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STRONG ANTIBODY REACTION AGAINST GLYCOSPHINGOLIPIDS INJECTED IN LIPOSOME-EMBEDDED FORMS IN β3GN-T5 KNOCKOUT MICE

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ABSTRACT

It is known that mutant mice of the β-1,3-*N*-acetylglucosaminyltransferase gene (β3Gn-T5) respond well to T-cell dependent and independent antigens. Here, we examined the effectiveness of anti-ganglioside antibody generation by immunization of β3Gn-T5 mutant mice with liposome-embedded glycosphingolipids such as GD1a and GT1b. Consequently, the mutant mice showed a more efficient generation of anti-GD1a or anti-GT1b antibodies than wild-type mice in an enzyme-linked immunosorbent assay using sera during immunization. Thus, the β3Gn-T5 deficient mutant mice proved more responsive than wild-type mice to not only protein antigens, but also to carbohydrates in glycolipids. Furthermore, about 50% of monoclonal antibodies generated using splenocytes of the immunized mutant mice were of the IgG class. Besides general high responsiveness to proteins and glycolipids, it could be expected that the mutant mice of β3Gn-T5 would be useful in the generation of monoclonal antibodies towards lacto-/neolacto-series glycolipids, since these mutants lack lacto-/neolacto-series glycolipids. In fact, they showed a good serum response in immuno-fluorescence assay with cultured living cells when immunized by glycolipids extracted from ovarian cancer cell lines. These results suggested that β3Gn-T5 mutant mice are useful for the generation of anti-glycolipid antigens with lacto-/neolacto-core structures expressed in cancer cells.

Key Words: Immunization, Antibody, Knockout, Glycolipids, Gangliosides, Liposome

INTRODUCTION

Glycosphingolipids are ubiquitously expressed on cell membrane in various organs and tissues, and have been considered to mediate cell-cell recognition and to modulate signals transduced via membrane, regulating cellular functions and fates.[1)] They are amphopathic molecules that are implicated in a variety of fundamental cell events.[2,3)] They are also involved in the pathogenesis of neurodegeneration by triggering aggregations of aberrant protein fragments[4,5)] or in the modula-

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tion of receptor signals such as epidermal growth factor receptor,[6] platelet-derived growth factor receptors,[7] insulin receptor[8] and complement-regulatory proteins.[9]

To examine the expressions of glycosphingolipids in cell membranes and tissues, monoclonal antibodies (mAbs) reactive with particular epitopes present in particular species of glycosphingolipids with high affinity have proven very useful. However, it has not been easy to generate antibodies selectively reactive with common glycosphingolipids.[10] Therefore, gene-engineered mice lacking some series of glycosphingolipids have been used for the generation of mAbs toward structures which are absent in those mutant mice.

The Lc3-synthase (β1,3-N-acetylglucosaminyltransferase-V: β3Gn-T5) synthesizes GlcNAc β1,3Galβ1,4Glc-ceramide to initiate the formation of lacto-/neolacto-series glycosphingolipids by transferring GlcNAc in a β1,3-linkage to lactosylceramide (Fig. 1).[11] The β3Gn-T5 is detected in the developmental stages as well as in the spleen and placenta of adult mice.

The present study demonstrated that the β3Gn-T5 knockout (KO) mice are more responsive to gangliosides than the wild-type when immunized by purified gangliosides. Then, we immunized β3Gn-T5 KO mice with glycolipid extracts from human ovarian cancer cell lines, showing successful generation of antibodies in the β3Gn-T5 KO mice. These results suggest that the β3Gn-T5 KO mice are useful in generating mAbs reactive not only with Lc3-derived structures but with a wide variety of structures related to the enzyme products.

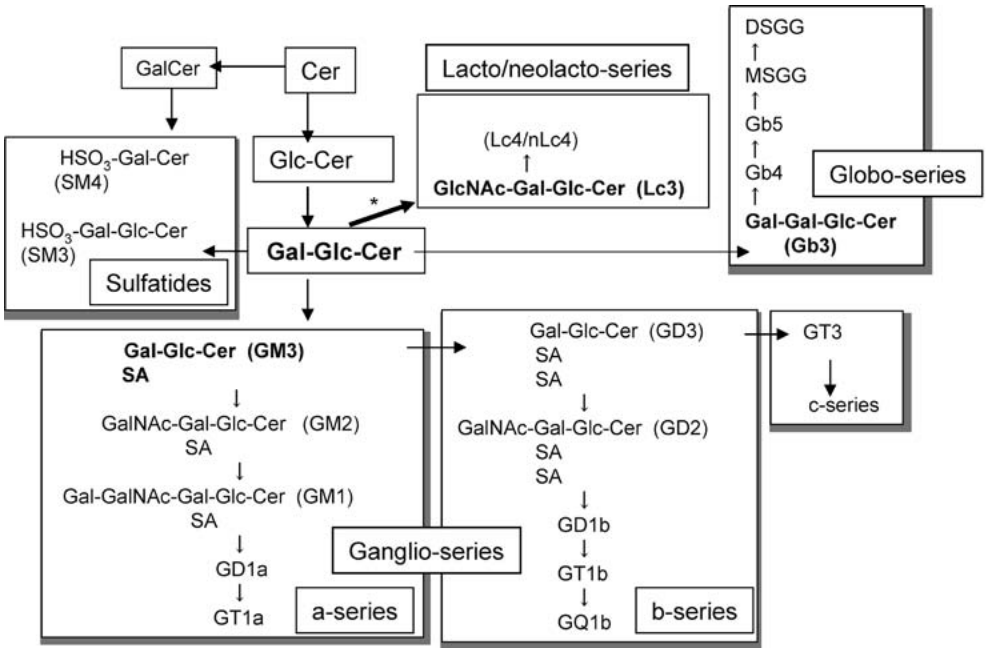


Fig. 1 Synthetic pathway of glycosphingolipids. Majority of glycosphingolipids are synthesized via GlcCer (and LacCer), while a minor portion is via GalCer. SA, N-acetyl-neuraminic acid. GlcCer, glucosylceramide; LacCer, lactosylceramide; GalCer, galactosylceramide. β3Gn-T5 catalyzes conversion of LacCer to Lc3.

MATERIALS AND METHODS

Cell culture

A multiple myeloma cell line, NS-1, was maintained in RPMI1640 containing 10% fetal calf serum (FCS). All human ovarian cancer cell lines were obtained in the Nagoya University Hospital. SKOV3, OVCA3, HEY, K2, HRA, KOC-7C, ES2, RMG-1, RMG-2, TOV21G, and SAOV were maintained in RPMI 1640 containing 10% FCS in a humidified 5% CO_2 atmosphere at 37°C. K2CR, K2CBR, K2VR, K2TR, K2AR were maintained in RPMI 1640 containing 10% FCS and an appropriate concentration of anti-cancer drugs in a humidified 5% CO_2 atmosphere at 37°C. Added drugs were as follows: Cisplatin™ (Wako, Osaka, Japan) (10 μg/ml), Carboplatin™ (Wako) (2.5 μg/ml), Etoposide™ (Sigma) (10 μM), Paclitaxel™ (Sigma) (10 μM), and Doxorubicin™ (Sigma) (0.8 μg/ml).

Animals

C57BL/6J, $KSN^{nu/nu}$ mice were purchased from Japan Charles River. β3Gn-T5 knockout mice were established by A. Togayachi at the Research Center for Medical Glycoscience, the National Institute of Advanced Industrial Science and Technology. The synthetic pathway of glycosphingolipids was shown in Fig. 1. The enzyme reaction catalyzed by β3Gn-T5 was depicted by a thick arrow with an asterisk (*). All mouse protocols were approved by the Committee of Laboratory Animals of the Nagoya University Graduate School of Medicine, along with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). All animals were maintained under specific pathogen-free conditions.

Gangliosides

Purified gangliosides and ganglioside mixture from bovine brain were purchased from Sigma.

Flow cytometry

The cell-surface expression of antigens was analyzed by FACS Caliver™ (Becton Dickinson, Mountain View, CA). Briefly, the $1x10^6$ cells were collected and incubated with diluted ascites or purified antibodies for 60 min on ice, then stained with FITC-conjugated goat anti-mouse IgG (H+L) (Cappel, Durham, NC) for 45 min on ice. Control cells for flow cytometry were prepared using the secondary antibody alone. For the quantification of positive cells, the CELLQuest™ program was used.

Immunization of mice

Three 8-week-old mice were immunized from the tail vein with liposomes containing glycolipid (10 μg) (Commercial GT1b and GD1a, or glycolipids extracted from ovarian carcinoma cell lines) Lipid A (10 μg) (Sigma), cholesterol (0.5 μmol) (Sigma) and dipalmitoylphosphatidylcholine (DPPC) (0.5 μmol) (Funakoshi, Tokyo, Japan) on days 1, 4, 8, 12, 16, 20 and 24. Antibody titer in mouse serum was determined using enzyme-linked immunosorbent assay (ELISA) or immuno-fluorescence assay (IF).

Generation of mAbs

β3Gn-T5 KO mice were immunized with liposomes containing glycolipids (10 μg) 7 times as described above. After a determination of antibody titer in mouse serum, the best responder was selected. Splenocytes were obtained three days after the last immunization, and were fused with NS-1 myeloma cells using polyethylene glycol (PEG). After selection with HAT medium

(Sigma), antibodies in culture supernatants were examined by ELISA and/or IF. Positive clones served for subcloning with limited dilution to establish stable clones.

ELISA

Commercial glycolipids in methanol (20 ng/10 µl) were plated in 60-well Terasaki plates™ (Greiner BioOne, Frickenhausen, Germany). After drying in air for 20 min, the plates were blocked with 5% BSA in PBS overnight at 4°C. After washing twice, serially diluted antibodies were added to the plates and then incubated at room temperature for 2 hr. After washing 5 times, horseradish peroxidase (HRP)-anti-mouse IgGs (Amersham Biosciences) was added as a secondary antibody. After washing 5 times, 10 µl of substrate solution [ortho-phenylene diamine (2 mg) (Wako) and H_2O_2 (8 µl) (Wako) in 5 ml of citrate-phosphate buffer] was added to the plates. After an appropriate period of incubation in the dark, the color development was recorded with a scanner.

IF assay

After plating target cells (K2, SAOV and ES2) on Terasaki plates at 1,000 cells/well and culturing overnight, the supernatants were removed and serially diluted sera or culture supernatants were added to the plates and incubated for 1 hr at room temperature. After washing twice, anti-mouse IgG (H+L) conjugated with FITC (Cappel, Durham, NC) was added, and plates were incubated for 30 min at room temperature in the dark. After washing twice, antibody binding was observed under fluorescence microscopy (BX51; OLYMPUS, Tokyo, Japan).

TLC of glycolipids

Glycolipids were extracted as previously reported.[12] TLC was performed using high-performance TLC plates (Merck, Darmstadt, Germany) and developed by solvent systems as follows: Neutral glycolipids were developed by a solvent system of chloroform/methanol/0.22% $CaCl_2$ (60:35:8), and acidic glycolipids by a solvent system of chloroform/methanol/0.22% $CaCl_2$ (55:45:10). Orcinol or resorcinol spray was used for the detection of neutral or acidic fractions, respectively.

Statistical analysis

Statistical significance of data was determined using Student's *t*-test.

RESULTS

Higher antibody response to immunized gangliosides in β3Gn-T5 KO mice

β3Gn-T5 KO mice and wild-type mice were immunized using liposome-embedded glycolipids as described in MATERIALS AND METHODS. Before immunization (pre-immune sera), no significant reaction was found in ELISA with either mutant mouse serum or normal mouse serum. After immunization with GD1a or GT1b embedded in liposome, sera were collected and examined for antibody activities to the individual glycolipids with ELISA. Generally, β3Gn-T5 KO mice showed 2-8 fold stronger activities than wild-type mice, as shown in Fig. 2. After five times injection of glycolipids, a definite reaction of up to 640x to GD1a was detected in the mutant mice, although wild-type mice showed up to 160x dilution. As for GT1b, the mutant sera showed a reaction at 640x, while the wild type did so at a 320x dilution. The intensities of reactions in ELISA were scored and plotted in Fig. 3.

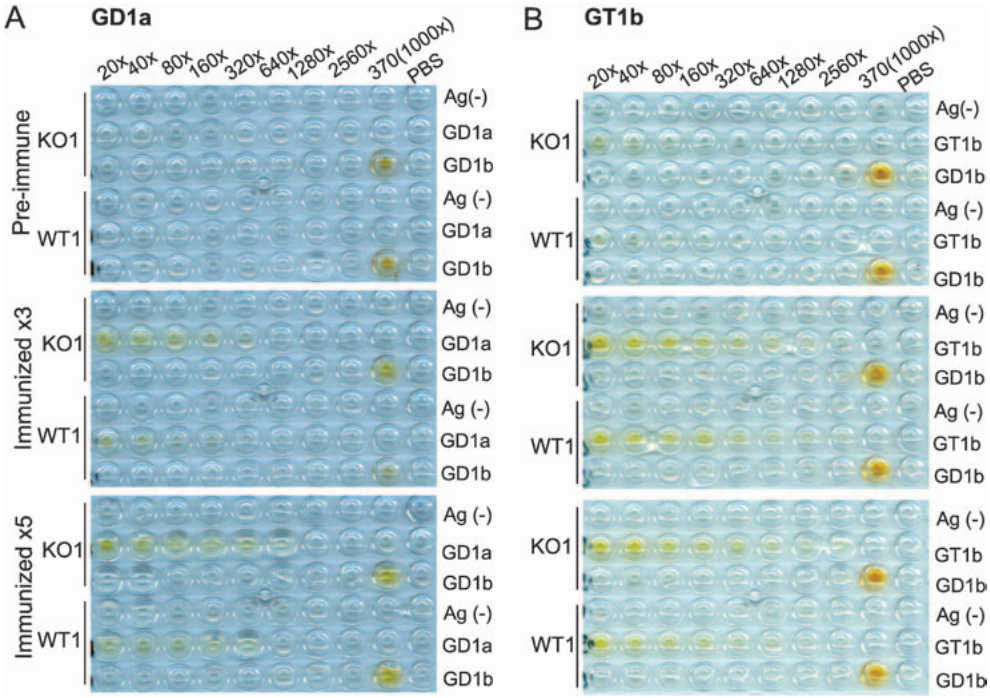


Fig. 2 Comparison of antibody responses between β3Gn-T5 KO and WT mice against two gangliosides. Four each of β3Gn-T5 KO and WT mice were immunized by gangliosides GD1a or GT1b embedded in liposome as described in MATERIALS AND METHODS. Sera at pre-immune and after immunization 3 times and 5 times were collected and used for ELISA as described in MATERIALS AND METHODS. (A) Results of anti-GD1a reactivity. (B) Results of anti-GT1b reaction. Gangliosides used for immunization were used as target antigens in the individual groups. mAb 370 reactive with GD1b was used as a positive control of ELISA.

Frequent generation of IgG class mAbs with β3Gn-T5 KO mice

After immunization of β3Gn-T5 KO mice with GD1a or GT1b embedded in liposome, spleen cells were removed and fused with NS-1 myeloma cells. After HAT selection, a number of mAbs reactive with gangliosides used for immunization were generated. More than 20 clones each were established. Out of ten clones examined, 6 of 10 of anti-GD1a mAbs and 4 of 10 of anti-GT1b mAbs were of IgG class, indicating that this condition could induce a high frequency of mAbs with IgG class. All remaining mAbs were of IgM class.

Profiles of glycolipid expression on human ovarian cancer cell lines

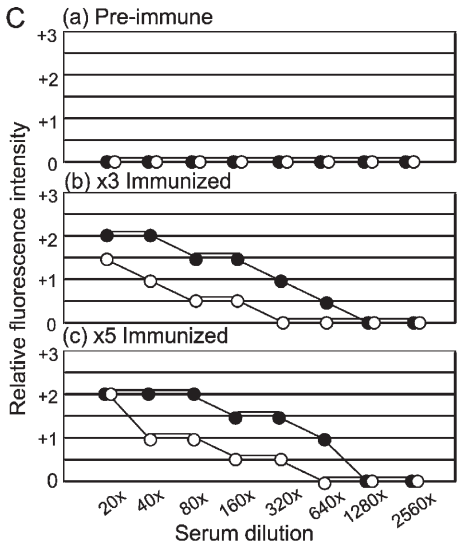
Using 11 ovarian cancer cell lines and five drug-resistant sublines, expression profiles of glycolipid antigens were examined with anti-ganglioside antibodies, anti-globo-series antibodies, and anti-blood group antigen antibodies. Many ovarian cancer cell lines expressed a-series gangliosides such as GD1a and GM1. Some of them also expressed globo-series glycolipids such as Gb3 and Gb4. As for blood group antigens, Lewis b, Lewis y, sialyl-Lewis a and sialyl-Lewis x were frequently found. Results for 3 cell lines were shown in Fig. 4A. These data suggested that β3Gn-T5 KO mice could be suitable for immunization with ovarian cancer-derived glycolipids to obtain mAbs reactive with lacto-/neolacto-series glycolipids.

A Anti-GD1a

Time/mice \ Dilution of sera		20	40	80	160	320	640	1280	2560
pre	KO	-	-	-	-	-	-	-	-
	WT	-	-	-	-	-	-	-	-
x3	KO	++	++	++~+	++~+	+	±	-	-
	WT	++~+	+	±	±	-	-	-	-
x5	KO	++	++	++	++~+	++~+	+	-	-
	WT	++	+	+	±	±	-	-	-

B Anti-GT1b

Time/mice \ Dilution of sera		20	40	80	160	320	640	1280	2560
pre	KO	±	±	-	-	-	-	-	-
	WT	±	±	-	-	-	-	-	-
x3	KO	+++ ~ ++	++	++	++~+	+	+	±	-
	WT	++	++~+	++~+	++~+	+	±	-	-
x5	KO	+++ ~ ++	+++ ~ ++	++	++	++~+	+	±	±
	WT	++	++~+	++~+	++~+	+	±	-	-



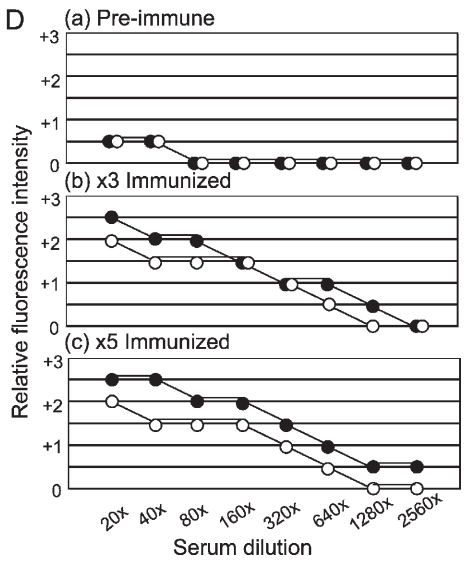


Fig. 3 Increased response of serum antibody against immunized gangliosides in β3Gn-T5 KO mice. Color intensity in each well of ELISA shown in Fig. 2 was visually estimated and scored in (A and B). Note increased reaction of each serum against injected ganglioside antigen together with repeated immunization. β3Gn-T5 KO mice showed 2-8-fold stronger reaction than WT mice. Relative fluorescence intensity was scored from - to +++ and plotted. (C and D) Scores in A and B were plotted. Closed circle, KO; open circle, WT.

Antibody response of β3Gn-T5 KO mice to immunized glycolipids

Using extracts from three representative ovarian cancer cell lines, i.e. K2, SAOV and ES2, liposome-embedded glycolipids (TLC patterns were presented in Fig. 4B) were injected into β3Gn-T5 KO mice. Serum reaction in the immunized mice with the 3 cultured cell lines was checked at pre-immune, after x3 immunization, x5 immunization and x7 immunization. A gradual increase in binding intensity was observed in all immunized KO mice with the highest titer of 1:2430 or more (Results of IF were shown in Table 1).

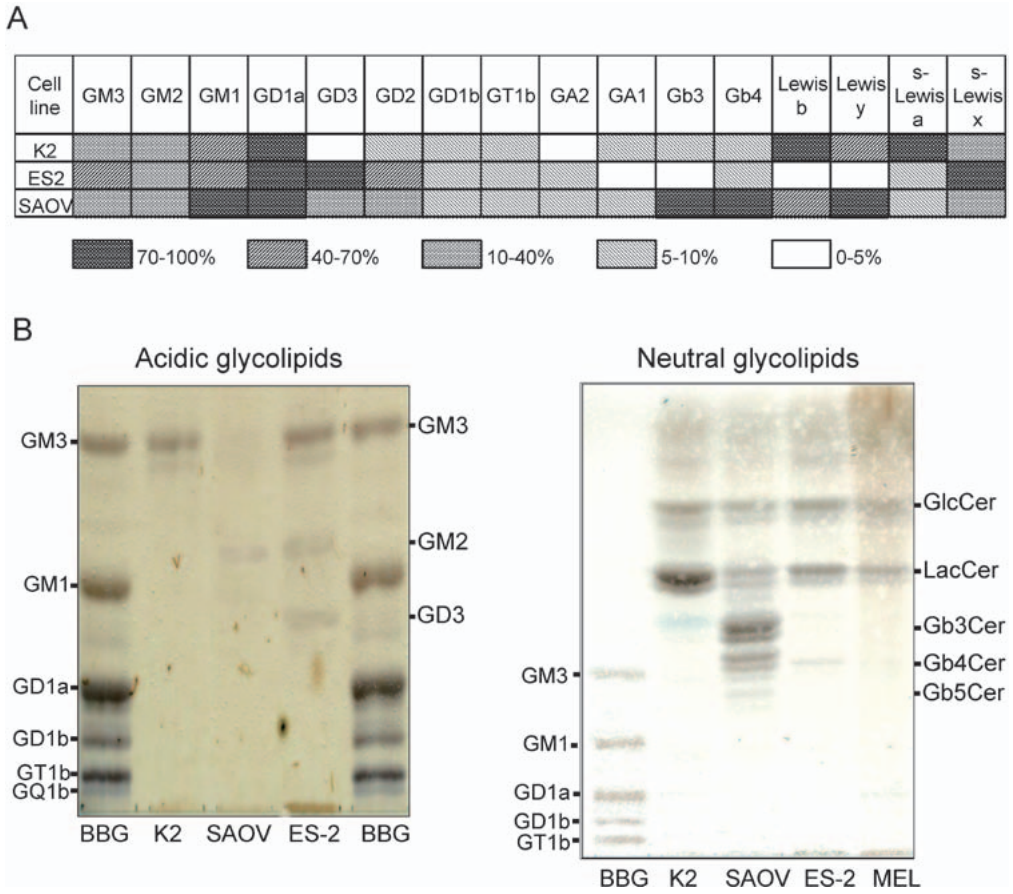


Fig. 4 Summary of flow cytometric analyses and TLC of glycolipids. Results of flow cytometry of 3 ovarian cancer cell lines were summarized in (A), showing different patterns among the three. (B) Extracts from these lines were used to immunize β3Gn-T5 KO mice. Glycolipids were separated by TLC as described in MATERIALS AND METHODS. Acidic glycolipids were detected by resorcinol spray (left), and neutral ones were by orcinol spray (right). BBG, bovine brain gangliosides as standards. MEL, neutral fraction from a melanoma cell line, SK-MEL-28 used as a standard.

Table 1 Antibody titer examined by IF after immunization of β3Gn-T5 KO mice

Mouse (6102)		10x	30x	90x	270x	810x	2430x
Pre-immune	K2	-	-	-	-	-	-
	ES2	-	-	-	-	-	-
	SAOV	-	-	-	-	-	-
Immunized x3	K2	70 ++	70 +	50 +	20 ±	-	-
	ES2	90 ++	90 ++	70 +	70 +	50 +	50 ±
	SAOV	80 ++	70 +	40 +	40 ±	-	-
Immunized x5	K2	90 +++	70 ++	70 +	40 +	30 ±	10 ±
	ES2	100 +++	100 ++	90 ++	80 +	80 +	40 ±
	SAOV	100 +++	100 ++	80 +	60 +	40 +	40 ±
Immunized x7 (before fusion)	K2	90 +++	90 ++	80 +	40 +	40 ±	-
	ES2	100 +++	100 ++	90 ++	80 ++	80 +	80 ±
	SAOV	100 +++	100 ++	90 ++	80 +	40 +	40 ±
Control (PC NC)		50 ++	60 ++	50 ++	-	-	-
		K2	ES2	SAOV	K2	ES2	SAOV

Results of IF with sera of a representative mouse (6102) were presented. Mice were immunized using glycolipids extracted from 3 ovarian cancer cells after embedding in liposome as described in MATERIALS AND METHODS. Numbers represent % of stained cells in IF, and grades of fluorescence intensity in positive cells were scored as -, ±, +, ++ and +++. Note increases in IF reaction with immunization times.

DISCUSSION

β3Gn-T5 KO mice have been reported to be highly reactive in the immunization with proteins.[13] In this study, we confirmed that KO mice are also more reactive to injected glycolipids, such as GD1a and GT1b, than are the wild-type mice. Compared with C57BL/6-background wild-type mice, β3Gn-T5 KO showed a titer two to 8 times higher in its serum reaction to the injected glycolipids in ELISA (Fig. 2). Thus, in addition to the general hyper-reactivity of β3Gn-T5 KO, the mice were expected to react strongly with lacto-/neolacto-series glycolipids such as Lewis and sialyl-Lewis antigen structures when immunized with glycolipids containing these epitopes. This is because these structures should act as "foreign" antigens to the KO mice. Actually, a high affinity IgG mAb reactive with 3'-isoLM1/3', 6'-isoLD1 was raised in β3Gn-T5 KO mice as reported recently.[14] These structures are disialyl- and monosialyl-forms of Lc4, synthesized via the action of β3Gn-T5 enzyme. Therefore, this mAb can be understood as a product based on the immune recognition of foreign antigens. Our study suggested that

they might be highly responsive not only to Lc3-derived glycolipid lineages, but also to general glycolipid structures including gangliosides.

In the application of antibodies to the treatment of cancers, their biological functions and accessibility to cancer tissues are crucial issues. Whether antibodies under investigation can bind with target molecules on the surface of living cells is a critical point for effective anti-cancer activity. In turn, if some "cancer antigens" are exposed on the cell surface only in cancer cells while being present in normal cells, they could be promising targets of antibody therapy. This study clearly demonstrated that the immunized mice injected with glycolipid fractions from ovarian cancer cell lines had high titers of serum antibodies reactive with surface molecules on the cancer cells, probably glycolipids (Table 1). These results strongly suggested that clones in the blood of immunized mice include B cells generating antibodies toward glycolipid antigens of lacto-/neolacto-series and ganglio-series. The establishment of mAbs using spleen cells from these mice is now on-going.

Not only exposure of glycolipids outside the cell membrane, but their density on the cell surface should be an important issue in determining the accessibility of mAbs. For the cluster formation, a very high density of glycolipids would be required.[15] Moreover, mAbs reactive with glycolipids exhibiting significant roles in cancer cells might be useful in the cancer therapeutics.[16] Further studies on the expression of these antigens in a wide variety of ovarian cancers and anti-cancer effects *in vivo* must be conducted.

In the application of mAbs reactive with cell surface molecules on cancer cells, IgG class mAbs are preferable, since they are easy to purify and possess superior biological functions in immunological actions such as antibody dependent cell mediated cytotoxicity (ADCC). Generally, it is not easy to generate high-affinity IgG antibodies reactive with glycosphingolipids.[10] In particular, ubiquitously expressed glycosphingolipids seemed to be less immunogenic than complex glycolipids such as sialyl-Lewis a and sialyl-Lewis x.[17] In this study, the frequency of acquisition of IgG class mAbs using β3Gn-T5 KO mice was about 50% as described in RESULTS. This is very high for mAbs reactive with carbohydrate antigens as reported so far.[17] Therefore, β3Gn-T5 KO mice might prove suitable to generate functionally useful mAbs whatever their final purposes are.

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