Density of GM3 with Normal Primary Structure Determines Mouse Melanoma Antigenicity; a New Concept of Tumor Antigen

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We attempted to induce anti-melanoma cytotoxic T cells (CTL) and suppressor T cells (Ts) inhibiting CTL generation by using liposomes carrying various densities of GM3 as tumor antigens. We found that liposomes carrying 6–16 mol% of GM3 with normal primary structure successfully generated anti-melanoma CTL and suppressor T cells, while liposomes with GM3 outside this range had little or no such activity. Anti-melanoma CTL induced by GM3(NeuGc)-liposomes belonged to CD4$^-$/CD8$^-$ double-negative CD3$^+$ CTL while GM3(NeuAc)-liposomes induced two types of T cells, CD4$^+$ T cells and double-negative I-J positive T cells which mediated inhibition of the induction of anti-melanoma CTL responses. These cell types were the same as those induced by mitomycin C-treated melanoma cells for CTL induction and soluble melanoma antigen for Ts generation. The results clearly demonstrate that even GM3 with normal primary structure can, at a certain density, generate melanoma antigenicity.

Key words: Density of GM3 — Mouse melanoma antigen — GM3-liposome — Anti-melanoma CTL — Anti-melanoma Ts

Many attempts have been made to identify tumor antigens with distinct primary structure from that on normal cells and tissues. In fact, changes in carbohydrate structure in tumor cells different from those of non-transformed cells are defined as tumor-associated antigens.[1-7] However, our previous studies have clearly demonstrated that the primary structure of a melanoma antigen recognized by a syngeneic immune system (analyzed by syngeneic monoclonal antibody, M2590) is GM3 with normal primary sequence, widely expressed on most normal cells and tissues.[8, 9] As M2590 shows melanoma specificity despite the fact that the melanoma epitope is the same as normal GM3,[10, 11] there is an apparent discrepancy with the generally accepted view that a tumor antigen is supposed to have distinct primary structure from that in normal cells.

In this paper, we show that liposomes carrying 6–16 mol% of normal GM3 effectively induce CD3$^+$, double-negative cytotoxic T cells (CTL)[5] against melanoma and also anti-melanoma suppressor T cells (Ts) which inhibit anti-melanoma CTL generation. The results thus clearly demonstrate that even GM3 with normal primary structure can, at a certain density, generate melanoma antigenicity. This represents a novel concept in the field of tumor antigens.

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[5] The abbreviations used are: CTL, cytotoxic T-lymphocytes; MMC, mitomycin C; Ts, suppressor T-lymphocytes; mAb, monoclonal antibody; TCR, T cell antigen receptor.

MATERIALS AND METHODS

Monoclonal antibodies Anti-Thy-1.2 (CMS-1) and anti-I-J mAb (E10)[12] were raised in our laboratory. Anti-L3T4 (CD4) mAb (GK1.5) was originally established by Dialynas *et al.*[13] Anti-CD3 (2C11) and anti-Lyt 2.2 (CD8) mAb (83-12-5) were generated by Blustone *et al.*[14, 15] Anti-FcR mAb (2.4G2)[16] raised by Unkless was a kind gift from Dr. T. Saito, NIH.

GM3-liposomes GM3-liposomes were prepared as previously described.[17] In brief, dipalmityl phosphatidylcholine (dpPC) (4.4 mmol), cholesterol (4.4 μmol), diacetylphosphate (0.18 μmol) and various amounts of GM3(NeuGc) or GM3(NeuAc) were dried and resuspended in 100 μl of 0.1 *M* phosphate-buffered saline. The resultant liposomes were washed twice with buffer 20 m*M* Tris-HCl, pH 8.0, and 150 m*M* NaCl.

CTL induction and assay Syngeneic anti-melanoma or anti-EL-4 CTL were induced in the *in vitro* primary culture system as previously described.[18-20] In brief, 4×10^7 spleen cells from unprimed C57BL/6 mice were cultured alone as a control or with 8×10^5 of mitomycin C (MMC)-treated B16 melanoma or EL-4 lymphoma cells or with 0.3 μg/ml GM3(NeuGc)-liposomes in 5 ml RPMI-1640 containing 10% fetal calf serum (lot no. 8178, Filtron, Australia), 5×10^{-5} *M* 2-ME, 20 m*M* HEPES, 1/100 dilution of MEM amino acids (Gibco Laboratories, Grand Island, NY), 1 m*M* sodium pyruvate

and 0.3 g/liter L-glutamine in a 6-well plate (Corning no. c-25810, Corning Glass Works, Corning, NY) at 37°C in humidified 5% CO_2 in air. Four days later, cells were harvested, washed extensively, and adjusted to 6×10^6/ml viable nucleated cells. To assay the activity of the cultured cells, the cells (6×10^5) were mixed with 1.5×10^4 ^{51}Cr-labeled target cells at a target/responder ratio of 1/40 in 0.2 ml of RPMI-1640 in a 96-well flat-bottomed microplate (Costar no. S3599, Costar Corporation, Cambridge, MA) in triplicate. B16 melanoma and EL-4 lymphoma cells were used as targets. The plates were incubated for 12 h at 37°C and the radioactivity in 0.1 ml of supernatant from each well was counted by a well-type γ-counter (LKB-Wallac CliniGamma 1272, Wallac Oy, Turuk, Finland). Specific precent lysis was expressed as precentage of specific ^{51}Cr release calculated by the following formula;

% specific ^{51}Cr release

$$= \frac{\text{cpm experimental release} - \text{cpm control release}}{\text{cpm maximum release} - \text{cpm control release}} \times 100.$$

Control release was determined by incubating ^{51}Cr-labeled target cells with naive spleen cells of C57BL/6 mice cultured for 4 days without stimulator cells or GM3-liposomes. Maximum lysis was obtained by disrupting the target cells with saponin. Spontaneous release from target cells was generally less than 25% of total lysis.

Ts induction and assay Ts induction and the assay system were described elsewhere.[19] In brief, C57BL/6 naive spleen cells (1×10^7/ml) were cultured in 5 ml of 10% FCS RPMI 1640 medium in the presence of 0.3 μg/ml GM3(NeuAc)-liposomes for 36 h at 37°C in 5% CO_2 in air. After incubation, cells were harvested, washed extensively, and used as Ts. For the assay of suppressor activity, cells thus cultured were added to the *in vitro* primary CTL induction system at the start of the culture at a Ts/responder ratio of 1/8. Ts activities were measured by ^{51}Cr-release assay and expressed as suppression of CTL activities.

Cytotoxic treatment In order to analyze phenotypes of cells involved in anti-melanoma CTL or Ts responses, CTL or Ts induced in the *in vitro* culture were treated with mAb at room temperature for 30 min, followed by treatment with 2-week-old rabbit complement at 1:10 dilution for 40 min at 37°C.

Blocking of CTL activity with anti-CD3 mAb The anti-melanoma CTL population induced by GM3-liposomes (9 mol% GM3) was mixed with ^{51}Cr-labeled B16 melanoma as a target. Before mixing, both components had been preincubated with anti-FcR (2.4G2) mAb for 30 min at room temperature. Anti-CD3 (2C11) mAb or irrelevant control mAb at various concentrations (18.5–167 μg/ml) was added to the mixture of CTL and target cells in the effector phase of CTL responses.

RESULTS AND DISCUSSION

Our previous studies on melanoma antigen have demonstrated that syngeneic mouse anti-melanoma CTL kill human melanomas as well as mouse melanoma cell lines in a genetically non-restricted fashion, suggesting that melanoma cells express cross-species melanoma epitopes.[18] We have successfully raised a syngeneic anti-melanoma mAb (M2590) reactive with a cross-species melanoma epitope, whose structure has turned out to be GM3.[10, 11] Further biochemical analyses have demonstrated that the structure of the GM3 epitope is the same as that of normal GM3,[8] but M2590 distinguishes cell-surface GM3 on melanoma and normal cells. As M2590 also blocks anti-melanoma CTL activity,[20] the cross-species melanoma epitope is found to be composed of GM3 with normal primary sequence. However, M2590 reacts with normal GM3 only if it is bound on a silica plate.[8, 9] This suggests that M2590 recognizes the surface density or conformational changes of GM3; a high density of GM3 even with normal primary structure may generate melanoma antigenicity.

To test this possibility in relation to anti-tumor T cell responses, especially anti-melanoma CTL responses, which play decisive roles in tumor rejection as well as Ts responses inhibiting CTL generation, we prepared liposomes carrying GM3(NeuGc) or GM3(NeuAc) gangliosides at various densities, and used GM3-lipo-
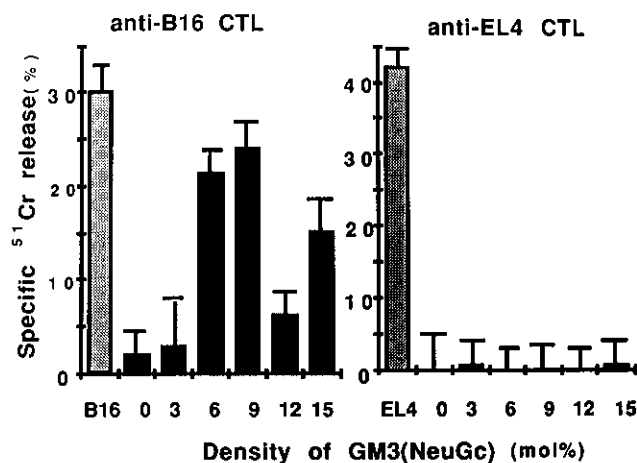

Fig. 1. Induction of anti-melanoma CTL by GM3(NeuGc)-liposomes. The activity of CTL induced by either B16 melanoma or EL-4 lymphoma (▨) as well as liposomes alone or liposomes carrying GM3 at various densities (■) was measured and expressed as specific ^{51}Cr-release. The columns indicate arithmetic means of the percent specific lysis of three cultures ± SD.

somes as an artificial melanoma antigen in order to induce anti-melanoma CTL or Ts. In our first experiments, C57BL/6 naive spleen cells were cultured with GM3(NeuGc)-liposomes instead of MMC-treated B16 melanoma cells as stimulators. The activity was assessed on B16 melanoma or EL-4 lymphoma as effector target cells. The reason why we used GM3(NeuGc) for anti-melanoma CTL induction is that epitopes for anti-melanoma CTL are suggested to be common between GM3(NeuGc) and GM3(NeuAc) by CTL inhibition assay, whereas the Ts epitope on melanoma antigen was found to be GM3(NeuAc) but not GM3(NeuGc).[19] Figure 1 demonstrates that liposomes carrying GM3 at 6–9 mol% and 15 mol% but not at other densities are effectively able to induce anti-melanoma CTL. The cytotoxic activity of CTL induced by GM3-liposomes is specific for melanoma, because the CTL killed B16 melanoma but not EL-4 lymphoma cells, both of which are derived from C57BL/6 mice. Similar results were also obtained by using GM3(NeuGc)-BSA complex as a stimulator (data not shown).

The activity of GM3-liposome-induced CTL is abrogated by cytotoxic treatment with anti-Thy-1 but not with anti-CD4 or anti-CD8, suggesting that the CTL

belong to the category of double-negative T cells (Fig. 2a). The cytotoxic activity is also specifically blocked in a dose-dependent manner by addition of various doses of anti-CD3 antibodies (Fig. 2b). Thus, an artificial antigen GM3(NeuGc)-liposomes, indeed induced double-negative CD3$^+$ CTL with anti-melanoma specificity. The results in Fig. 2b also demonstrate that the cytolytic activity of double-negative anti-melanoma CTL is mediated through T cell receptor (TCR) but not by other nonspecific mechanisms, since the CTL activity was blocked by anti-CD3 (2C11) but not by control antibody. We do not know whether they use $\alpha\beta$ or $\gamma\delta$ TCR as yet. These data agree with our previous results obtained in experiments using MMC-treated B16 melanoma cells as stimulating antigens for CTL induction.[20] Therefore, it is clear that liposomes carrying normal GM3 at a certain density can serve as a melanoma antigen. This again confirms previous findings that the primary structure of melanoma antigen determined by gas chromatography is the same as that of normal GM3 ganglioside.[8, 9]

A similar phenomenon, that a certain surface density of GM3 generates melanoma antigenicity, was also observed in the induction of anti-melanoma Ts. In these experiments liposomes carrying various densities of GM3(NeuAc) were incubated with naive C57BL/6 spleen cells in order to induce Ts. The incubated cells were then added to the in vitro primary anti-melanoma CTL induction system. As shown in Fig. 3, the induction of anti-melanoma CTL was completely suppressed by the Ts induced by 16.4 mol% GM3(NeuAc)-liposomes. The
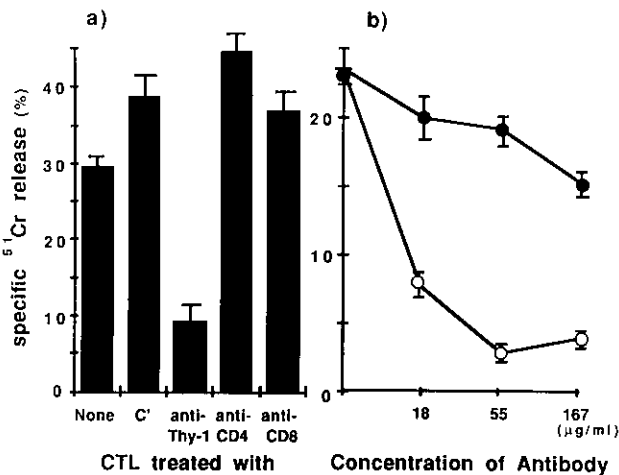
Fig. 2. Surface markers of anti-melanoma CTL induced by GM3(NeuGc)-liposomes. Surface phenotypes of anti-melanoma CTL were determined either by cytotoxic treatment (a) or by blocking of CTL activity with mAb (b). (a) Anti-melanoma CTL induced by GM3-liposomes (9 mol% GM3-(NeuGc)) under the same conditions as described in Fig. 1 were treated with mAb and rabbit complement. (b) CD3 expression and TCR-mediated cytolytic activity of anti-melanoma CTL were investigated by blocking with anti-CD3 mAb (2C11) (○) or control mAb (●). The assay was carried out under the same conditions as described in (a). Results are expressed as arithmetic means of percent specific lysis of three cultures ±SD.
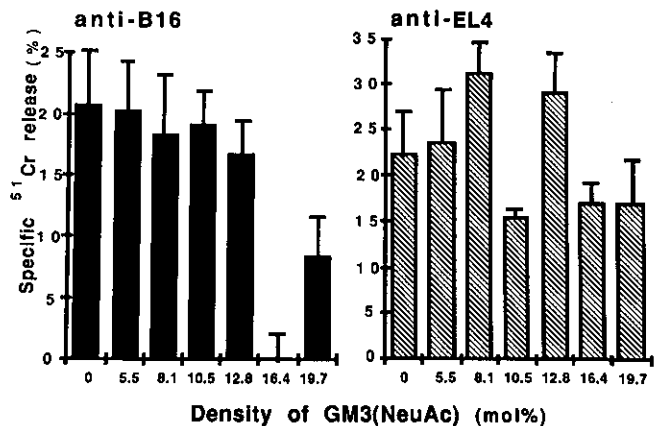
Density of GM3(NeuAc) (mol%)

Fig. 3. Induction of anti-melanoma Ts by GM3(NeuAc)-liposomes. Ts activity of the cells incubated with liposomes carrying various densities of GM3(NeuAc) (0–19.7 mol%) was added to the CTL induction system. Three days later CTL activity was measured. The columns indicate arithmetic means of the percent specific lysis of three cultures ±SD.

Specific ^{51}Cr release (%)

Fig. 4. Phenotypes of anti-melanoma Ts induced by GM3-(NeuAc)-liposomes. Anti-melanoma Ts were induced by GM3-(NeuAc)-liposomes (16.4 mol%) *in vitro* and treated with mAb with complement. Their Ts activity was then assessed in the culture for induction of the *in vitro* primary anti-melanoma CTL responses, followed by ^{51}Cr-release assay on B16 melanoma as target cells. The columns indicate arithmetic means of the percent specific lysis of three cultures ± SD.

suppression was specific because no significant suppressive effects were observed in anti-EL-4 CTL responses. Moreover, the cells incubated with GM3 liposomes at less than 12 mol% did not show any suppressor effect on either anti-melanoma or anti-EL-4 CTL response. Therefore a GM3 density at around 16.4 mol% is critical to generate the epitope for anti-melanoma Ts. At a GM3 density of 18 mol%, GM3-liposomes also possessed moderate ability to induce anti-melanoma Ts.

Two types of cells, CD4$^+$ and double-negative I-J$^+$ T cells, were demonstrated to be involved in this suppression because cytotoxic treatments of GM3-induced Ts populations either with anti-CD4 or anti-I-J antibody completely abrogated the suppressive activity, while a mixture of these two populations without activity recovered the suppressive activity (Fig. 4). Anti-CD8 treatment did not affect any Ts activity. Thus, both types, CD4$^+$ and double-negative I-J$^+$ T cells, involved in the suppression of anti-melanoma CTL generation were indeed induced by GM3(NeuAc)-liposomes. Our data here agree with our previous results obtained in experiments using soluble melanoma antigen for Ts induction.[19]

The question arises, how does GM3 with normal primary structure generate the melanoma antigenicity? Several possible mechanisms can be considered. 1) A protein may be associated with GM3 and may modify the tertiary structure of GM3 to generate melanoma antigenicity. 2) The protein may function just to assemble GM3,

resulting in an increased density of GM3 and making it immunogenic. In fact, the idea that the melanoma antigen is not simply GM3 itself is supported by the results that most of the anti-melanoma activity of conventional CTL and CTL clones were blocked by either anti-GM3 M2590 or anti-melanoma M562 antibody recognizing 80 kd glycoprotein with mouse melanoma specificity.[20, 21] Moreover, our recent studies demonstrate that soluble materials purified by M 562 antibody induce anti-melanoma suppressor T cells that inhibit CTL generation.[22] Therefore it is likely that the protein molecules associated with ganglioside contribute to the assembly or modification of the GM3 tertiary structure, making normal GM3 immunogenic.

We do not have any method available to analyze the fine tertiary structure of GM3 with melanoma antigenicity on the tumor cell surface or on GM3-liposomes with ability to induce CTL or Ts at present. However, Nores *et al.* have provided some ideas regarding the melanoma epitope perhaps having a "GM3-lactone" like structure, because anti-melanoma M2590 showed a high affinity with an approximate Kd value of 0.3 to 0.5 μg/ml for GM3-lactone, whereas it showed low affinity (Kd value of 6 to 15 μg/ml) for GM3.[17] Therefore, it is possible that GM3 at a certain surface density creates a GM3-lactone-like conformation with melanoma antigenicity.

It is intriguing that a completely artificial antigen, GM3(NeuGc)-liposomes, is able to induce anti-melanoma CTL but not Ts responses. These results clearly demonstrate that GM3(NeuGc)-liposomes work as an artificial tumor antigen instead of melanoma cells, suggesting the feasibility of developing an anti-tumor vaccine in the future. In any event, the above results raise a basic question about the concept of a tumor antigen that is widely accepted at present, and introduce the novel idea that the tertiary structure of glycolipid with a normal primary structure can generate tumor antigenicity under some conditions.

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