte co-mitogenic activity eluted in two regions, 20-10 KD and 5 KD. IL 2 activity was not detected in these fractions by the lack of proliferation inducing activity on an IL 2-dependent cell line, CTLL-2 (data not shown). Third monokine, TNF eluted in 55-35 KD position. As we previously observed that purified human IL 1α, and β augmented differentiation of Ml cells into macrophage-like cells (9), we added human recombinant IL 1α to these fractions, and then measured the D-factor activity. As demonstrated in Figure 1, in the presence of IL 1α new D-factor activity appeared in a region of 60-40 KD. The D-factor fractions of 60-35 KD were pooled, and then were further fractionated on HPLC DEAE column. After chromatography, however, we could not detect the D-factor activity in any fractions. In contrast, in the presence of IL 1α one peak of D-factor activity appeared at 0.08 to 0.11 M NaCl. The D-factor ac-

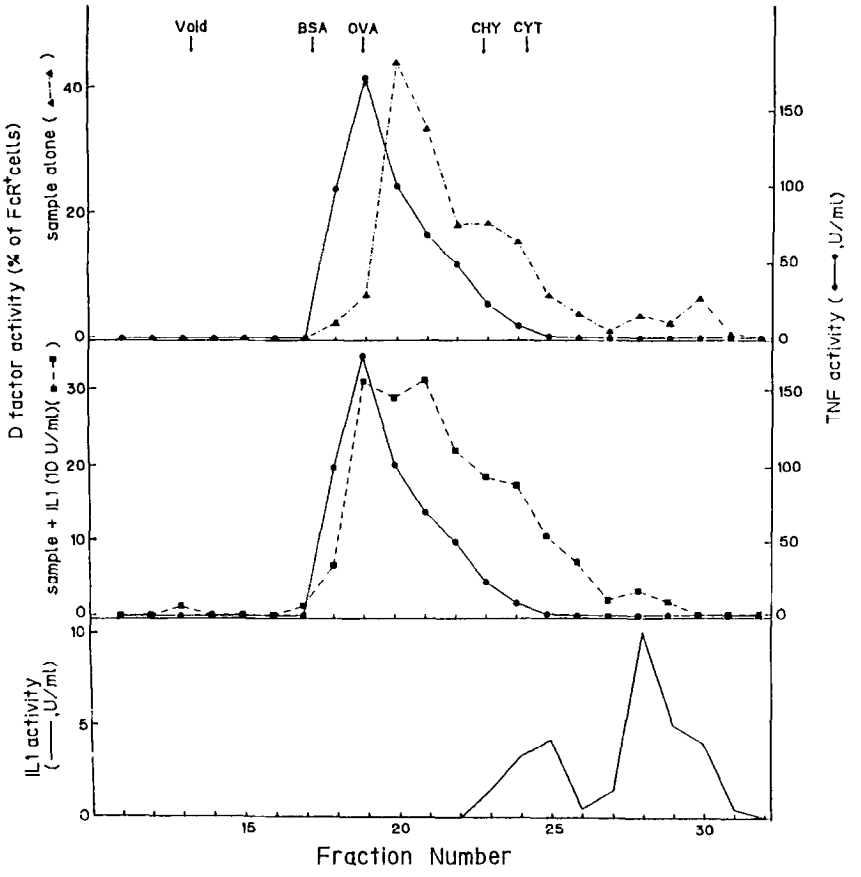

Fig. 1. Gel filtration HPLC of a conditioned medium of macrophages. The sample was applied to a 7.5 mm x 60 cm column of TSK gel G3000 SW equilibrated with PBS. The flow rate was 0.5 ml/min, and 1.0 ml fractions were collected. After chromatography, D-factor, IL 1 and TNF activities were determined. The D-factor activity was determined with or without human IL 1α (10 U/ml). Arrows indicate the position where reference proteins eluted: BSA, bovine serum albumin; OVA, ovoalbumin; CHY, chymotrypsinogen A; CYT, cytochrome C.

tivity was co-eluted with TNF. Peak fractions of the D-factor from DEAE column were pooled, and then were applied to HPLC hydroxyapatite column chromatography. Similarly to DEAE column chromatography, in the absence of IL 1α there was no D-factor activity. Again the D-factor activity appeared at 0.13 to 0.15 M NaPB after addition of IL 1α. Even by the separation on this column, the D-factor and TNF were completely co-chromatographed (Figs. 2 and 3).

These results therefore suggest that D-factor activity found in the conditioned medium resulted from the coordinated effects between TNF and IL 1. In order to evaluate this possibility cells were treated with mixtures of human recombinant TNF, the partially purified guinea pig TNF, IL 1 and human

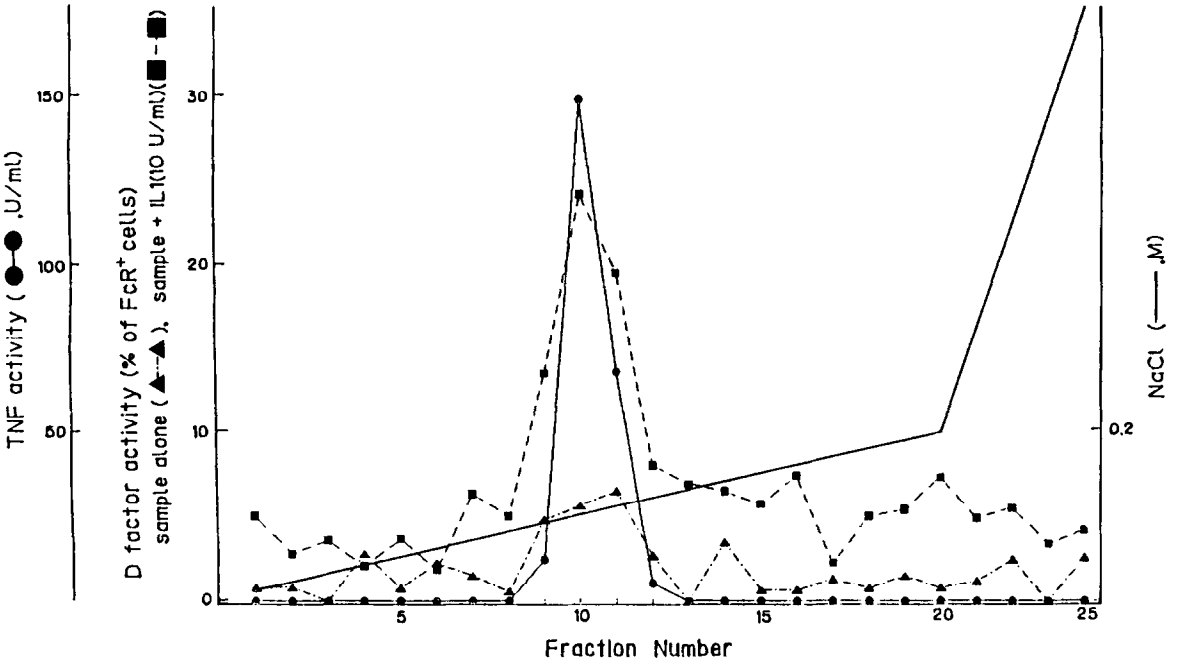

Fig. 2. DEAE HPLC of D-factor(s) (fractions 18-22 in HPLC gel filtration) from guinea pig macrophages. The samples from gel filtration HPLC were dialysed against 0.02 M sodium phosphate buffer (NaPB), pH 7.4,, and applied to a 7.5 x 75 mm column of TSK gel DEAE-5PW equilibrated with the same buffer. The starting buffer was 0.02 M NaPB and limiting buffer was 0.02 M NaPB, 1.0 M NaCl. The flow rate was 1.0 ml/min, and 4.0 ml fractions were collected. After chromatography, the D-factor and TNF activities were determined.

recombinant IL 1α, and then their effects on FcR expression or phagocytosis activity of Ml cells were determined. As shown in Table 1, all these cytokines by themselves did not affect on either FcR expression or phagocytosis activity. In contrast, mixture of IL 1 and TNF either recombinant human preparations or obtained from guinea pig macrophages induced both of FcR expression and phagocytosis activity. Morphologically the cells treated with both of IL 1 and TNF for 5 days exhibited macrophage-like characteristics, such as enlargement of cytoplasms, enhancement of adherence to culture plate and appearance of cytoplasmic granules (data not shown). Thus, the D-factor detectable only in the presence of IL 1α was shown to be TNF or closely related molecule. The shoulder of D-factor activity observed in fractions 23-25 on HPLC gel filtration may be attributable to the effects between TNF and IL 1 because both factor activities overlapped in these fractions. As shown in Table 1,

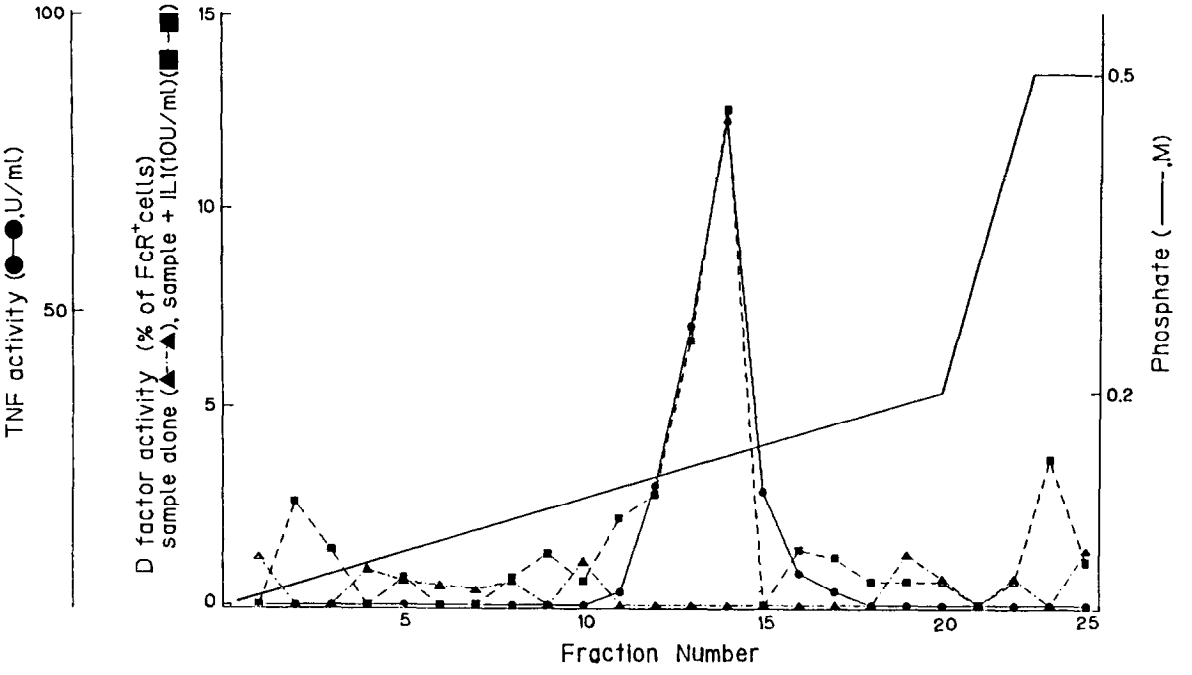

Fig. 3. Hydroxyapatite HPLC of D-factor(s) (fractions 9-12 in DEAE HPLC) from guinea pig macrophages. The samples from DEAE HPLC were dialysed against 0.01 M NaPB, pH 7.0, 0.3 mM $CaCl_2$, and applied to a 7.8 x 100 mm hydroxyapatite column (BIO-RAD, Richmond, CA) equilibrated with the same buffer. The starting buffer was 0.01 M NaPB, pH 7.0, 0.3 mM $CaCl_2$, and the limiting buffer was 0 5 M NaPB, pH 7.0, 0.1 mM $CaCl_2$. The flow rate was 1.0 ml/min, and 4.0 ml fractions were collected. After chromatography, D-factor and TNF activities were determined.

1 U/ml of IL 1 was sufficient to induce differentiation in conjunction with TNF. However, we did not rule out the possibility that other factor(s) may contribute to inducing differentiation because fractions 40-42 on gel filtration induced differentiation even in the absence of exogenous IL 1α irrespective of the lack of IL 1 activity.

Recently, human natural and recombinant TNFs were reported by itself to induce differentiation of human myeloid leukemic cell lines (6,7). However, as demonstrated in this study we could not observe any differentiation-inducing activity in guinea pig TNF or recombinant human TNF. This may be because of the difference of target cells. Ml cells may be more primitive than other human leukemic cells, and thus they may require two distinct signals given by TNF and IL 1 for differentiation. Indeed, human myeloid leukemic cell line, HL60, expressed FcR without stimulation (data not shown).

Table 1. Effects of human or guinea pig TNF and IL 1 on FcR expression and phagocytosis activity of Ml cells

Treatment	Medium	G.P. TNF (U/ml)		Hr TNF (U/ml)	
		1000	100	1000	100
FcR expression					
Medium	0.7±1.0	1.0±0	0.8±1.1	0±0	0.4±0.6
G.P. IL 1 (1 U/ml)	0 7±0.9	26.5±4.9	5.5±2.4	15.2±2.3	13.9±0.1
Hr IL 1α (1 U/ml)	0.9±1.2	23.9±1.6	8.0±1.3	19.7±5.7	16.8±0.4
Phagocytosis activity					
Medium	1.1±1.6	0.5±0.6	1.0±0.1	0.6±0.8	0±0
G.P. IL 1 (1 U/ml)	2.3±0.6	13.9±1.9	7.2±1.6	19.0±5.9	10.7±0.6
Hr IL 1α (1 U/ml)	1.3±1.8	20.3±0.4	12.4±2.8	19.9±3.2	13.7±0.8

Ml cells were cultured with or without guinea pig (G.P.) partially purified IL 1, human recombinant (Hr) IL 1α, G.P. partially purified (on DEAE HPLC) TNF and human recombinant TNF at 37°C for 3 days, and then the percentage of FcR positive cells or phagocytosis active cells were determined. Each value represents mean±S.D. based on triplicate cultures.

Alternatively even in the case of human leukemic cells, TNF effect may be mediated through the endogenous production of IL 1. Further study is necessary to determine these possibilities. Recent study revealed that TNF and IL 1 possess a lot of similar biological activities, such as antiproliferative effects for some tumor cells (8,12), stimulation of normal fibroblast growth (13,14), inducing fever (15), osteoclast activation (16,17) and induction or augmentation of human monocyte-mediated tumor cytotoxicity (18,19). Our findings therefore presented a new similar biological activity between these two cytokines.

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