APPEARANCE OF ASIALO GM1 GLYCOSPHINGOLIPID ON THE CELL SURFACE DURING LYMPHOKINE-INDUCED DIFFERENTIATION OF M1 CELLS

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Received 13 March 1981; revised version received 29 May 1981

1. Introduction

It has been reported that the M1 cell line, established from a myeloid leukemia of a mouse of strain SL [1], can be induced to differentiate into macrophages and granulocytes by exposure to various inducers, such as conditioned media from various cell sources, serum from mice injected with endotoxin, ascitic fluid from tumor-bearing animals, lipopolysaccharides (LPS), glucocorticoid hormones, inhibitors of DNA and RNA synthesis and synthetic single-stranded polyribonucleotide [1–5]. We had shown that culture supernatants of macrophage-like cell line (J774.1) cells or of the matured cells of M1 itself also can induce the differentiation of M1 cells [6], and suggested the possibility that a factor(s) produced by mature macrophages stimulates differentiation of premature macrophages into mature cells. Here, we show that products of lymphocytes stimulated either specifically or nonspecifically can induce differentiation of M1 cells. We also show that the differentiation of M1 cells is accompanied by the appearance of asialo GM1, gangliotetraosyl ceramide, on the cell surface.

2. Materials and methods

Specific pathogen-free (DBA/2 × BALB/c)F1 (CDF1) and ICR female mice, 6–8 weeks old, were purchased from the Shizuoka Experimental Animal Cooperative (Hamamatsu).

The clone of M1 cells used was M1/436-7 that had been established by Y. Ichikawa (Chest Disease Research Institute, Kyoto University) and provided through M. Saito (Central Research Laboratory, Morinaga Milk Industry, Tokyo). This cell line can be induced to differentiate into macrophages, but not into neutrophil granulocytes [7]. Cells were suspended in RPMI 1640 medium supplemented with 10% newborn calf serum inactivated by heating at 56°C for 30 min, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. For induction of cell differentiation, M1 cells (3.5×10^5 cells/ml) were incubated with various inducers at 37°C for 48 h.

The appearance of Fc receptors and phagocytosis of latex beads were assayed by the methods in [6].

Lymphokine (LK)-rich murine sera were obtained by the method in [8]. Briefly, ICR mice were immunized intravenously (i.v.) with 10^7 viable BCG cells and, 3 weeks later, injected i.v. with 50 mg of old tuberculin (OT). From 3–4 h after the injection of OT, the mice were bled and the sera were pooled as LK. Serum from unimmunized mice injected with OT alone or from BCG-immunized mice given no OT were used as controls. To obtain concanavalin A-induced LK (con A-LK), normal mouse spleen cells (1×10^7 cells/ml) were stimulated with 100 μg con A–agarose (Sigma Chemicals, St Louis MO)/ml at 37°C for 48 h following [9] with a slight modification. The culture supernatants of spleen cells alone and of con A–agarose alone were used as controls without further purification.

Peritoneal exudate cells (PEC) were obtained from CDF1 mice that had received an intraperitoneal injection of 1 ml 10% proteose peptone 4 days before. Adherent PEC were incubated with the serially diluted LK at 37°C for 4 h, washed thoroughly, and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum inactivated by heating at 56°C for 30 min. These cells were mixed with P815 masto-

cytoma cells maintained by passage through CDF1 mice at an effector to target cell ratio of 10, and incubated at 37°C for 40 h. [^{3}H]TdR (0.1 μCi) was added to the culture 16 h before the termination of incubation: Macrophage-activating factor (MAF) activity was indicated by the percentage of inhibition of tumor growth calculated by the following formula:

$$\left[1 - \frac{\text{cpm (effector cells + target cells)} - \text{cpm (effector cells)}}{\text{cpm (target cells)}}\right] \times 100\%$$

Lipopolysaccharides (LPS) (*Escherichia coli* 0111:B4, Westphal method) was purchased from Difco Labs (Detroit MI).

The ammonium sulfate precipitate of specific rabbit antiserum to asialo GM1 (anti-asialo GM1) was provided by M. Kasai (Dept. of Tuberculosis, National Institute of Health, Tokyo). The antiserum reacted with asialo GM1, but not with the related glycosphingolipids, such as GM1, GM2, GD1b and asialo GM2 [10].

Cells suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) at 1–2 × 10^6 cell/tube were incubated for 20 min on ice with 20 μl appropriately diluted anti-asialo GM1, washed repeatedly, and then incubated for 20 min on ice with 20 μl appropriate dilution of the fluoresceinated (FITC)-goat anti-rabbit immunoglobulin G (IgG), which was provided by T. Kamiyama (Dept. Veterinary Science, National Institute of Health, Tokyo). After being washed, the cells were suspended in PBS and stored on ice until examined. Non-specific or background staining was determined by using an ammonium sulfate precipitate of normal rabbit serum instead of anti-asialo GM1 or by staining with only FITC-goat anti-rabbit IgG, and was <1%.

Lac–Cer, $GbOse_3$–Cer and $GbOse_4$–Cer were prepared from human erythrocytes. IV^3-α-GalNAc–$GbOse_4$–Cer (Forssman glycosphingolipids) and $LcnOse_4$–Cer (Paragloboside) were prepared from sheep erythrocytes [11]. Asialo GM1 ($GgOse_4$–Cer) was obtained from GM1 by formic acid hydrolysis (1 M formic acid, 100°C for 1 h) and the desired compound was isolated by silicic acid column chromatography. These glycosphingolipids were tritiated by KB^3H_4 (113 mCi/mmol) (Amersham) in the presence of $PdCl_2$ [12] and used as standards.

M1 cells were incubated together with 5% LK, 10 μg LPS/ml or medium alone at 37°C for 48 h and washed thoroughly. Glycosphingolipids were obtained from the cells and subjected to tritiation and to analysis by high performance silica gel thin-layer chromatography and autoradiofluorography according to [13].

3. Results and discussion

LK induced in vivo with OT and LK induced in vitro with con A were tested for their ability to induce the differentiation of M1 cells into macrophages. As shown in fig.1A,B, the LKs induced Fc receptors and latex particle phagocytosis of M1 cells, while controls never induced such markers. These results suggest that the LKs contain a factor(s) inducing the differentiation of M1 cells into macrophages.

The differentiation-inducing factor(s) (D-factor) present in conditioned media from mouse embryonal fibroblasts and Yoshida sarcoma cells and in endotoxin serum have been reported to be different from the colony-stimulating factor [14–16]. To know whether or not the differentiation-inducing factor contained in LK is different from MAF, we tried to absorb con A-LK with 10^8 M1 cells by mixing them at 4°C for 3 h. The incubation mixture was centrifuged and the supernatant was assayed for MAF activity and differentiation-inducing activity for M1 cells. The results are shown in table 1. MAF activity of LK after absorption with M1 cells was the same as that of unabsorbed LK. In contrast, the differentiation-inducing activity of LK for Fc receptors and phagocytosis of latex particles of M1 cells was reduced significantly by absorption with M1 cells. The results suggest strongly that the molecule which is capable of inducing the differentiation of M1 cells is not the same as MAF and that the former can bind to M1 cells but MAF cannot. LKs obtained from BCG-immunized guinea pig lymphocytes stimulated with PPD in vitro and also from normal guinea pig spleen cells stimulated with con A–agarose in vitro induced the differentiation of M1 cells in the same way as did LK obtained from mice, suggesting that D-factor contained in LK acts beyond the species barrier (not shown). It is not yet

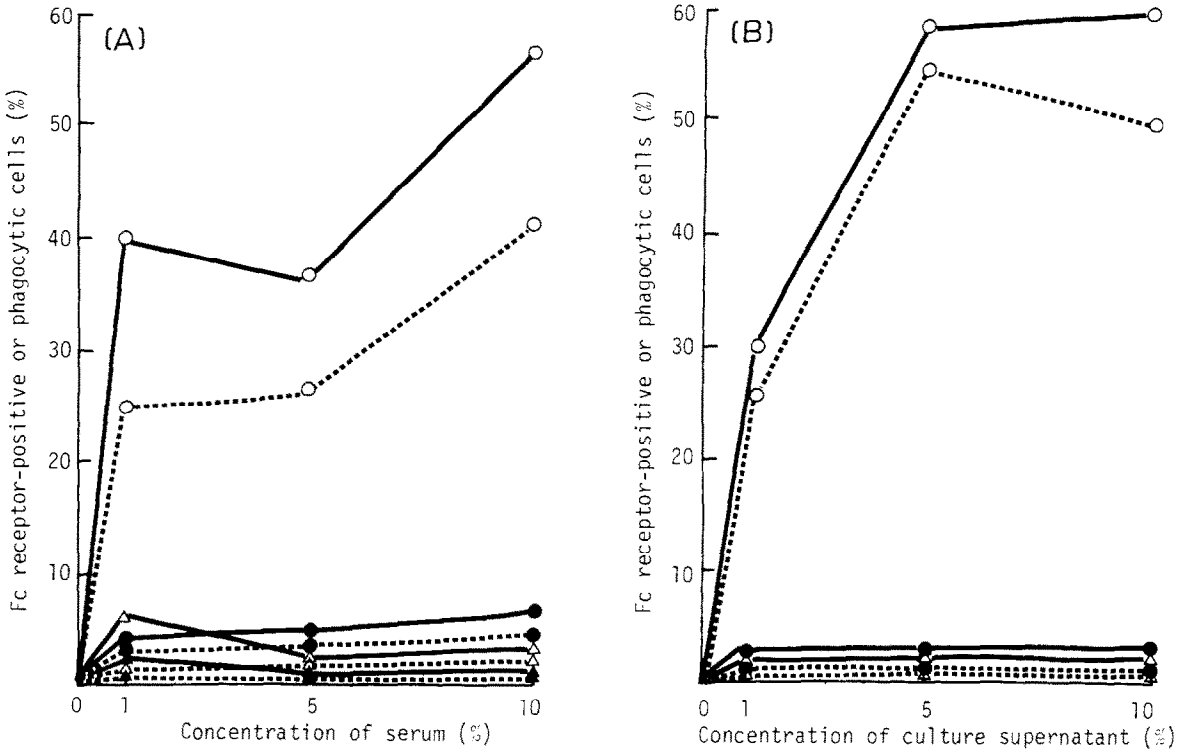

Fig.1. Induction of Fc receptor- or phagocytosis-positive M1 cells by LK-containing mouse serum (A) or con A-LK (B): (A) M1 cells (3.5×10^5/ml) were incubated at 37°C for 48 h with serum from mice immunized with BCG and challenged with OT (○), from mice immunized with BCG alone (△), from mice injected with OT alone (●), or from normal mice (▲); (B) M1 cells (3.5×10^5/ml) were incubated with culture supernatants from mouse spleen cells incubated with (○) or without (●) con A–agarose or con A–agarose alone (△) for 48 h. These cells were washed and assayed for Fc receptors (——) and phagocytic activity (···).

Table 1
Differentiation-inducing activity and MAF activity of LK with or without absorption by M1 cells[a]

LK	Conc. (%)	Differentiation of M1 cells		MAF activity[b]
		Fc receptors (% ± SD)	Phagocytosis (% ± SD)	
Without absorption	0	0.6 ± 0.2	3.1 ± 0.5	−3.8
	1.25	43.5 ± 1.4	20.8 ± 0.2	20.5
	2.5	47.8 ± 0.3	25.3 ± 0.3	90.1
	5	72.0 ± 0.3	32.3 ± 0.2	96.0
With absorption	1.25	15.2 ± 1.2	5.8 ± 0.2	37.5
	2.5	33.6 ± 0.3	9.6 ± 0.3	95.5
	5	44.9 ± 0.2	12.5 ± 0.5	95.1

[a] Con A–agarose-induced LK was incubated with or without M1 cells (1×10^8) at 37°C for 3 h and centrifuged, and the supernatants diluted serially were assayed for the ability to induce differentiation of M1 cells and the activity as MAF

[b] Adherent PEC incubated with various concentrations of LK at 37°C for 4 h were mixed with P815 mastocytoma cells at an effector to target cell ratio of 10, and incubated at 37°C for 40 h. MAF activity was expressed by inhibition (%) of [^{3}H]TdR incorporation into P185 cells (1×10^4 cells/well) according to the formula described in the text

known whether or not D-factor contained in LK is identical with D-factor reported from various other sources [1–3,6].

Apparently, the differentiation of M1 cells is accompanied by some changes in plasma membrane components, as recognized by the changes in cell morphology, Fc and C3 receptors, adhesion. However, little is known about the chemical basis of these cell surface changes. In [17] changes in membrane proteins, particularly glycoprotein, of M1 cells during differentiation were reported. Glycolipids are also known as good cell surface markers of various cell types [18,19]. Among them asialo GM1, gangliotetraosyl ceramide, has been reported to exist on the cell surface of mouse natural killer (NK) cells [10], mouse immature T cells [20] and on subpopulations of rat T lymphocytes and macrophages [21–23]. We found that this glycosphingolipid is expressed also on mouse lung macrophages [24] and mouse peritoneal exudate cells activated with LK in vitro (unpublished). Therefore, we investigated whether or not asialo GM1 exists on M1 cells before and after differentiation with LK.

As shown in table 2, assay by indirect immunofluorescence microscopy showed that ~50% and 10% of the cells incubated with con A-LK and LPS, respectively, for 48 h reacted with anti-asialo GM1, while none of the M1 cells incubated with medium alone reacted. These findings with the immunofluorescence technique were confirmed by autoradiofluorographic thin-layer chromatography. As shown in fig.2, a band comparable to that of asialo GM1 was clearly visible in the glycosphingolipid of M1 cells incubated with LK and weakly in that incubated with LPS. However, the asialo GM1 band was only faintly detected in the glycosphingolipid of M1 cells incubated with medium alone. In addition, bands corresponding to $GbOse_3$–

Table 2
Appearance of anti-asialo GM1-reacting cells after differentiation of M1 cells with LK[a]

Incubation with	Percent of cells positive for		
	Anti-asialo GM1	Fc receptors	Phagocytosis
None	0	0.9	0.9
LK 5%	47.3	71.6	34.3
LPS 10 μg/ml	13.2	26.9	8.1

[a] M1 cells were incubated with LK or LPS for 48 h and assayed for the presence of Fc receptor, phagocytosis of latex particles, and reactivity with anti-asialo GM1 by immunofluorescence microscopy

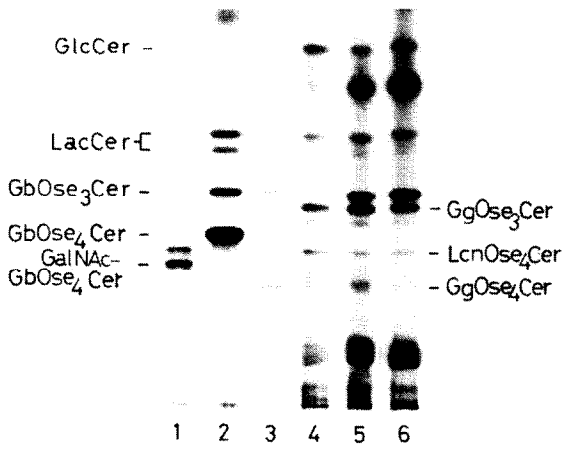

Fig.2. Autoradiofluorography of [^{3}H]glycosphingolipids from untreated M1 cells and M1 cells differentiated by LK. [^{3}H]Glycosphingolipids from 10^6 cells were applied on a high-performance silica gel thin-layer plate (Merck, Darmstadt), which was developed with chloroform–methanol–water (60:30:5, by vol.): (1) $LcnOse_4$–Cer and IV^3-α-GalNac–$GbOse_4$–Cer (Forssman glycosphingolipid); (2) Lac–Cer, $GbOse_3$–Cer; (3) $GbOse_3$–Cer and asialo GM1 ($GgOse_4$–Cer); (4) glycosphingolipids of M1 cells; (5) glycosphingolipids of LK-treated M1 cells; (6) glycosphingolipids of LPS-treated M1 cells.

Cer and to monohexosylceramide were much more strongly visible in the fraction from M1 cells incubated with LK or LPS than in the fraction from control M1 cells. Asialo GM1 was also detected on M1 cells incubated with the culture supernatant of J774.1 cells (not shown) a macrophage-like cell line, obtained as in [6].

In [25] GM1b, the sialylated glycosphingolipid of asialo GM1, was induced on M1 cells by LPS treatment. The results in [25] and ours strongly support the hypothesis that the pathway of asialo GM1 synthesis is essentially present in M1 cells and that asialo GM1 synthesis-related glycosyl transferase is primarily induced during the differentiation. We think that asialo GM1 should be a useful cell surface marker to study ontogeny, differentiation, and macrophage subsets.

We found that LK can induce the differentiation of M1 cells. The factor contained in LK which induces the differentiation of M1 cells is different from MAF. Indirect immunofluorescence microscopic assay of the cells and autoradiofluorographic thin-layer chromatography of their glycosphingolipids showed that asialo GM1 appears on the surface of M1 cells during differentiation induced by LK.

Acknowledgements

The authors express their gratitude to Dr Masataka Kasai for providing anti-asialo GM1 antibody. This work was partly supported by research grants from the Ministry of Health and Welfare, Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

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