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Efficient generation of useful monoclonal antibodies reactive with globotriaosylceramide using knockout mice lacking Gb3/CD77 synthase

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Abstract Efficient generation of useful monoclonal antibodies (mAbs) with high performance in cancer therapeutics has been expected. Generation of mAbs reactive with globotriaosylceramide (Gb3/CD77) was compared between A/J mice and Gb3/CD77 synthase-deficient (A4GalT-knockout) mice by immunizing Gb3-liposome. Specificity and functions of established antibodies were examined by ELISA, TLC- immunostaining, cytotoxicity of cancer cells and immunoblotting. Compared with results with conventional mice, better generation of mAbs with higher functions has been achieved with A4GalT-knockout mice, *i.e.* acquisition of IgG class antibodies, activities in antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and aggregation activity toward a Burkitt's lymphoma line Ramos. Binding of mAb k52 induced tyrosine phosphorylation of several proteins in Ramos cells. One of the strongest phosphorylation bands turned out to be c-Cbl. Pretreatment of B cell lines with mAbs resulted in the attenuation of BCR-mimicking signaling. All these results suggested that A4GalT-knockout mice are very useful to generate mAbs against globo-series glycolipids, and that suppressive signaling pathway driven by endogenous Gb3-ligand molecules might be present in B cells.

Keywords Monoclonal antibody · Globotriaosylceramide · IgG · ADCC · Glycolipid · Knockout

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Introduction

Monoclonal antibodies (mAbs) are now widely used in various biological experiments, disease diagnosis, and therapy of malignant tumors [1, 2]. Techniques for generation of mAbs have been markedly developed, and various forms of immunogens are now being used to immunize animals for stimulation of immune cells that will be used for fusion with myeloma cell lines. On the other hand, animals to be immunized have been fixed as mice and rats in almost all situations. Theoretically, mutant mice that lack antigen molecules to which mAbs are being generated should be the best as a source of lymphocytes for the generation of hybridomas.

During analyses of gene knockout (KO) mice, mAbs were generated using KO mice of GM2/GD2 synthase gene in our group [3] and others [4]. Anti-GD3 mAbs were also generated very efficiently by our colleagues [5]. Generally, these mAbs showed high specificity to particular gangliosides, and were often biologically functional [3–5]. These

results have indicated that it should be very efficient to immunize mutant mice of glycosylation machineries to generate anti-carbohydrate mAbs with high quality. Thus, we expected that immunization of mutant mice lacking certain lineage of glycolipids might result in efficient production of mAbs toward known and unknown carbohydrate structures deleted in the mutant mice.

Globotriaosylceramide (Gb3)/CD77 is known to be mainly expressed in a subset of germinal center B cells, kidney, small intestine and vascular endothelial cells in human [6] and mice [7], and is also overexpressed in certain tumors such as Burkitt's lymphomas [8] and colon cancers [9, 10]. Although a number of studies reported functions of Gb3/CD77 as a sole receptor for bacterial verotoxins [11], little is known about physiological roles of Gb3/CD77.

In this study, we tried to immunize Gb3/CD77 synthase gene (A4GalT) KO mice with Gb3 to examine whether they respond to Gb3 more efficiently than wild type mice. Consequently, the mutant mice lacking globo-series glycolipids generated more and better mAbs than wild type mice, *i.e.* many IgG class mAbs with functionally useful properties such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and aggregation activity of cancer cells could be established.

Materials and methods

Animals

A/J, BALB/c$^{nu/nu}$ mice were purchased from Japan Charles River. A4GalT-deficient mice were established in our laboratory [11]. All mouse protocols were approved by the committee of Laboratory Animals in Nagoya University Graduate School of Medicine along with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1966). All animals were maintained under specific pathogen-free conditions.

Cell culture

A Multiple myeloma cell line NS-1, a Burkitt lymphoma cell line Ramos used in this study were maintained in RPMI1640 containing 10% fetal calf serum (FCS). cDNA-transfected L cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% FCS and G418 (300 μg/ml). Burkitt's lymphoma cell lines, Ramos, HS-Sultan, Daudi and Raji were obtained from the Japan Health Science Foundation. A4GalT-transfected L cells (LVTR-1) and a vector control line (Lneo-2) were established as described previously [12].

Comparison of antibody titer in serum

To compare antibody titer to Gb3/CD77 in sera between A4GalT-deficient mice and wild type mice, four 6-10-week-old mice from A4GalT-deficient and wild type mice were intravenously immunized with liposome containing Gb3/CD77 glycolipid and other lipids as described below. Antibody titer in sera of the individual mice was determined by enzyme-linked immuno-solvent assay (ELISA) using horseradish peroxidase (HRP)-anti-mouse IgGs (Amersham Biosciences). Isotype specific secondary antibodies, *i.e.* HRP-anti-mouse Igμ (Southern Biotech) for IgM or a mixture of HRP-anti-mouse Igγ1, γ2a, γ2b and γ3 (Southern Biotech) for IgG, respectively, were also used to determine the antibody reaction of each class immunoglobulin.

Generation of mAbs to Gb3/CD77

Three 8-week-old A/J mice were subcutaneously immunized with liposomes containing Gb3/CD77 glycolipid (100 μg) (Larodan AB, Malmo, Sweden), Lipid A (10 μg) (Sigma), cholesterol (0.5 μmol) (Sigma) and dipalmitoylphosphatidylcholine (DPPC) (0.5 μmol) (Funakoshi, Tokyo, Japan) [13] every 2 weeks. Antibody titer in mouse serum was determined using immuno-fluorescence assay (IF). Three days after fifth immunization, splenocytes were obtained and fused with NS-1 myeloma cells using polyethylene glycol. After selection with HAT medium (Sigma, St Louis, MO), antibodies in culture supernatants were examined by IF and/or enzyme-linked immuno-solvent assay (ELISA). Positive clones were served for subcloning with limited dilution, and their specificities were further confirmed by thin layer chromatography (TLC)-immunostaining. In the case of Gb3/CD77 synthase-null mutant mice, eight-week-old mice were subcutaneously immunized with Gb3 in liposomes four times and boosted with intravenous injection from the tail vein. Antibody titer of each mouse was determined with IF assay using anti-mouse IgG conjugated with FITC (Zymed Lab. Inc., SanFrancisco, CA). After fifth immunization, spleen cells were fused with NS-1 as described above. Antibodies in culture supernatants of hybridomas were examined by ELISA and/or IF, and positive clones were subcloned, then their activity was further confirmed with TLC-immunostaining. Antibodies were purified using protein A-sepharose 4 fast flow affinity column according to the manufacturer's protocol (Amersham Biosciences, Bucks, UK).

ELISA

Commercial glycolipids in methanol (20 ng/10 μl) were plated in 60-well Terasaki plates (Greiner BioOne,

Frickenhausen, Germany). After dry up in air for 20 min, the plates were blocked with 5% BSA in PBS for 2 h at room temperature. After washing twice, serially diluted antibodies were added to the plates and then incubated at room temperature for 2 h. After washing five times, horseradish peroxidase (HRP)-anti-mouse IgG (Amersham Biosciences) as a secondary antibody was added. After washing five times, 10 μl of substrate solution [ortho-phenylene diamine (2 mg) (Wako Junyaku, Osaka, Japan) and H_2O_2 (8 μl) (Wako) in 5 ml of citrate-phosphate buffer] was added to the plates. After appropriate time of incubation in the dark, the color development was recorded with a scanner.

IF assay

After plating LVTR-1 or Lneo-2 cells in Terasaki plates at 500 cells/well overnight, the supernatants were removed and serially diluted serum, culture supernatant or ascites were added to the plates and incubated for 1 h at room temperature. After washing twice, anti-mouse IgG (H+L) conjugated with FITC (Cappel, Durham, NC) was added, and plates were incubated for 1 h at room temperature in the dark. After washing twice, antibody binding was observed under a fluorescence microscope (BX51, OLYMPUS, Tokyo, Japan).

TLC-immunostaining

TLC was performed using a high performance TLC plates (Merck, Darmstadt, Germany) using a solvent system of chloroform/methanol/0.22% $CaCl_2$ (60:35:8). The specificity and reactivity for Gb3/CD77 was examined by TLC-immunostaining using an aluminum-backed silica plate (Merck) as described previously [14]. After TLC, the plate was blotted onto polyvinylidene difluoride membrane as described previously. After blocking with 5% BSA in PBS, the plate was incubated with culture supernatant, and then antibody binding was detected with ABC kit™ (Vector Laboratories, Burlingame, CA) and Konica Immunostaining HRP-1000™ (Konica, Tokyo).

Further analyses of mAb specificity with Gb3-related glycolipids

To examine the reactivity of mAbs with α(1-4)-galactosyl-paragloboside (P1 antigen) and iGb3, TLC-immunostaining and flow cytometry were performed, respectively. For P1 antigen, a neutral glycosphingolipid fraction derived from A-blood type human erythrocytes, which contains large amounts of P1 antigen was used in TLC- immunostaining. A solvent system of chloroform/methanol/water (60:35:8) was used for TLC, and separated glycolipids were stained by mAbs using a human mAb HIRO59 (3D4) as a control of anti-P1 Ab [15]. For iGb3, A3GalT2 cDNA was isolated using RT-PCR products from mouse lung cDNAs. A catalytic-dead mutant of A4GalT cDNA was also isolated. Amplified cDNAs of A3GalT2 and catalytic-dead A4GalT were digested with BamHI and EcoRI, then cloned into BamHI and EcoRI sites of a pMXs-GFP retroviral expression vector. They were expressed in L cells. The following primers, 5′-GGG GAT CCG CTC TGG GGA CAG AGT TGG G-3′ and 5′-CCG AAT TCC TAG TTT CGC ACC AGC GTA T-3′ were used for the cloning of mouse A3GalT2. iGb3-expressing stable cell lines were isolated based on negative staining by an anti-LacCer antibody (Huly-M13) using FACS Aria II ™ (Becton-Dickinson, Mountain View, CA). Gb3-expressing stable cell lines were isolated with a similar method. Reactivity of mAbs to iGb3 was analyzed by FACSCaliver™ (Becton Dickinson).

Flow cytometry

Cell surface expression of Gb3/CD77 was analyzed by FACSCaliver™ (Becton Dickinson). Briefly, the cells were incubated with diluted ascites or purified antibodies for 60 min on ice and 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 second antibody alone. For quantification of positive cells, CELLQuest™ program was used.

Proliferation assay and proliferation suppression assay

To examine growth suppression activity of generated antibodies, WST-1 assay was performed by assessing the reduction of WST-1 to formazan based on the absorbance at 450 nm using an ELISA reader Immuno Mini NJ-2300 (System Instruments, Tokyo) as described previously [12]. Briefly, cells (1×10^4) were seeded in 48-well plates and cultured overnight in RPMI1640 containing 10% FCS. Then, diluted ascites or purified antibodies were added to the wells and cultured. WST-1 assay was performed every 24 h by triplicate for Ramos cells. Growth suppression activity of anti-Gb3 mAbs was compared with that of control antibodies.

CDC assay

Cells were once washed with PBS containing 5% FCS to remove RPMI1640 medium, and plated in 48-well plates (1×10^4/50 μl in PBS containing 5% FCS). Fifty μl of PBS containing 5% FCS and various amounts of ascites were added to the individual wells and incubated for 1 h at room temperature. Then, 200 μl of PBS containing 5% FCS and rabbit serum (30 μl:1/10 or 150 μl:1/2) were added to the individual wells and incubated for 1 h at 37°C in a final volume of 300 μl/well. After CDC reaction, cell proliferation reagent WST-1 (Roche, Manheim, Germany) was

added to the individual wells (30 μl/well). After incubation for 2 h, the plates were shaken thoroughly for 1 min and the absorbance of the samples at 450 nm was measured using a microtiter plate reader (ImmunoMini NJ-2300, Immuno Mini NJ-2300, InterMed, Tokyo, Japan).

ADCC activity

Target cells ($1x10^6$) were incubated with diluted ascites in falcon tubes for 1 h on ice, and then washed with RPMI1640 containing 10% FCS twice, and plated in individual wells. Human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient (Amersham Biosciences Inc.) centrifugation from heparinized blood (25 ml) of healthy donors according to the manufacturer's instructions. These cells were added to microtiter plates at the indicated ratios (target/effector) and incubated for 4 h at 37°C. The amounts of LDH released due to ADCC-mediated cell lysis were measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay™ (Promega, Madison, WI) according to the manufacturer's instructions.

Immunoblotting and immunoprecipitation

Cells were lysed with a lysis buffer (20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 5 μg/ml aprotinin). Cell lysate (derived from 4.0×10^6 cells) was applied for SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with PBS containing 5% BSA for 1 h at room temperature, the membrane was incubated with the first antibody for 1 h at room temperature and then washed with PBS containing 0.05% Tween 20 three times, and incubated with HRP-conjugated anti-mouse IgG (H+L) (Zymed Laboratories Inc. San Francisco, CA) for c-Cbl. Detection was performed using an ECL system™ (Amersham Biosciences Inc.) according to the manufacturer's instructions. As for immunoprecipitation, cell lysates (derived from $4x10^6$ cells) were immunoprecipitated by PY20 or polyclonal antibodies to c-Cbl and protein-G sepharose 4 fast flow™ (Amersham Biosciences). After washing the immune complexes, SDS-sample buffer was added and boiled for 3 min.

B cell stimulation with anti-BCR antibody or mAb k52

Ramos cells were resuspended in RPMI1640 containing 10% FCS at a concentration of 10^7/ml. Prior to the stimulation, cells were left for 10 min at 37°C. Cells were then stimulated with goat anti-human IgM (10 μg/ml) (Fc5μ fragment specific, Jackson Immuno-Laboratory, West Grove, PA) or mAb k52 (10 μg/ml) for indicated time.

Results

Higher antibody reaction to immunized Gb3 in A4GalT-deficient mice

To examine whether A4GalT-deficient mice were more responsive to Gb3/CD77 than wild-type mice, we immunized them with Gb3-containing liposomes, and antibody titers were determined using sera from both types of mice.

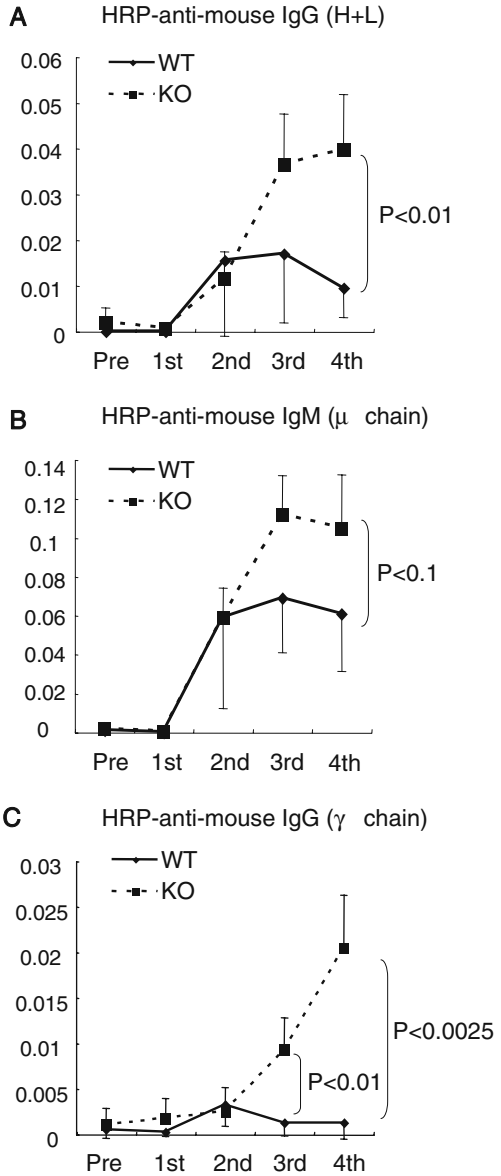


Fig. 1 Comparison of antibody titer to Gb3/CD77 in sera from A4GalT-deficient mice with those from wild type mice. Forty-folds diluted serum titers of antibodies to Gb3 obtained at time points indicated from wild type mice (n=4, solid line) and A4GalT-deficient mice (n=4, dashed line) were determined by ELISA using HRP-anti-mouse IgG (H+L) (**a**), HRP-anti-mouse Igμ (**b**) and a mixture of HRP-anti-mouse Igγ1, γ2a, γ2b and γ3 (**c**)

As expected, we found that the antibody titers to Gb3/CD77 were higher in A4GalT-deficient mice than in wild type mice after 3rd immunization (Fig. 1a). When isotype specific secondary antibodies were used, IgG titer to Gb3/CD77 was significantly high in A4GalT-deficient mice compared to those in wild type mice after 3rd immunization (Fig. 1c), while no significant differences in IgM titer to Gb3/CD77 were observed between two types of mice (Fig. 1b). Taken together, A4GalT-deficient mice were indeed beneficial for production of IgG class antibodies to Gb3/CD77.

Generation of anti-Gb3/CD77 mAbs with a conventional mouse line

First, we immunized wild type A/J mice with Gb3-containing liposome. Out of immunized mice, a mouse, which had the

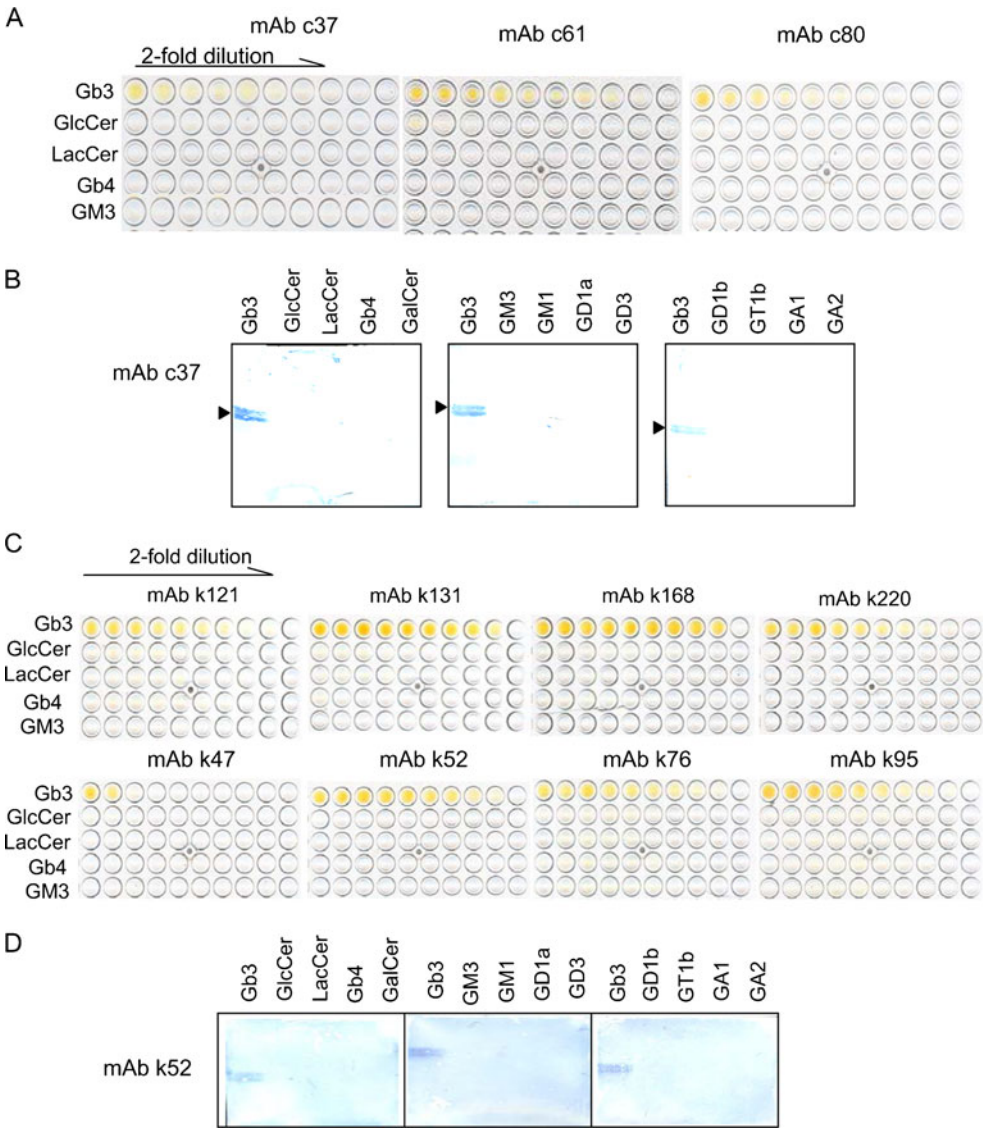


Fig. 2 Specificity and reactivity of mAbs obtained from A/J mice with Gb3/CD77. **a** An example of ELISA performed with obtained mAbs (hybridoma supernatants) with 2-fold dilution toward purified glycolipids (20 ng/well). After washing of supernatants, HRP-conjugated secondary Ab was added and incubated for 1 h. After washing, binding of mAbs was detected by adding the substrate solution. Color development was recorded by a scanner after 10–20 min. **b** Specificity of mAbs obtained from A/J mice with various glycolipids as analyzed by TLC-immunostaining. TLC-immunostaining was performed with obtained mAbs (hybridoma supernatants at 1: 2 dilution) as described under "Materials and methods". TLC was done by a solvent system: C:M:0.22% $CaCl_2$ (60:35:8). Glycolipids (0.5 μg/well) were examined. Antibody binding was detected with ABC complex^{TM} and Konica immunostain-HRP 1000^{TM}. Results of mAb c37 were shown. Results of other mAbs were essentially same. (C, D) Specificity and reactivity of mAbs obtained from Gb3/CD77 synthase KO mice. **c** Specificity and reactivity of mAbs as analyzed by ELISA. ELISA was performed for various glycolipids as in A. **d** TLC-immunostaining was performed as described in B. Results of mAb k52 was shown. Results of other mAbs were essentially same

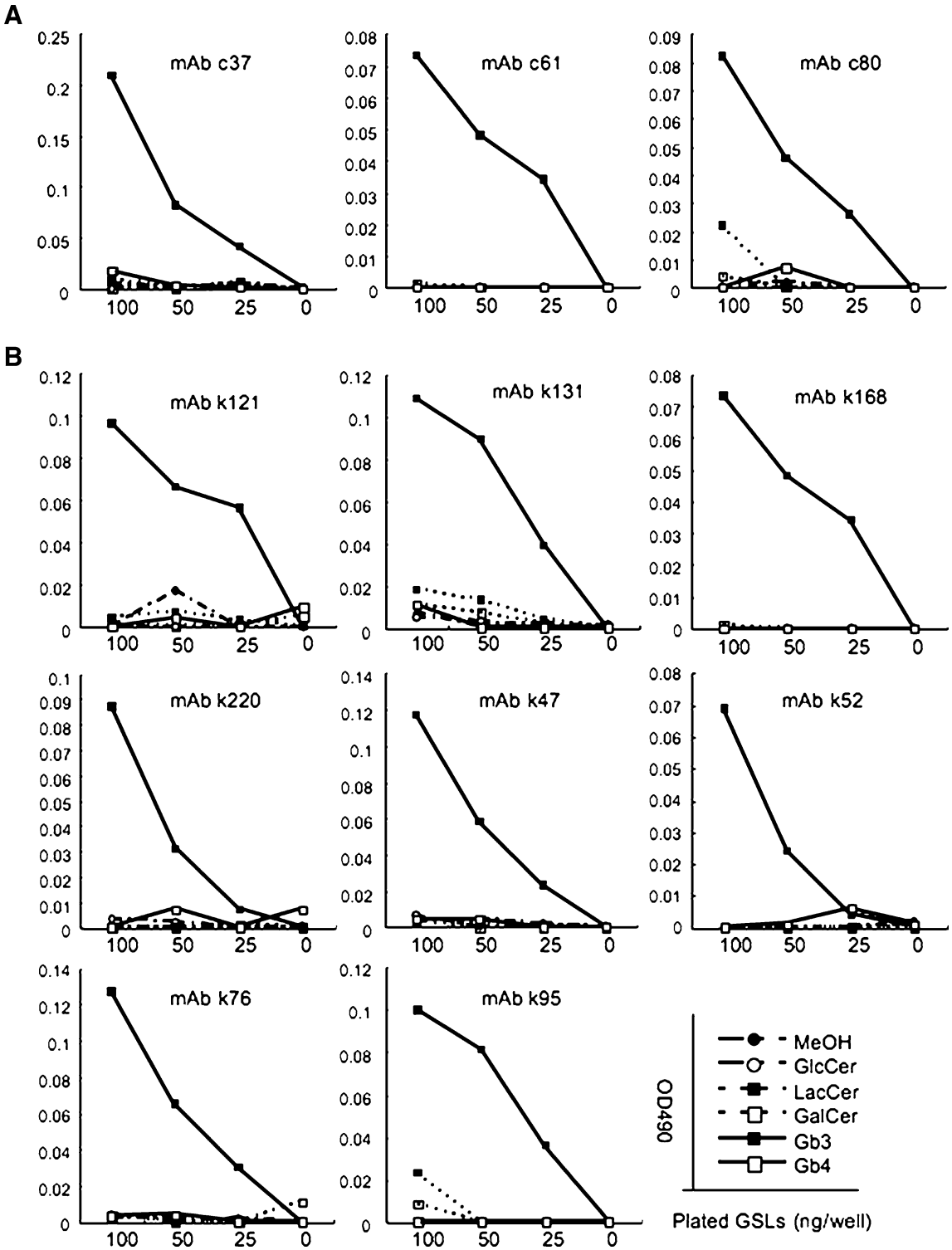


Fig. 3 Reactive patterns of mAbs with serially diluted glycolipids. ELISA was performed with mAbs using serially diluted purified glycolipids (0, 25, 50, 100 ng/well). After washing of mAbs, HRP-conjugated secondary Ab was added and incubated for 1 h. After washing, binding of mAbs was detected by adding the substrate solution. Color development was recorded by an ELISA reader Immuno Mini NJ-2300^{TM} (System Instruments, Tokyo) at 490 nm after 10–20 min. Ascites of hybridomas obtained from wild type mice (**a**) and of those from A4GalT-deficient mice (**b**) were used at the dilution of 1:100. Dashed line/filled circle, MeOH alone; dashed line/open circle, GlcCer; dashed line/filled square, LacCer; dashed line/open square, GalCer; solid line/filled square, Gb3; solid line/open square, Gb4 were fixed on each well

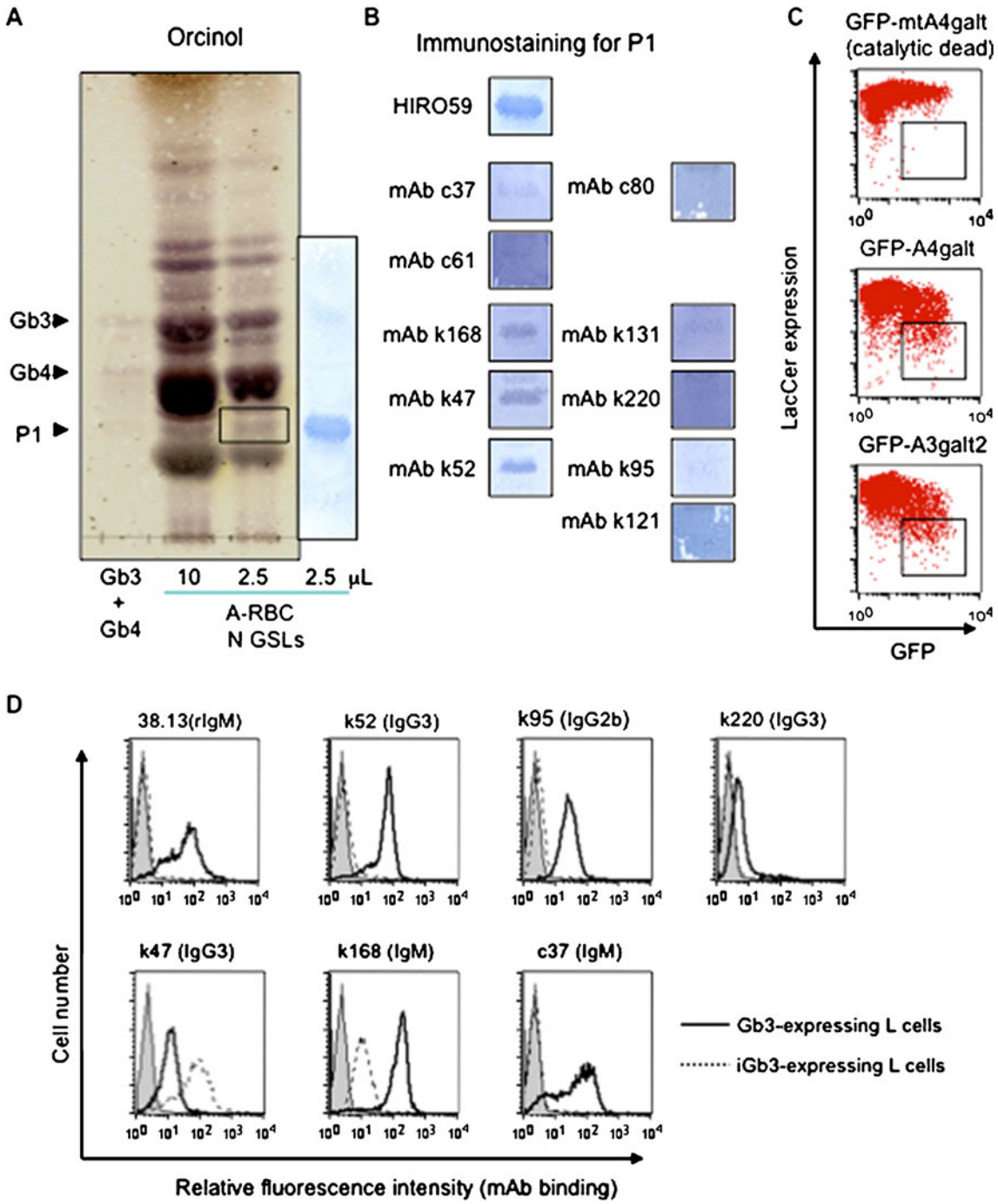
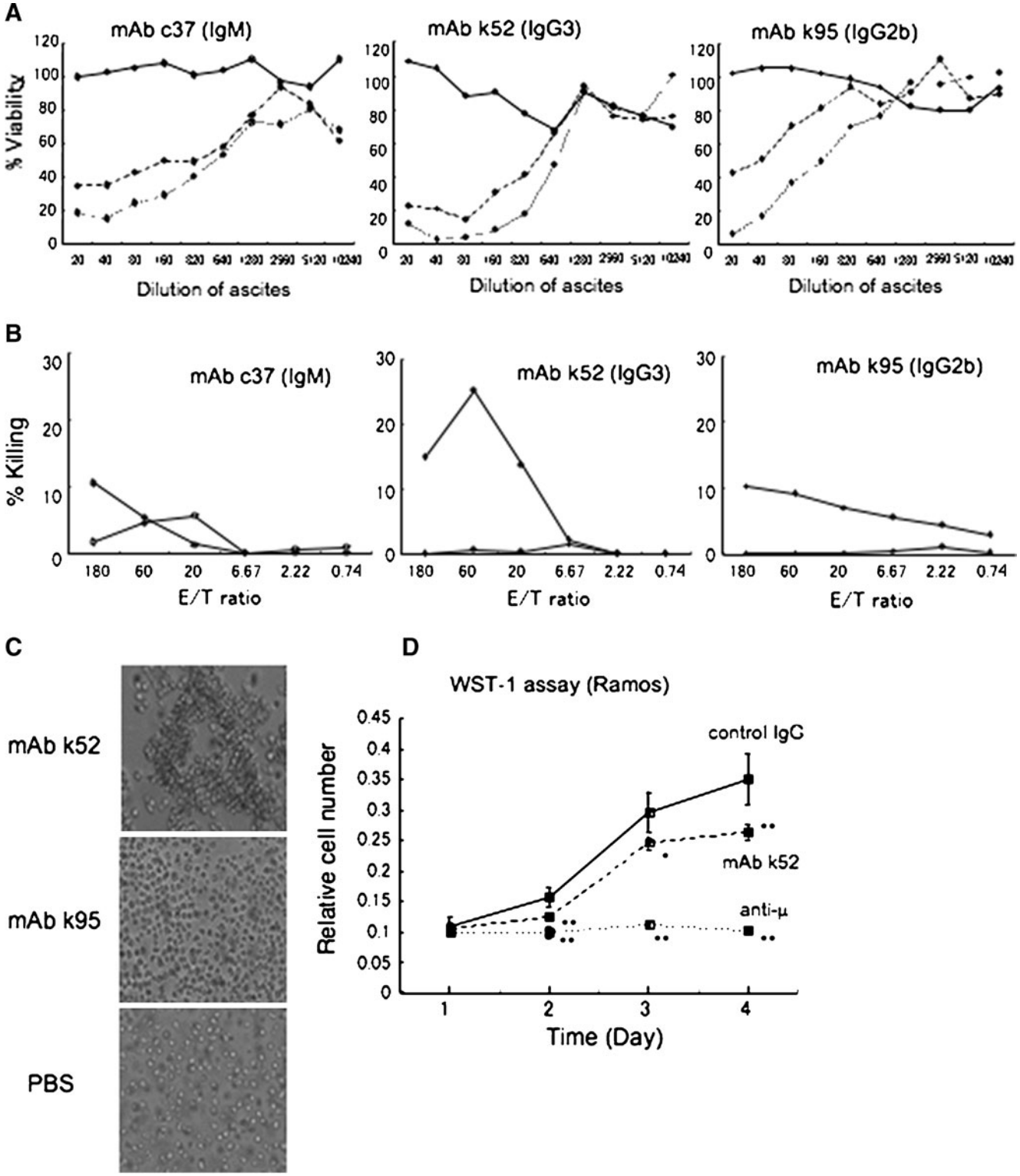


Fig. 4 Further specificity analyses of mAbs with Gb3-related glycolipids. To examine the reactivity of mAbs with neutral glycosphingolipids such as P1 antigen and iGb3. Neutral glycolipids derived from A-blood type human erythrocytes were analyzed by TLC-immunostaining. Separated glycolipids were visualized by orcinol spray (**a**). TLC-immunostaining was performed with obtained mAbs described under "Materials and methods" (**a**, **b**). A human mAb, HIRO59 (3D4) was used for detection of P1 as positive control. To examine the reactivity of mAbs with iGb3, flow cytometry was performed using GFP-A4GalT- and GFP-A3GalT2-expressing cells. The transfectant cells were sorted as a negative fraction stained by an anti-LacCer antibody Huly-M13. GFP-A4GalT catalytic-dead mutant-expressing cells were used as a negative control (**c**). Reactivities of mAbs with cells expressing GFP-A3GalT2 as well as those expressing GFP-A4GalT were analyzed by flow cytometry (**d**) as described under "Materials and methods". MAbs used in this experiments were k52, k95, k220, k47, k168 and c37. A rat mAb 38.13 was used for detection of Gb3 as positive control

highest titer of antibody for Gb3, was used for the donor of splenocytes to be fused with mouse NS-1 myeloma cells. Using ELISA- and IF-based screening, 3 hybridoma clones (c37, c61 and c80) were established after screening 400



clones. The specificity of established mAbs to Gb3/CD77 was determined by ELISA and TLC-immunostaining (Fig. 2a and b). For 13 different glycolipids, all 3 clones exclusively reacted to Gb3/CD77. All 3 clones did not react to lactosylceramide, GlcCer, GM3 or Gb4 (Fig. 2a), suggesting that non-reducing terminal α4-galactose is necessary to react with established antibodies. The remaining glycolipids, *i.e.* GalCer, GD1b, GD3, GA1, GT1b, GA2, GM1 and GD1a

◀**Fig. 5** Anti-tumor activity of mAbs against Ramos cells. **a** Results of CDC. Ramos cells were incubated with an array of obtained mAbs (ascites diluted x20~×10,240) followed by addition of rabbit serum (x2~×10 dilution) as a source of complement, and incubated. CDC activity was determined by WST-1 assay as described in "Materials and methods". Lines used are: —, no serum; ... , serum (1:10 dilution); ... , serum (1:2 dilution). **b** Results of ADCC. Ramos cells were incubated with mAbs c37, k52 or k95, followed by addition of PBMCs, and were incubated for 1 h. ADCC activity was determined by measuring released LDH using CytoTox 96 Non-Radioactive Cytotoxicity Assay™ as described in "Materials and methods". E/T ratio, effector/target ratio. Symbols used are : open circle, control IgG (or IgM) ; closed circle, anti-Gb3/CD77 mAbs. (**c**, **d**) Effects of anti-Gb3/CD77 mAbs on morphology and cell growth of Ramos cells. **c** Ramos cells were incubated with mAb k52, mAb k95 or PBS as a negative control. Cell aggregation occurred only in mAb k52-treated cells by 1 min incubation. **d** Ramos cells (1×10^7 cells /ml) were treated with mAb k52 at 20 μg/ml. WST-1 assay was performed to examine the effects of mAb k52 on the cell growth at the time points indicated. *$P<0.05$, **$P<0.01$ compared with control IgG-treated samples. Similar results were obtained by at least three times repeated experiments. For mAb k220 and mAb k95, similar results were obtained. Anti-lgμ was used at 10 μg/ml to induce apoptosis

were also non-reactive (data not shown). Similar reaction specificity was observed in TLC-immunostaining (Fig. 2b). MAbs c61 and c80 were also reactive exclusively with Gb3 (data not shown). Isotype of 3 clones was determined by ELISA and revealed to be of IgM class (data not shown).

Generation of anti-Gb3/CD77 mAbs using A4GalT-deficient mice

To generate another antibodies against Gb3/CD77 with high performance in terms of anti-tumor activity, we immunized A4GalT-deficient mice lacking all globo-series glycolipids with Gb3-containing liposome as we did for wild type mice. Out of immunized mice, a mouse showing the highest titer for Gb3/CD77 was sacrificed and the splenocytes were fused with mouse NS-1 myeloma cells. Using ELISA- and IF-based screening, 8 hybridoma clones were established with reactivity to Gb3/CD77 after screening 323 clones. For 13 different glycolipids, all clones exclusively reacted to Gb3/CD77 (Fig. 2). Results of ELISA with various glycolipids were shown in Fig. 2c (results for GalCer, GD1b, GD3, GA1, GA2, GT1b, GM1 and GD1a were not shown). The specificity of established mAbs to Gb3/CD77 was also determined by TLC-immunostaining (Fig. 2d). ELISA was performed to determine isotypes of 8 clones and revealed that they were of IgM (4 clones), IgG3 (3 clones) and IgG2b (1 clone) (data not shown). To further confirm the specificity of obtained mAbs, ELISA was performed, in which serially diluted doses of several glycolipids were fixed on ELISA plates. For any dilutions of fixed glycolipids, all mAbs from wild type mice were exclusively bound to Gb3/CD77 (Fig. 3a). mAbs from A4GalT-deficient mice also showed similar specificities (Fig. 3b). Structurally related glycolipids to Gb3/CD77, such as α(1-4)-galactosyl-paragloboside (P1 antigen) and iGb3, were examined for their reactivities with mAbs. As known well, a neutral glycolipid fraction from A blood type human erythrocytes contains high amount of P1 antigen. As shown in Fig. 4a, HIRO59 (3D4) specifically bound to P1 antigen. In TLC-immunostaining with mAbs, about half of obtained mAbs were reactive with P1 antigen more or less (Fig. 4b). Thus, mAbs from KO mice were not necessarily more specific to Gb3/CD77 than those from wild type. To examine whether mAbs bind to iGb3, iGb3 synthase-expressing cells were established from L cells (Fig. 4c). After transfection of cDNA of A3GalT2 or A4GalT into L cells, respectively, binding of anti-LacCer antibody to those cells was gradually decreased accompanied by appearance of GFP signal in both types of cells. On the other hand, transfection of cDNA of catalytic mutant of A4GalT showed no differences in the binding of an anti-LacCer antibody between GFP-negative and positive population, indicating that the decrease of binding of the anti-LacCer antibody found in A3GalT2-transfected GFP-positive cells depends on their catalytic activity and conversion of LacCer to iGb3. After cell sorting of this fraction (gated fraction found in Fig. 4c), verotoxin sensitivity in those cells were examined, showing that A3GalT2-expressing cells were not killed by verotoxin (data not shown), although A4GalT-expressing cells were easily killed by verotoxin. Therefore, we concluded that A3GalT2-expressing cells were presumably positive for iGb3, and then those cells were used for specificity analyses. In contrast to the case of P1 antigen, almost all mAbs specifically bound to Gb3/CD77 but not to iGb3. Interestingly, mAb k47 bound to iGb3 more strongly than to Gb3 (Fig. 4d).

Anti-tumor activities of mAbs reactive with Gb3/CD77

To assess anti-tumor activities of these clones, we examined CDC activity of them using a Burkitt's lymphoma, Ramos as a target cell. All three clones with IgM class obtained from A/J mice splenocytes and an IgM class anti-Gb3 mAb from A4GalT-KO showed strong CDC activity, while antibody alone did not induce cell lysis (Fig. 5a). Although 4 IgG class antibodies obtained from A4GalT-KO mice also induced obvious CDC, the intensities were weaker than those of IgM type mAbs. Then, we examined ADCC activity of these mAbs. MAb k52 definitely induced ADCC for Ramos cells using human peripheral mononuclear cells (PBMCs) as effecter cells (Fig. 5b). IgM or IgG2b class mAbs showed no or minimal ADCC activities (Fig. 5b).

Biological effects of binding of mAbs on B cell lines

Since Gb3/CD77 was expressed in a subset of germinal center B lymphocytes and it might be required in the process of antibody affinity maturation, we speculated that unknown ligands for Gb3/CD77 might exist and induce signals in germinal center B cells. To test this hypothesis, we treated Ramos cells with mAbs and examined morphological changes or intracellular protein phosphorylation. Among clones tested, mAb k52 induced cell aggregation in a calcium dependent manner, whereas mAb k95 did not (Fig. 5c). MAb k52 also showed a mild but significant growth suppression activity against Ramos cells (Fig. 5d).

Binding of anti-Gb3 mAb induced tyrosine-phosphorylation in Ramos cells

Immunoblotting of phosphorylated proteins using cells after addition of these mAbs revealed that drastic changes in intracellular protein phosphorylation patterns occurred (Fig. 6a). These changes were also observed in mAb k220 (IgG3)-treated cells (data not shown), while phosphorylation levels were less than those by mAb k52. Since mAb k220 did not induce cell aggregation, the phosphorylation by mAb k52 might not be mediated by cell aggregation. Furthermore, anti-Gb3 mAbs with IgG3 isotype were not necessarily capable to induce phosphorylation (data not shown). When compared to the BCR-mediated signaling via an anti-μ-specific antibody, the protein phosphorylation patterns caused by mAb k52 were similar to those by anti-BCR crosslinking with some differences (Fig. 6b). Among several phosphorylated proteins, a 120 kDa protein seemed to be a common phosphorylated molecule in both types of stimulation (Fig. 6b). As described above, mAb k52 showed a mild but significant growth suppression activity against Ramos cells, suggesting that Gb3/CD77-mediated signals might introduce negative effects on pre-existing signals such as BCR-mediated signals or survival signals. To identify the 120 kDa band as c-Cbl [16] , and to clarify whether c-Cbl might be phosphorylated due to the ligation of Gb3/CD77 with mAb k52, immunoprecipitation (IP)-immunoblotting (IB)-analysis was performed. The results demonstrated that the 120 kDa band was, at least partly, c-Cbl, and it was phosphorylated by the ligation of Gb3/CD77 with mAb k52 (Fig. 6c).

Implication of Gb3-mediated signals in the signaling of B cells

To test whether Gb3/CD77 might play a role as a negative regulator of BCR signaling, we examined the effects of pretreatment of Ramos cells with mAb k52 before addition of an anti-μ-antibody on the protein phosphorylation patterns. As expected, pretreatment with mAb k52 significantly suppressed the protein phosphorylation mediated by an anti-μ-antibody compared to that with control IgG (Fig. 7a and b).

Discussion

Since Kohler and Milstein established methods for generation of hybridomas to produce mAbs [17], monoclonal techniques have been dramatically distributed and being used widely in various fields of biology in all over the world. Not only B cell hybridomas, but also T cell hybridomas have also been developed, and been utilized in a wide range of immunological analyses [2]. MAbs have been one of the most useful technologies to promote modern biology and medical researches [1]. In the processes for generation of mAbs, immunization of animals and screening of hybridomas have been the most critical steps. For the diagnosis of diseases and immunotherapy of cancers, specificity in the antibody binding and biological functions of antibodies including binding affinity, CDC and ADCC are very critical. To improve the efficiency of immunization, various trials for the preparation of immunogens have been performed, *i.e.* conjugation to haptens, addition of adjuvants to antigens, inclusion into liposomes, and usage of peptides determined from topographic structures of individual proteins. However, no particular mice for efficient generation of mAbs have been reported.

To generate mAbs reactive with glycosphingolipids, injection of tumor cells into conventional mice have been a popular way of immunization as experienced in many laboratories. Immortalization of B cells with Epstein-Barr-virus was efficient [18, 19] particularly for generation of human mAbs due to lack of useful fusion-partner cell lines. Purified or recombinant proteins were, then, widely used for the production of mAbs. Peptides derived from various functional molecules have also been used as immunogens for various bio-molecules. To generate a useful array of mAbs against gangliosides, purified glycosphingolipids were used for immunization [20–22], using some particular lines of mice based on the results of the past studies [23].

To generate anti-ganglioside mAbs, utilization of mutant mice lacking complex gangliosides have been tried, and showed excellent antibody production [4]. We also reported production of useful mAbs reactive with GD1a [3] or GD3 and GD1b [5] using GM2/GD2 synthase KO or GD3 synthase KO mice, respectively. In these cases, anti-ganglioside mAbs showed better specificity and binding affinity toward structures that were deficient in the mutant mice used for the immunization. However, there have been no reports on the utilization of KO mice

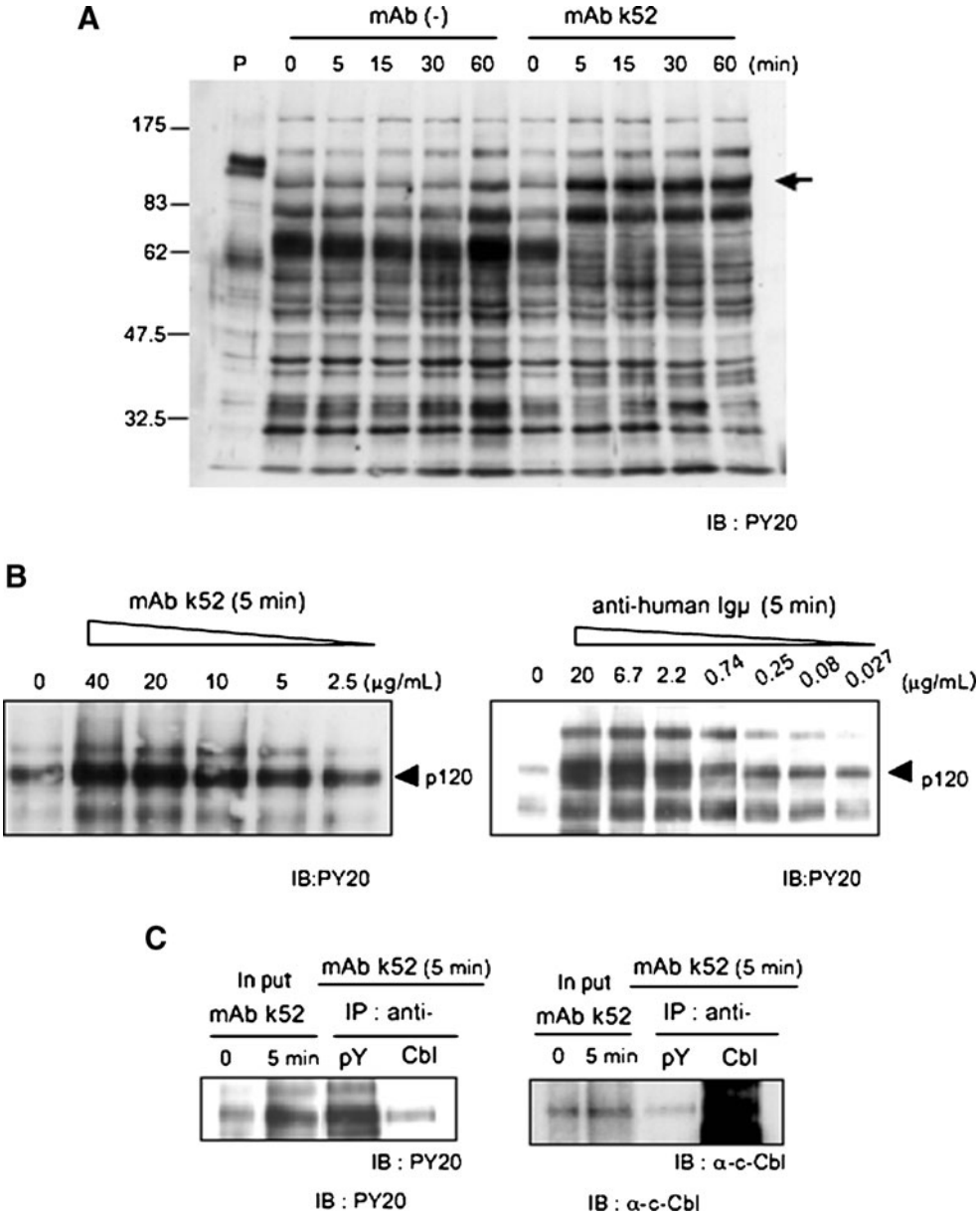


Fig. 6 Tyrosine phosphorylation of proteins induced by mAb k52. **a** Tyrosine phosphorylation induced by mAb k52 was analyzed. Ramos cells (10^7 cells /ml) were stimulated with mAb k52 (20 μg/ml) or mAb k220 (20 μg/ml) at 37°C for indicated time. Cell lysates were prepared and analyzed with immunoblotting using PY20. Results with mAb k52 were shown. **b** Tyrosine phosphorylated proteins induced by mAb k52 were similar to those by an anti-μ Ab. Ramos cells were incubated in the presence of mAb k52 (0~40 μg/ml) for 5 min, and the lysate was applied for immunoblotting with PY20. Tyrosine-phosphorylated proteins in Ramos by treatment with an anti-μ Ab (0~20 μg/ml) were also analyzed. **c** Identification of p120 as c-Cbl. Immunoprecipitation was performed with PY20 or an anti-c-Cbl antibody after the treatment with mAb k52 for 5 min. Then, the immunoprecipitates were analyzed with immunoblotting using HRP-PY20 (left) or anti-c-Cbl antibody (right) combined with HRP-conjugated anti-rabbit IgG. In put showed immunoblotting with lysates of Ramos before or after the treatment with mAb k52 for 5 min using PY20 or an anti-c-Cbl antibody

lacking neutral glycosphingolipids such as globo-series glycolipids.

In this study, highly efficient generation of anti-Gb3 mAbs was demonstrated when A4GalT-KO mice were used for immunization. The efficiency of antibody generation and quality of functions of mAbs produced were apparently better when compared with mAbs raised in conventional mice. In a previous trial, they reported exclusive production of IgM mAbs using purified Gb3 glycolipids as antigens [24]. In this study, IgG-class mAbs reactive with Gb3 were obtained with relatively higher rate, although IgM class mAbs are usually dominant in anti-glycolipid mAbs with current methods. This was also the case in our studies.

Gb3 is a starting molecule for the synthesis of all globo-series glycosphingolipids, and have been paid attention by researchers as a Burkitt's tumor marker [8], a verotoxin specific receptor [11, 25] or a cell death marker of B-cell lineage [26]. Roles of globo-series glycosphingolipids in physiological and pathological situations are now on-going in our laboratory using the mutant mice lacking globo-series glycolipids [11]. Consequently, anti-Gb3 mAbs with various biological functions should be very useful in these functional studies of glycosphingolipids.

In terms of biological functions of mAbs, anti-Gb3 mAbs obtained in this study were very interesting, since some of them appeared to cause activation of B cell signaling pathway when bound to the cell surface in a

similar manner to that of anti-μ cross-linking [27]. There were several components undergoing tyrosine-phosphorylation after treatment with mAb k52, including c-Cbl. c-Cbl has been known as an E3 ubiquitin ligase [28] and to attenuate BCR-mediated signaling by leading molecules such as Syk to degradation by the proteasome system [29]. In the analyses of roles of Gb3/anti-Gb3 mAb-mediated signals in BCR-mediated signals, pretreatment of Ramos cells with mAb k52 resulted in the mild attenuation of levels of tyrosine-phosphorylation triggered by an anti-μ antibody. This result indicated that c-Cbl activation actually plays roles for regulation of BCR-mediated signals. Therefore, most intriguing issue here should be whether endogenous ligands reactive with Gb3 exist, and what they are?

Since Gb3/CD77 was expressed in a subset of germinal center B lymphocytes, and binding of a mAb triggered cell aggregation and tyrosine-phosphorylation of proteins in Burkitt's lymphoma cells, we speculated that endogenous ligands for Gb3/CD77 might exist and regulate signaling in germinal center B cells.

The fact that pretreatment of Ramos cells with mAbs resulted in significant growth suppression supported our speculation that Gb3/CD77 might mediate signals with negative effects on pre-existing signals such as BCR signaling by BCR ligation. It was well known that c-Cbl, E3 ubiquitin ligase is negative regulator of BCR signals [29, 30] based on ubiquitin-proteasome mediated degradation of the signaling molecules located upstream involved in the transduction of BCR-mediated signals such as Syk, SFK or Tec. They are phosphorylated when activated through BCR ligation. As shown in this study, c-Cbl was phosphorylated by mAb k52, and pretreatment of Ramos with mAb k52 caused reduced cell growth as well as attenuation of BCR-mediated signals. These results might link to the general functions of c-Cbl in the development [31] and significances in pathogenesis [32].

It was also well known that BCR complex was translocated from non-lipid raft to the lipid raft after BCR ligation [33, 34] with cognate antigens or an anti-μ-antibody (Supplemental Fig. S1). As Gb3/CD77 also located in lipid raft, we hypothesized that Gb3/CD77 might play a role as a negative regulator of BCR signaling at lipid rafts [35]. Actually, pretreatment with mAb k52 significantly suppressed the phosphorylation mediated by an anti-μ-antibody. Furthermore, binding of mAb k52 induced translocation of BCR to the lipid rafts in Ramos cells (Supplemental Fig. S1), suggesting that translocation of BCR from non-lipid rafts to lipid rafts might mean not only efficient transduction of BCR-signals, but initiation of negative signaling such as ubiquitin-proteasome-mediated degradation or dephosphorylation of key molecules, being regulated by Gb3/CD77-mediated signals.

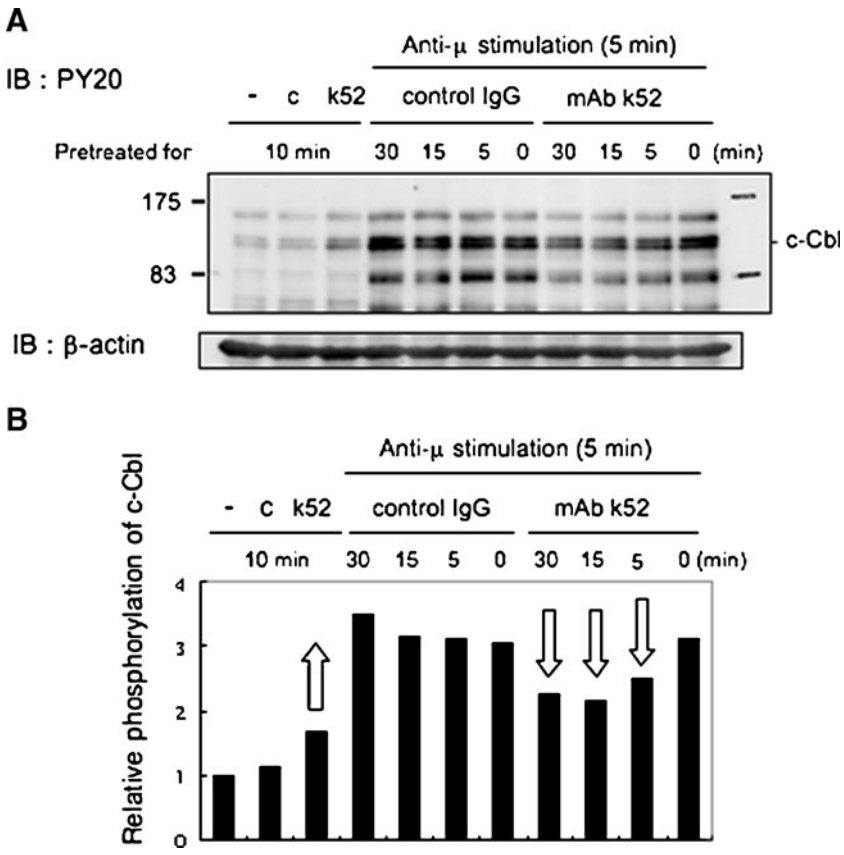


Fig. 7 Effects of pretreatment with mAb k52 on the protein phosphorylation pattern mediated with an anti-μ antibody. **a** Western immunoblotting of lysates. Ramos cells were pretreated with mAb k52 or control IgG for times indicated, then incubated in the presence of an anti-μ antibody (0.5 μg/ml) for 5 min. Lysates were prepared and applied for immunoblotting with PY20. Ramos lysates treated with none (−), control IgG (indicated as c) or mAb k52 for 10 min were used as controls. **b** Relative intensity of bands of p120 in A was measured using NIH image 1.61 and plotted after correction with that of β-actin. Similar results were obtained in repeated experiments

It is not clear at this moment why only mAb k52, among the obtained antibodies, showed such a significant effect on cell morphology, ADCC, phosphorylation and signaling. It might not be due to its Ig isotype, nor its affinity. It may be possible that fine structures recognized by individual mAbs are different, and those by mAb k52 are somewhat unique. The cross-reaction of mAb k52 with P1 antigen as shown in Fig. 4b, might induce some change in the cell membrane. Relevance of fine specificities of the mAbs to their functional effects remains to be investigated.

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There is no conflict of interest.

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