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Induction of Mouse Anti-melanoma Cytotoxic and Suppressor T Cells *in vitro* by an Artificial Antigen, GM3-lactone

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We investigated the ability of GM3-lactone liposomes to induce anti-melanoma T cell responses in mice. GM3-lactone liposomes, like murine B16 melanoma cells, induced anti-melanoma cytotoxic T cells (CTL) and also suppressor T cells (Ts). A small dose of GM3-lactone (0.0003 μg/ml) was enough to generate CTL in the *in vitro* primary response, whereas relatively large amounts of the antigen (0.03–0.3 μg/ml) were required for anti-melanoma Ts induction. As the epitope for anti-melanoma Ts is NeuAc but not NeuGc residue on GM3, and anti-melanoma CTL are effectively induced by either GM3(NeuAc) or GM3(NeuGc)-lactone liposomes, GM3(NeuGc)-lactone or GM3(NeuGc) liposomes have potent activity as an artificial melanoma antigen to induce anti-melanoma CTL *in vitro*.

Key words: GM3-lactone — Artificial melanoma antigen — Anti-melanoma CTL — Anti-melanoma Ts

It has recently been established that carbohydrate moieties serve as tumor antigens in the syngeneic immune system. In fact, Irie *et al.* have successfully established tumor-specific monoclonal antibodies from patients with melanoma and the antigens recognized by these antibodies were found to be GM2 or GD2 gangliosides.[1,2] Thus, in human, epitopes on GM2 and GD2 seem to be immunodominant and to act as tumor antigens. Similarly, our previous biochemical and physicochemical analyses of mouse melanoma antigen using anti-murine melanoma monoclonal antibody (M2590) raised by syngeneic immunizations have also shown that melanoma antigen in mice is GM3 and its primary structure is the same as that of normal GM3, even though the monoclonal antibody reacts only with melanoma but not normal cells expressing GM3 on the surface.[3,4] The tertiary structure of GM3 on melanoma cells is thus considered to be distinct from that on normal cells. We have further demonstrated that the density of GM3 is critical for generation of melanoma antigenicity because the monoclonal anti-melanoma antibody (M2590) recognizes GM3 with a density of more than 10 mol%, while no reactivity was observed toward GM3 at a low density (less than 7.5 mol%).[5] Thus, it is possible that a local increase of density causes GM3 with normal primary structure to undergo conformational changes, resulting in the generation of melanoma antigenicity.

In addition to the above evidence, Nores *et al.*[5] reported that minute amounts of GM3-lactone are present in melanoma cells. Nores *et al.*[5] and Dohi *et al.*[6] have also demonstrated that the lactone form of GM3 is more immunogenic than GM3 itself. Moreover, the affinity of anti-melanoma M2590 antibody for GM3-lactone is about 10 times higher than that for GM3.[5] Therefore, lactone-like conformation of GM3 or GM3-lactone itself is suggested to have melanoma antigenicity.

Based on the above evidence, we investigated the melanoma epitopes important in the responses of T cells, such as anti-melanoma CTL[2] and Ts, involved in the positive and negative regulation of tumor cell growth. In this paper, we demonstrate that GM3-lactone has potent activity to induce anti-melanoma CTL and Ts.

MATERIALS AND METHODS

Animals Specific pathogen-free (SPF) C57BL/6 mice, 7–8 weeks old, were purchased from Shizuoka Experimental Animal Co. Ltd., Hamamatsu.

Preparation of GM3 conjugates GM3 was extracted from dog erythrocytes and purified by DEAE-Sephadex chromatography as previously described.[5] GM3-lactone was prepared by treating GM3 with glacial acetic acid. Then, GM3 and GM3-lactone were incorporated into liposomes, which were composed of 2 μmol of dipalmytoylphosphatidylcholine, 2 μmol of cholesterol and 80 nmol of diacetylphosphate in 1 ml of phosphate-buffered saline.

Induction of *in vitro* primary CTL and assay system The *in vitro* primary culture of C57BL/6 spleen cells for generation of melanoma-specific syngeneic CTL and the assay for the CTL activity were described previously.[7] In brief, 4×10^7 naive spleen cells of C57BL/6 were cultured alone as a control or together with 8×10^5 mitomycin C

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[2] Abbreviations: CTL, cytotoxic T lymphocytes; Ts, suppressor T lymphocytes; MMC, mitomycin C; GM3(NeuGc), NeuGcα2-3Galβ1-4Glc; GM3(NeuAc), NeuAcα2-3Galβ1-4Glc.

(MMC)-treated B16 melanoma cells or artificial antigen (GM3 liposomes or GM3-lactone liposomes) as the stimulating antigen in 5 ml of medium (RPMI1640 supplemented with 10% FCS, 5×10^{-5} *M* 2-mercaptoethanol, 20 m*M* Hepes, 1 m*M* sodium pyruvate, 0.3 g/dl L-glutamine, 0.2 g/liter kanamycin and 1/100 dilution of MEM amino acids (Gibco Laboratories)) in 6-well plates (Corning C25910, Corning Glass Works, Corning, NY) at 37°C in 5% CO_2 in air. After 4 days, the lymphoid cells (4.8×10^5) were mixed with 1.2×10^4 ^{51}Cr-labeled B16 melanoma or EL-4 lymphoma cells (control) (CTL/target ratio of 40:1) in a final volume of 0.2 ml of medium in 96-well flat-bottomed microplates (Costar 3599, Costar Corporation, Cambridge). The plates were incubated for 12 h at 37°C in 5% CO_2. The specific ^{51}Cr-release was assayed as described.[5]

Induction of Ts and assay system Anti-melanoma suppressor T cells (Ts) were induced by the same method as previously described.[8] In brief, naive C57BL/6 spleen cells (4×10^7) were cultured in 5 ml of medium in the presence or absence of GM3 or GM3-lactone liposomes at various concentrations in Petri dishes (60 mm diameter, Nunclon 1-50326, Nunc Products, Roskild, Denmark) for 24 h in 5% CO_2 in air. After incubation, cells were harvested, washed extensively and used as Ts. For the assay of suppression activity, cells were added to the *in vitro* primary CTL induction system using B16 melanoma or EL-4 lymphoma as the stimulating antigen at the start of the culture at the Ts/responder ratio of 1:4.

RESULTS

Induction of anti-melanoma CTL by GM3-lactone Our previous experiments have indicated that anti-melanoma CTL recognize the GM3 sugar moiety but could not distinguish GM3 molecular species.[9, 10] As the blocking efficiency of GM3(NeuAc) and GM3(NeuGc) liposomes was similar, the results strongly suggested that anti-melanoma CTL recognize a common structure between GM3(NeuAc) and GM3(NeuGc). It was also demonstrated that GM3-lactone is more immunogenic than GM3, suggesting that melanoma antigen may have a "lactone-like" moiety.[5, 10]

Here, we used GM3-lactone liposomes as an artificial melanoma antigen in the *in vitro* primary anti-melanoma CTL response in order to investigate whether GM3-lactone, like GM3, acts as an antigen for induction of anti-melanoma T cell responses. In the first experiment, naive C57BL/6 spleen cells were cultured *in vitro* with GM3(NeuAc)-lactone or GM3(NeuGc)-lactone liposomes at various concentrations instead of MMC-treated B16 melanoma cells as the stimulator under usual conditions. The cytotoxic activity and specificity of CTL induced by GM3-lactone liposomes were assessed by ^{51}Cr-release assay using ^{51}Cr-labeled B16 melanoma or EL-4 lymphoma cells as target cells. As shown in Fig. 1a, both GM3(NeuAc)-lactone and GM3(NeuGc)-lactone are potent inducers of anti-melanoma CTL response. Even low doses of GM3-lactone liposomes (0.003 μg/ml), like melanoma cells themselves, have the ability to induce specific anti-melanoma CTL, supporting the previous findings that anti-melanoma CTL recognize the common structure between GM3(NeuGc) and GM3(NeuAc).[9, 10] GM3(NeuGc)-bovine serum albumin also acts as a stimulator to induce anti-melanoma CTL (data not shown).

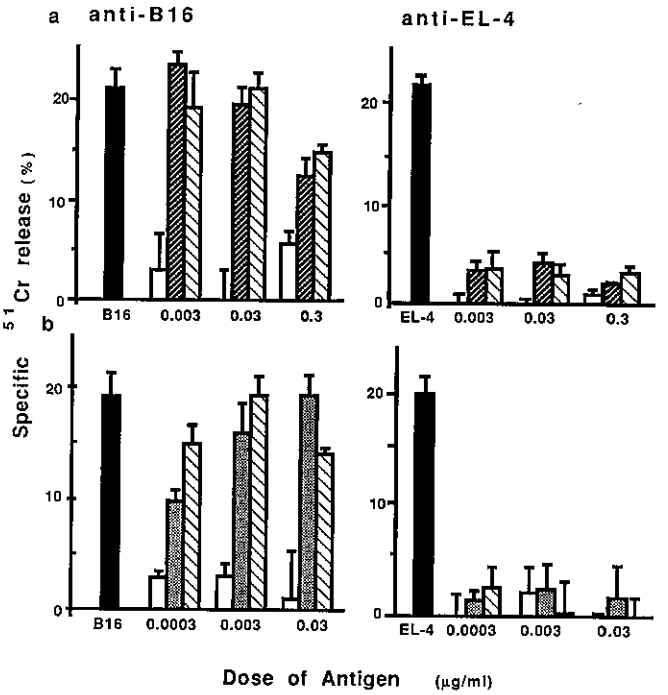

Fig. 1. Induction of anti-melanoma CTL by GM3-lactone liposomes. Various doses of liposomes carrying GM3(NeuAc)-lactone (▨), GM3(NeuGc)-lactone (▧) or GM3(NeuGc) (▩) at 9 mol% were used as stimulating artificial antigens for *in vitro* primary CTL response. B16 melanoma cells (■), EL-4 lymphoma cells (■) and liposomes (□) alone were also used as positive or negative controls. The CTL induced were assayed on ^{51}Cr-labeled B16 melanoma or EL-4 lymphoma cells. The columns and bars indicate arithmetic means of 3 cultures and SD, respectively. a) GM3(NeuAc)-lactone (▨) and GM3-(NeuGc)-lactone liposomes (▧) were used. b) GM3(NeuGc) (▩) and GM3(NeuGc)-lactone liposomes (▧) were used.

In the next experiment, we compared GM3 and GM3-lactone as inducers of anti-melanoma CTL. The results shown in Fig. 1b clearly demonstrated that GM3-lactone and GM3 liposomes both induce anti-melanoma CTL. About 20% specific anti-melanoma CTL activity was observed by cells induced by either GM3-lactone lipo-

somes or GM3 liposomes at concentrations of 0.0003–0.03 μg/ml in culture. There was no difference in the ability to induce CTL with anti-melanoma activity between GM3 and GM3-lactone. Moreover, the CTL-inducing ability was comparable to that of B16 melanoma cells.

The CTL activities induced by the artificial antigen, GM3-lactone or GM3 liposomes, were found to be specific for melanoma because control liposomes did not induce any CTL activity and also because the CTL induced by GM3 or GM3-lactone could not kill EL-4 lymphoma derived from C57BL/6, which has the same origin as B16 melanoma cells (Fig. 1).

Induction of anti-melanoma Ts by GM3-lactone Our previous studies have demonstrated that the Ts epitope is GM3(NeuAc) but not GM3(NeuGc).[7] In the present experiments, we compared the efficiency of GM3 and GM3-lactone as inducers of anti-melanoma Ts. For this purpose, we used GM3 liposomes and GM3-lactone liposomes as stimulating antigens. Naive C57BL/6 spleen cells were cultured for 24 h with GM3 liposomes (10 mol % GM3) or GM3-lactone liposomes (10 mol% GM3-lactone). The cells thus cultured were added to the CTL induction system with MMC-treated B16 melanoma or EL-4 lymphoma as a stimulator and their suppressor activity was investigated in terms of the suppression of CTL responses toward ^{51}Cr-labeled B16 melanoma or EL-4 lymphoma. The results are illustrated in Fig. 2. GM3-lactone at relatively high doses (0.3–3.0 μg/ml) successfully induced anti-melanoma Ts (Fig. 2). As our previous studies have shown that GM3 at a density of 10 mol% could not induce anti-melanoma Ts,[10] GM3-lactone was much more potent for induction of anti-melanoma Ts. The results in Table I also confirm the

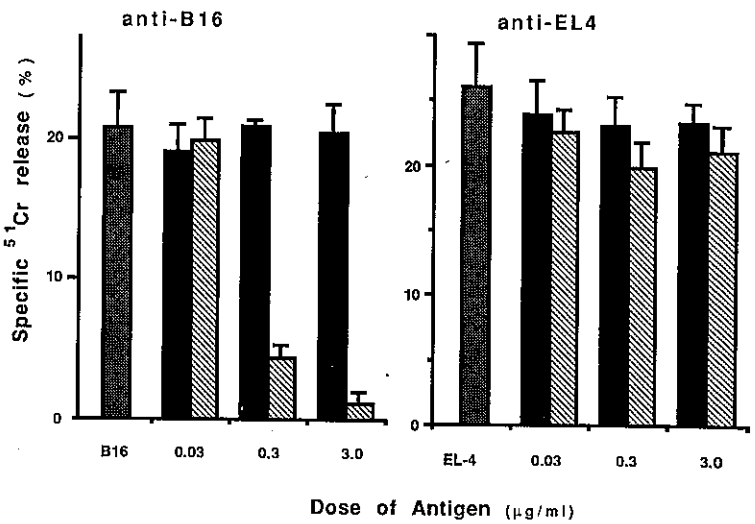

Fig. 2. Induction of anti-melanoma suppressor cells by GM3-lactone. Various doses of liposomes carrying GM3(NeuAc)-lactone (9 mol%) (▨) were used to induce suppressor T cells. As positive and negative controls, B16 melanoma cells (▩), EL-4 lymphoma cells (▩) and liposomes alone (■) were used. The columns and bars indicate arithmetic means of 3 cultures and SD, respectively.

Table I. Induction of Anti-melanoma Ts by GM3-lactone

Antigen	Specific ^{51}Cr release (%) on			
	Exp. 1		Exp. 2	
	B16	EL-4	B16	EL-4
B16	22.0±4.8[c]	—	19.1±1.7	—
EL-4	—	22.5±6.9	—	20.4±3.6
GM3 liposomes[a]	19.8±1.0	15.4±0.4	16.2±3.7	38.0±9.6
GM3-lactone liposomes[b]	4.3±1.0	19.8±2.0	3.5±3.1	27.9±3.7
Liposomes	20.3±2.0	23.3±1.5	18.1±1.7	22.4±6.1

a) GM3 liposomes (9 mol% GM3(NeuAc)) were used at a concentration of 0.3 μg/ml in the culture.
b) GM3-lactone liposomes (9 mol% GM3(NeuAc)-lactone) were used at a concentration of 0.3 μg/ml in the culture.
c) Arithmetic means of three cultures±SD.

above data. No significant difference was observed between GM3 and GM3-lactone as inducers of anti-melanoma Ts.

DISCUSSION

It has been well documented that CTL and Ts cells play a decisive role in anti-tumor immune responses.[11, 12] However, it is not easy to analyze in detail the mechanisms of anti-tumor immune responses against naturally occurring tumors, because the tumor antigens are hard to identify, and also because anti-tumor immune responses are difficult to induce in an *in vitro* primary culture system.

Despite the above difficulties, we have identified melanoma antigen as GM3 ganglioside by the use of monoclonal antibody (M2590), anti-melanoma CTL and Ts systems.[7-9] The structural analysis of melanoma GM3 purified by applying monoclonal antibody M2590 showed that the primary structure of melanoma GM3 is the same as that of normal GM3, even though the M2590 antibody reactivity was specific for melanoma.[4] We then found that the density of GM3 is important for the generation of melanoma antigenicity. Thus, a certain density seems to result in some conformational change of GM3, resulting in melanoma antigenicity.[10] In addition, Nores *et al.* have demonstrated the presence of small amounts of GM3-lactone in the membrane material from B16 melanoma.[5] The GM3-lactone was also found to be more immunogenic than GM3.[5, 6] Therefore, it is likely that a certain density of GM3 results in the formation of a GM3-lactone like conformation. The results shown in Figs. 1 and 2 as well as those in Table I support the above notion that GM3-lactone has potent activity to stimulate T cells involved in anti-melanoma responses. Moreover, GM3-lactone is much more effective than GM3, especially in the induction of anti-melanoma Ts responses. Thus at the T cell level as well as in antibody formation, a GM3-lactone-like conformation may serve as the dominant epitope for subsets of anti-melanoma T cells.

GM3 and GM3-lactone were also compared as inducers of anti-melanoma CTL. However, no significant difference was observed (Fig. 1). GM3-lactone induces anti-melanoma CTL even at a concentration as low as 0.0003 μg/ml. Moreover, two molecular species of GM3-lactone, GM3(NeuAc)-lactone and GM3(NeuGc)-lactone, as well as B16 melanoma cells, were equally effective in the induction of anti-melanoma CTL. The results in Fig. 1 are in agreement with the previous data demonstrating that the epitope for anti-melanoma CTL is a common determinant between GM3(NeuAc) and GM3(NeuGc).[9]

Another important point is that a small amount (0.0003 μg/ml) of GM3-lactone or GM3 liposomes is effective for induction of anti-melanoma CTL, whereas higher doses are required for anti-melanoma Ts induction (Figs. 1 and 2). Moreover, NeuGc residue is not the epitope for anti-melanoma Ts, but NeuAc is. Thus, both GM3(NeuGc) liposomes and GM3(NeuGc)-lactone liposomes in small doses *in vitro* seem to be able to act as artificial melanoma antigens in the *in vitro* induction of anti-melanoma CTL because they did not induce anti-melanoma Ts.

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REFERENCES

1) Tai, T., Paulson, J. C., Cahan, L. D. and Irie, R. F. Ganglioside GM2 as a human tumor antigen (OFA-I-1). *Proc. Natl. Acad. Sci. USA*, **80**, 5392–5396 (1983).
2) Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A. and Paulson, J. C. Identification of a human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2. *Proc. Natl. Acad. Sci. USA*, **79**, 7629–7633 (1982).
3) Taniguchi, M. and Wakabayashi, S. Shared antigenic determinant expressed on various mammalian melanoma cells. *Gann*, **75**, 418–426 (1984).
4) Hirabayashi, Y., Hamaoka, A., Matsumoto, M., Matsubara, T., Tagawa, M., Wakabayashi, S. and Taniguchi, M. Syngeneic monoclonal antibody against melanoma antigen with interspecies cross-reactivity recognizes GM3, a prominent ganglioside of B16 melanoma. *J. Biol. Chem.*, **260**, 13328–13333 (1985).
5) Nores, G. A., Dohi, T., Taniguchi, M. and Hakomori, S. Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: requirements for tumor-associated antigen and immunogen. *J. Immunol.*, **139**, 3171–3176 (1987).
6) Dohi, T., Nores, G. and Hakomori, S. An IgG_3 monoclonal antibody established after immunization with G_{M3}

lactone: immunochemical specificity and inhibition of melanoma cell growth *in vitro* and *in vivo*. *Cancer Res.*, **48**, 5680–5685 (1988).
7) Wakabayashi, S., Taniguchi, M., Tokuhisa, T., Tomioka, H. and Okamoto, S. Cytotoxic T lymphocytes induced by syngeneic mouse melanoma cells recognize human melanomas. *Nature*, **294**, 748–750 (1981).
8) Takahashi, K., Ono, K., Hirabayashi, Y. and Taniguchi, M. Escape mechanisms of melanoma from immune system by soluble melanoma antigen. *J. Immunol.*, **140**, 3244–3248 (1988).
9) Ono, K., Takahashi, K., Hirabayashi, Y., Itoh, T., Hiraga, Y. and Taniguchi, M. Mouse melanoma antigen recognized by Lyt-2$^-$ and L3T4$^-$ cytotoxic T-lymphocytes. *Cancer Res.*, **48**, 2730–2733 (1988).
10) Harada, Y., Sakatsume, M., Nores, G. A., Hakomori, S. and Taniguchi, M. Density of GM3 with normal primary structure determines mouse melanoma antigenicity; a new concept of tumor antigen. *Jpn. J. Cancer Res.*, **80**, 988–992 (1989).
11) Fujimoto, S., Greene, M. I. and Sehon, A. H. Regulation of the immune response to tumor antigens. II. The nature of immunosuppressor cells in tumor-bearing hosts. *J. Immunol.*, **116**, 800–806 (1976).
12) Burnet, F. M. Immunological surveillance in neoplasia. *Transplant. Rev.*, **7**, 3–25 (1971).