Glycoconjugate Journal 18, 347–353, 2001

Prominent immunogenicity of monosialosyl galactosylgloboside, carrying a stage-specific embryonic antigen-4 (SSEA-4) epitope in the ACHN human renal tubular cell line—a simple method for producing monoclonal antibodies against detergent-insoluble microdomains/raft

Yohko U. Katagiri[1], Kazuhiro Ohmi[1], Chihiro Katagiri[2], Takaomi Sekino[1], Hideki Nakajima[1], Tomohiko Ebata[1], Nobutaka Kiyokawa[1] and Junichiro Fujimoto*[1]

[1]*Department of Pathology, National Children's Medical Research Center, 3-35-31, Taisido, Setagaya-Ku, Tokyo 154-8509*
[2]*Biochemistry Laboratory, Institute of Low Temperature Science, Hokkaido University, N19W8, Kita-Ku, Sapporo 060-0819*

The binding of Shiga toxin (Stx) to Gb3Cer† in detergent-insoluble microdomains (DIM)/raft of the ACHN human renal tubular cell line causes the temporal activation of the Src-family kinase Yes [1]. As a strategy for examining signaling mechanisms in DIM/raft, monoclonal antibodies (MAbs) are reliable tools for characterizing the constituent molecules in these microdomains. Thus, we employed DIM/raft suspensions of ACHN cells as an immunogen to develop MAbs. Simply subcutaneous injections of ACHN DIM/raft could elevate the serum titer after several boosts. The first screening was performed using dot-blot immunostaining with culture supernatants on a polyvinylidene difluoride (PVDF) membrane, on which DIM/raft or their chloroform/methanol (C/M) (2:1, v/v) extracts were dot-blotted. The next screening was performed by flowcytometric analysis of ACHN cells treated with or without a permeabilizing reagent. Many of the clones (21/31 clones = 68%) thus obtained were also found to recognize to lipid fractions of the DIM/raft. Strikingly, all of the 21 clones that reacted to the lipid fraction were found to recognize monosialosyl galactosylgloboside (MSGG) or GL7, which carries the SSEA-4 epitope. Using DIM/raft as immunogens may enable us to easily obtain MAbs for glycolipids.

Keywords: detergent-insoluble microdomain (DIM), raft, monoclonal antibody, monosialosyl galactosylgloboside, stage-specific embryonic antigen-4 (SSEA-4)

Introduction

Researches on DIM/raft signaling microdomains [2] have recently undergone significant advances in various fields [3,4]. Rafts are lateral assemblies of glycosphingolipids (GSL) and cholesterol that associate with specific proteins. These rafts are viewed as platforms that serve as scaffolds to facilitate apical sorting or the association of signaling molecules, increase the rate of interactions, and enhance crosstalk networks. We have already demonstrated that the binding of Stx, an enterotoxin produced by *Shigella dysenteriae* serotype 1 and enterohemorrhagic *Escherichia coli*, to Gb3Cer in DIM/rafts causes the temporal activation of the Src family kinase Yes in the ACHN human renal tubular cell line [1]. To study the downstream signaling mechanism after Stx binding to Gb3Cer, it is critically important to characterize the constituent molecules in these microdomains. For this purpose, we attempted to produce MAbs against DIM/raft molecules.

In the present study, we report a simple and efficient method for producing MAbs against DIM/raft. DIM/raft with sufficient quantity and quality can be easily obtained by sucrose stepwise density-gradient centrifugation of Triton

*To whom all correspondence should be addressed: Junichiro Fujimoto, Department of Pathology, National Children's Medical Research Center, 3-35-31, Taisido, Setagaya-Ku, Tokyo 154-8509. Tel. and Fax: +81-3-3487-9669; E-mail: jfujimoto@nch.go.jp
†Glycosphingolipids are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature [19].

X-100 lysate. Since DIM/rafts are membranous fractions, oil droplet and liposome preparations are not necessary.

In addition, all the clones that reacted to lipid fractions of DIM/rafts also recognized MSGG, which is highly found in human renal cancer and deeply involved in metastasis [5]. This simple method enables a new approach for creating MAbs against glycolipids.

Materials and methods

Cell culture, MAbs and chemical reagents

ACHN, a human renal tubular cell line, was purchased from the American Type Culture Collection and maintained in Eagle's MEM (Sigma Chem. Co., St Louis, MO) supplemented with nonessential amino acids supplements (Gibco Laboratories, Grand Island, NY) and 10% fetal bovine serum (Sigma). P3U1, mouse myeloma cell line obtained from the American Type Culture Collection was maintained in RPMI medium (Sigma) supplemented with 10% fetal bovine serum. RM1and RM2 [6], MAbs for MSGG and disialosyl galactosylgloboside (DSGG), respectively, were kindly donated by Dr. Saito, S. of Tohoku University, Japan. Y111-13 and EpB5, MAbs for SSEA-3 and SSEA-4, respectively, were presented to us by Dr. Kannagi, R. of the Aichi Cancer Center, Japan. The GSL mixtures used as standards for the thin-layer chromatography (TLC) were purchased from Matreya, Inc., PA.

Preparation of DIM/raft microdomains

DIM/raft microdomains of ACHN cells were prepared as described by Katagiri *et al.* [1], with a slight modification. Briefly, confluent cultures of ACHN cells in a 15 cm culture-plate were lyzed in 1.5 ml of Tris-buffered saline (25 mM Tris-HCl buffer, pH 7.5/0.15 M NaCl) containing 1% TritonX-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and aprotinin (10 trypsin inhibitor units/ml) and homogenized using a Teflon glass homogenizer. Cell extracts were adjusted to 40% sucrose in Tris-buffered saline and placed in a 12-ml ultracentrifuging tube. A discontinuous 5–30% sucrose gradient was formed above the sample by layering onto 4 ml of 30% sucrose solution, followed by 4 ml 5% sucrose solution. Both were made in Tris-buffered saline. DIM/rafts were recovered as visible bands at the interphase between 5% and 30% sucrose solutions after centrifugation at 39,000 rpm for 18 h at 4°C in a Beckman SW 40Ti rotor. After several washes with PBS in a microcentrifuge at 15,000 rpm for 15 min, DIM/raft recovered from two 15 cm-plate cultures were used as an immunogen. DIM/raft injected per one immunization consisted of 12 μg proteins and 183 μg lipids/glycolipids, determined by BIO-RAD protein assay kit and an Iatroscanner TH-10 [Iatron Laboratories, Japan, reference 7 and 8], respectively.

Production of MAbs

Immunization was performed using a subcutaneous injection of DIM/raft suspension in PBS into 6 week-old Balb/c female mice, followed by four booster shots at 10-day intervals. Seven days later after the final injection, 38% polyethylene glycol HYBRI-MAX (Sigma) was used to fuse the spleen cells with the P3U1 mouse myeloma cell line, which had been cultured in the S-clone cloning medium CM-B (Sanko-Jyunyaku, Tokyo). Hybrid cells were selected in CM-B containing hypoxanthine, aminopterine, and thymidine (Sigma). After 2–3 weeks of incubation, the culture supernatant of the hybrid cells was screened, first by dot-blot immunostaining assay and next by flowcytometric analysis, as described below. The hybridoma clones whose culture supernatants were positive for DIM/raft were then selected and subcloned by the limiting dilution method.

Dot-blot immunostaining assay

DIM/rafts prepared from a 15 cm-plate culture were suspended in 2 ml of PBS and dot-blotted onto a PVDF membrane (Millipore Corp., Bedford, MA) at 10 μl (= ca. 60 ng protein) per dot. To screen for clones that recognized lipid, the C/M extract of the DIM/raft was dot-blotted. The dots were fenced off from each other with a waterproof pen (DAKO, A/S, Denmark). The membrane was blocked with 5% skimmed milk in PBS and washed with PBS containing 0.025% Tween 20 (Sigma) until the membrane was entirely wet. The fenced dots were probed with 20 μl of each culture supernatant for 1 h at room temperature in a moisture chamber. After washing for 1 h with 3 changes of wash solution, the membranes were treated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) at a 1:2000 dilution ratio and visualized with enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech. UK Ltd., Buckinghamshire).

Flowcytometric analysis

ACHN cells were harvested and incubated with each culture supernatant before and after treatment with Cytofix/Cytoperm (Pharmingen, San Diego, CA). These cells were subsequently stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Laboratory, West Grove, PA) at 1:50 dilution ratio and analyzed by flow cytometer (EPICS-XL, Beckman Coulter).

TLC immunostaining

C/M extracts of DIM/raft were separated on plates precoated with Silica Gel 60 (HPTLC plate, Merck, Darmstadt). The developing solvent system was a mixture of chloroform/methanol/0.5% $CaCl_2$ (5:4:1, v/v/v). TLC blotting was performed according to Taki *et al.* [9]. The immunostaining was done as described above.

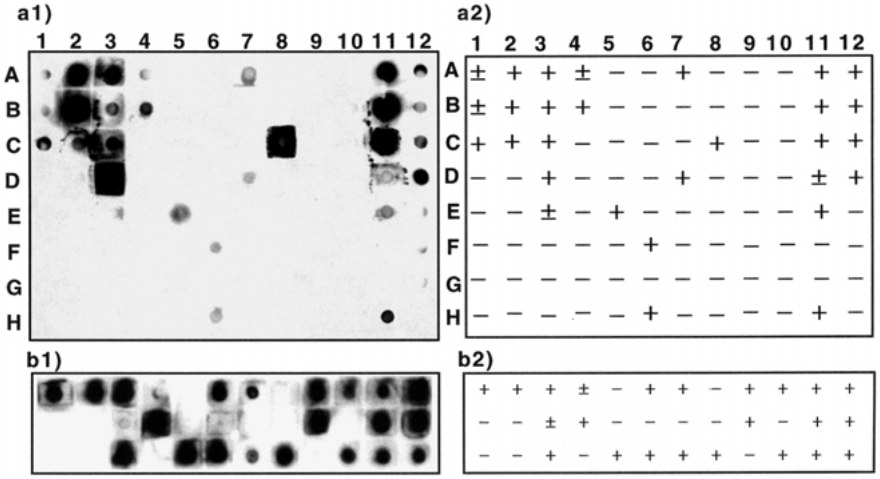

Figure 1. Detection of hybridoma clones that reacted with DIM/raft (a) and its C/M extracts (b). a1,b1) X-ray film after a several-second exposure to an immunostained PVDF membrane. a2,b2) Evaluation of the X-ray film. +; positive, −; negative, ±; not determined.

Separation of raft lipid components with two-dimensional (2-D) TLC

2-D TLC was carried out according to the method of Bouhours *et al.* [10]. The first chromatographic run was performed as described above. The second run was performed using chloroform/methanol/2.5 N NH_4OH (5:4:1, v/v/v) at a rotation of 90° from the first direction. The plate was sprayed with 0.03% primuline (Aldrich, Chem. Co. Inc., Milwaukee, WI) in acetone/water (4:1, v/v) and photographed under UV light. After the lipids were marked and transferred to PVDF membranes, the marked spots were cut-off and extracted in 100 µl of methanol, as described by Isobe *et al.* [11].

Quantification of MSGG present in DIM/raft

The amount of MSGG was determined from TLC plates by densitometric scanning and comparison with GM1[3] after Resorcinol staining.

Results

Production of the Hybridomas

The dot-blot immunostaining system used for the first screening in this study enabled the positive and negative clones to be clearly discriminated. Whether or not the culture supernatants were reactive to the DIM/raft was evaluated using an X-ray film visualized with the ECL detection system. In an example shown in Figure 1a, several wells were evaluated as positive. A fusion of P3U1 mouse myeloma cells with the spleen cells of mice immunized against the DIM/raft of ACHN cells resulted in 8.0% of the hybrid cells testing positive in the first assay after fusion. Twenty-one clones also bound to C/M extracts of DIM/raft (Figure 1b), showing that these antigens are highly hydrophobic or lipids. Further examination of these positive wells with flowcytometry confirmed the 31 clones of MAbs recognizing DIM/raft. Twenty-eight clones were reactive to intact ACHN cells, whereas 3 clones were definitely reactive to permeabilized cells after treatment with Cytofix/Cytoperm. Figure 2 shows two representative clones (Clone #1 and Clone #2) that were reactive to intact ACHN cells and one representative clone (Clone #3) that was reactive to permeabilized ACHN cells. Clone #1 in Figure 2a reacted to C/M extracts, whereas Clone #2 did not.

Raft2 was one of the subclones, which were obtained from Clone #1 by the limiting dilution method. Figure 3 shows flowcytometric analysis and TLC immunostaining with Raft2. None of the standard glycolipids simultaneously developed were stained with Raft2.

Identification of antigenic lipid by immunostaining

ACHN cells express a large amount of globo-series GSL and some of them are sialylated Gb5Cer, or MSGG [12]. We tried to identify the antigenic lipid by immunostaining with MAbs against Gb5Cer, MSGG and DSGG. Five lanes shown in Figure 4 contained equal amounts of C/M extracts of ACHN DIM. The Rf value of the Raft2-reacting glycolipid (lane 1) was the same as that of RM1 (lane 2) and EpB5 (lane 5), both of which recognize SSEA-4 antigen, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 3Galα1 → 4 Gal1β → 4Glc, *i.e.* sugar chain of MSGG. A clear band was seen in lanes 2, 4 and 5, but not in lane 3. These results suggest that ACHN cells express Gb5Cer, but not DSGG, as described by Saito [12].

2-D TLC was successfully used to separate the C/M extracts into several spots (Figure 5a). Spots #1 ~ #15 were stained with Orcinol-sulfuric reagent and both spots of #5 and #6 were also stained with Resorcinol-HCl reagent (data not shown).

[3]Gangliosides are abbreviated according to Svennerholm [20].

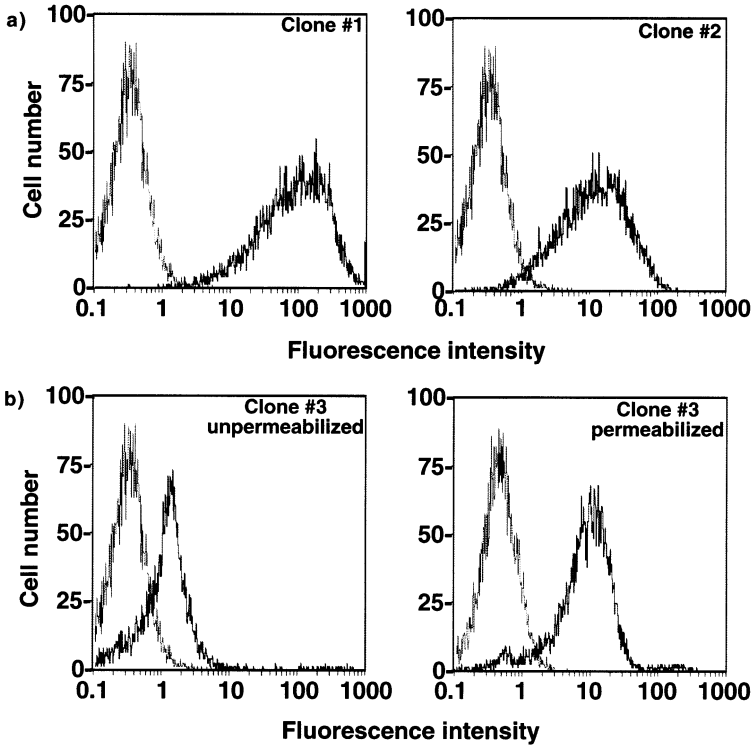

Figure 2. Flowcytometric analysis of culture supernatants that tested positive for DIM/raft by dot-blot immunostaining. - - - -; negative control (non-immune serum), - - - -; culture supernatant. a) clones reactive to intact ACHN cells. b) clones reactive to permeabilized ACHN cells. left; unpermeabilized, right; permeabilized.

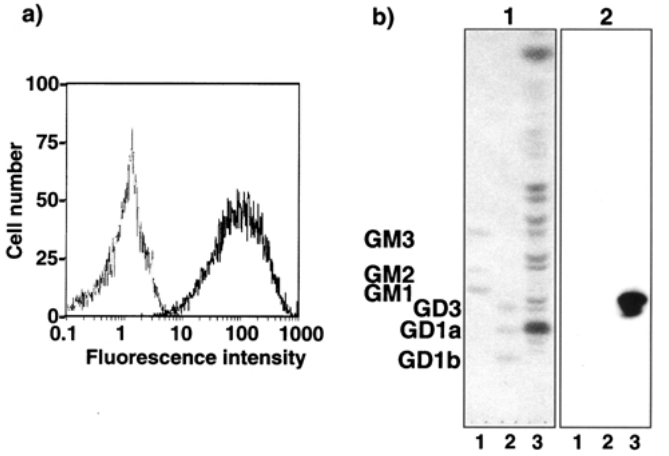

Figure 3. Flowcytometric analysis and TLC immunostaining with Raft2. a) see legend for Figure 2. b) Duplicates of standard gangliosides (1 μg each, lane 1 and 2) and C/M extract of the raft microdomains from 10^7 cells (lane 3) were separated on TLC. After developing, the TLC plate was cut into two pieces, "b1" and "b2". b1) sprayed with Orcinol-H_2SO_4 reagent. b2) TLC blotted to a PVDF membrane and immunostained with Raft2. From top to bottom, the bands of lane 1 correspond to GM3, GM2, and GM1; lane 2 bands correspond to GD3, GD1a, and GD1b. The amount of C/M extract spotted on "b2" was 1/100 of that on "b1".

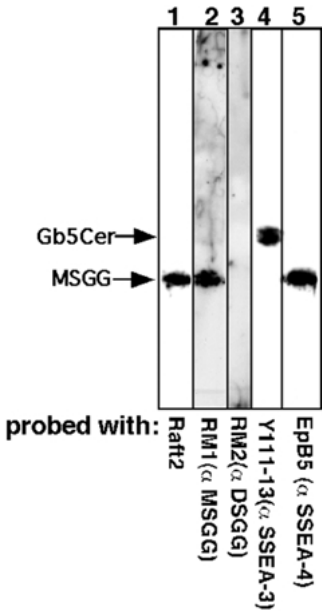

Figure 4. TLC immunostaining with MAbs recognizing SSEA-4. Each strip, where equal amounts of C/M extracts were separated by TLC and blotted, was probed with Raft2, RM1, RM2, Y111-13, and EpB5.

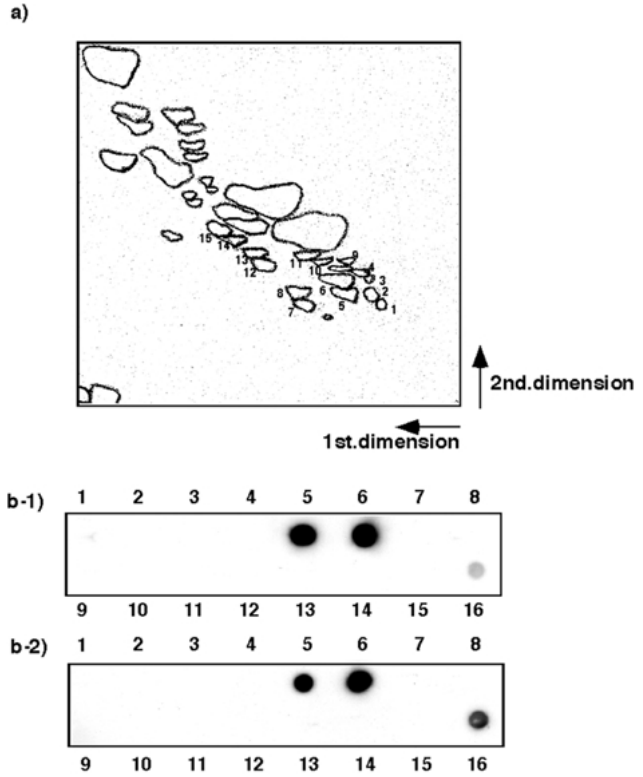

Figure 5. 2-D TLC analysis of Raft2 recognizing antigen. a) illustration of the primuline-staining pattern of the lipids separated by 2-D TLC. The C/M extract obtained from 5×10^7 cells of DIM/raft was applied. b) dot-blot immunostaining with Raft2 b-1) and RM1 b-2). Methanol extracts (0.5 μl) from each spot (equivalent to 2.5×10^5 cells) was dot-blotted on a PVDF membrane and immunostained. Spot #16 contains the C/M extract of raft microdomains, namely, total lipid.

Glycolipids separated on the 2-D plate were marked with a soft drawing pencil after spraying of primuline reagent and transferred to PVDF membrane. Spots #1 ~ #15 on PVDF membrane were extracted in methanol and dot-blotted. The blots were stained with Raft2 (Figure 5b-1) and RM1 (Figure 5b-2). Both MAbs reacted to the exact same glycolipids, #5 and #6. These results clearly show that Raft2 recognizes MSGG. A binding assay for the Stx-1B subunit on a PVDF membrane [13] and immunostaining with Y111-13 revealed spots #14 and #15 to be Gb3Cer and spots #7 and #8 to be Gb5Cer, respectively (data not shown).

Antigen analysis of the other lipid-reactive clones

The C/M extract of the ACHN raft was TLC-blotted and probed with the culture supernatants of 21 lipid-reactive clones. All the clones bound to the same band as that recognized by Raft2 (Figure 6a). To further confirm this finding, MSGG (#5 and #6 in Figure 5a were pooled) was dot-blotted and probed with these culture supernatants as Figure 1. Figure 6b shows that all the clones reacted to MSGG.

Discussion

Many important molecules in signal transduction have recently been shown to be associated with raft microdomains in a highly ordered way. We attempted to produce MAbs against DIM/raft to catalog the constituent molecules of DIM/raft microdomains and resolve signaling mechanisms. We obtained 31 clones, 10 of which seemed to recognize proteins and 21 of which recognized glycolipids. Surprisingly, all 21 clones that

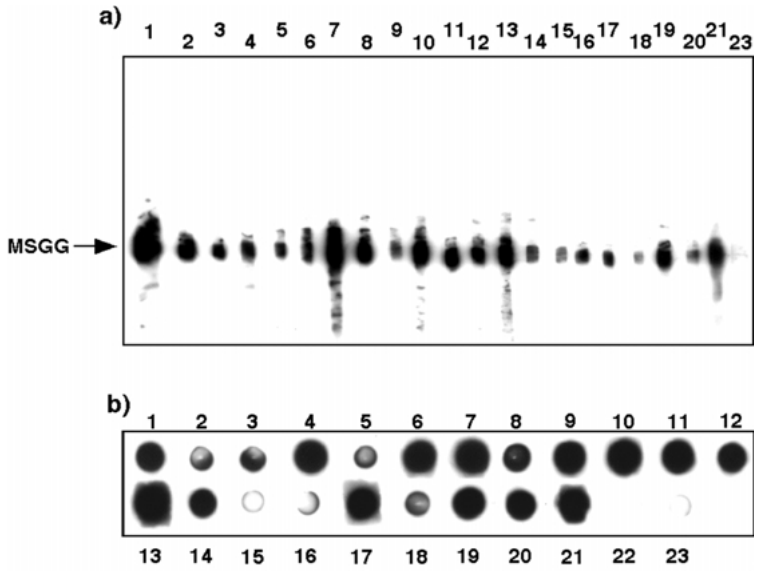

Figure 6. Immunostaining with MAbs that reacted with lipid fractions of ACHN DIM/raft. a) Each strip was immunostained with a culture supernatant of clones (lane 1 ~ 21) that reacted with lipids, as shown in Figure 6a. Lane 13, Raft2; lane 23, immune serum. b) MSGG (#5 and #6 in Figure 5a were pooled) was dot-blotted and immunostained with each culture supernatant as shown in Figure 1a. Spots 1 ~ 21 and 23 correspond to the above lane. Spot 22, preimmune serum.

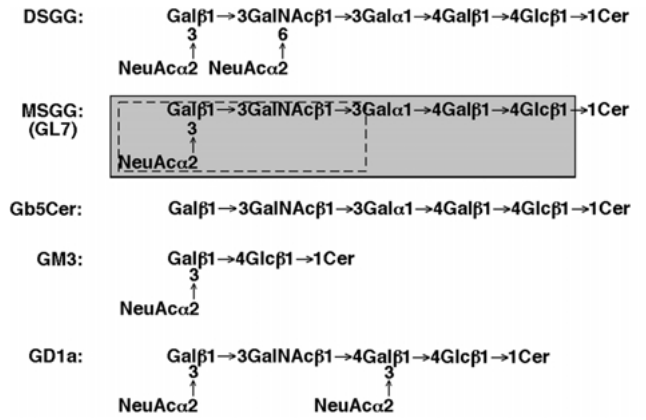

Figure 7. Epitope map of Raft2. Shadowed and hatched rectangle represents the SSEA-4 epitope and the minimum epitope of Raft2, respectively.

reacted to lipid fractions of ACHN DIM/raft were found to recognize MSGG.

It is not clear why MSGG was selected as only one antigen among lipids/glycolipids, although DIM/raft was not particularly rich in MSGG (4.85 μg per 183 μg total lipids per one injection). The expression of globo-series gangliosides is associated with the metastatic potential of renal cell carcinoma [5]. Growth suppression and an increase in GM3 with a simultaneous decrease in MSGG have been observed in human renal tubular cell line, ACHN [12]. The high expression and prominent immunogenicity of MSGG might define the characteristics of ACHN cells.

Unreactiveness of Raft2 to Gb5Cer, which carries the SSEA-3 epitope, indicates that sialylation is needed for recognizing the antigen. Furthermore, Raft2 did not bind to either GM3 or GD1a. Taken together, the minimum epitope is thought to be NeuAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAcβ1 $\rightarrow$ 3 (Figure 7). Shevinsky *et al.* [14] established SSEA-3 and -4 antigens by injection of a huge number of four-cell stage embryos into syngeneic mice. Subsequently, Kannagi *et al.* [15] identified these antigens as extended globo-series structures linked to globoside. SSEA-4 shows a high antigenicity and a high affinity for its antibody [16]. The MAbs described in this study also displayed a strong binding to MSGG in TLC-immunostaining and could be used to detect SSEA-4 even in normal renal cells when examined by flowcytometry (data not shown).

Immunization with a glycolipid combined with a foreign protein or included in a liposome or cell membrane induces a significant antibody response. In particular, glycolipids noncovalently adsorbed onto acid-treated *Salmonella minnesota* can be used to effectively immunize mice against a number of glycolipids [17]. This method requires at least 100 μg of glycolipid antigens to immunize one mouse. The immunization method employed in this study does not require either adsorbents or large amounts of glycolipid antigens. However, the use of DIM/raft microdomains does not enable the specific glycolipid to be selected as an antigen. Human renal cancer cell lines contain high quantities of globo-series glycolipids, and glycolipids with long sugar chains are often sialylated [18]. Since the immune-serum obtained just prior to the splenectomy reacted only to MSGG (data not shown), SSEA-4 might be the strongest antigen among ACHN raft lipids. Thus, the finding that all of the established clones reacted to the same molecule is not surprising. These results suggest that MAbs for glycolipids with predefined specificity can be produced if suitable cells are selected as sources of rafts.

A dot-blot immunostaining procedure on a PVDF membrane in which each dot is fenced separately is a highly sensitive and timesaving screening method. In addition, smaller amounts of antigens are sufficient to detect positive clones, compared to the amounts required by enzyme-linked immunosorbent assays.

The advantages of the present method are its simplicity, rapidity and high efficiency. The method will help us to easily produce MAbs against DIM/raft and study the signaling mechanisms that occur in DIM/raft microdomains.

Acknowledgments

The authors thank Dr. Tatsuya Yamagata (Japan Institute of Leather Research, Tokyo) for his helpful discussion.

Grant sponsors: The Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan; Grant number: 96-5; The ministry of Health and Welfare of Japan; Grant number: 12C-01; Science and Technology Promotion Bureau for Organized Research Combination System of Japan; Grant number: 6070.

References

1 Katagiri, YU, Mori, T, Nakajima, H, Katagiri, C, Taguchi, T, Takeda, T, Kiyokawa, N, Fujimoto, J, Activation of Src family kinase yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains, *J Biol Chem* **274**, 35278–82 (1999).

2 Simons, K, Ikonen, E, Functional rafts in cell membranes, *Nature* **387**, 569–72 (1997).

3 Simons, K, Toomre, D, Lipid rafts and signal transduction, *Nature Reviews* **1**, 31–9 (2000).

4 Katagiri, YU, Kiyokawa, N, Fujimoto, J, A role for lipid rafts in immune cell signaling, *Microbiol Immunol* **45**, 1–8 (2001).

5 Satoh, M, Handa, K, Saito, S, Tokuyama, S, Ito, A, Miyano, N, Orisaka, S, Hakomori, S, Disialosyl galactosylgloboside as an adhesion molecule expressed on renal cell carcinoma and its relationship to metastatic potential, *Cancer Res* **56**, 1932–8 (1996).

6 Saito, S, Levery, SB, Salyan, ME, Goldberg, RI, Hakomori, S, Common tetrasaccharide epitope NeuAc alpha 2 $\rightarrow$ 3Gal beta 1 $\rightarrow$ 3(Neu-Ac alpha 2 $\rightarrow$ 6)GalNAc, presented by different carrier glycosylceramides or O-linked peptides, is recognized by

different antibodies and ligands having distinct specificities, *J Biol Chem* **269**, 5644–52 (1994).
7 Ackman RG, Flame ionization detection applied to thin-layer chromatography on coated quartz rods, *Methods Enzymol* **72**, 205–52 (1981).
8 Hiraoka T, Katagiri C, Treatment of low density lipophorin with lipoprotein lipase: diacylglycerol content has no effect on dissociation of apolipophorin III from low-density lipophorin, *J Biochem* **112**, 689–93 (1992).
9 Taki, T, Handa, S, Ishikawa, D, Blotting of glycolipids and phospholipids from a high-performance thin-layer chromatogram to a polyvinylidene difluoride membrane, *Anal Biochem* **221**, 312–6 (1994).
10 Bouhours, JF, Bouhours, D, Hansson, GC, Developmental changes of gangliosides of the rat stomach. Appearance of a blood group B-active ganglioside, *J Biol Chem* **262**, 16370–5 (1987).
11 Isobe, T, Naiki, M, Handa, S, Taki, T, A simple assay method for bacterial binding to glycosphingolipids on a polyvinylidene difluoride membrane after thin-layer chromatography blotting and in situ mass spectrometric analysis of the ligands, *Anal Biochem* **236**, 35–40 (1996).
12 Saito, S, Nojiri, H, Satoh, M, Ito, A, Ohyama, C, Orisaka, S, Inverse relationship of expression between GM3 and globo-series ganglioside in human renal cell carcinoma, *Tohoku J Exp Med.* **190**, 271–8 (2000).
13 Nakajima, H, Katagiri, YU, Kiyokawa, N, Taguchi, T, Suzuki, T, Sekino, T, Mimori, K, Saito, M, Nakao, N, Takeda, T, Fujimoto, J, Single-step method for purification of Shiga Toxin-1 B subunit using receptor-mediated affinity chromatography by globotriaosylceramide-conjugated octyl sepharose CL-4B, *Protein Expr Purif* **22**, 267–275 (2001).
14 Shevinsky, LH, Knowles, BB, Damjanov, L, Solter, D, Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells, *Cell* **30**, 697–705.
15 Kannagi, R, Cochran, NA, Ishigami, F, Hakomori, S, Andrews, PW, Knowles, BB, Solter, D, Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells, *EMBO J* **2**, 2355–61 (1983).
16 Thomson, JA, Itskovitz-Eldor, J, Shapiro, SS, Waknitz, MA, Swiergiel, JJ, Marshall, VS, Jones, JM, Embryonic stem cell lines derived from human blastocysts, *Science*, **282**, 1145–7 (1998).
17 Kannagi, R, Monoclonal anti-glycosphingolipid antibodies, *Method Enzymol* **312**, 160–79 (2000).
18 Saito, S, Orikasa, S, Satoh, M, Ohyama, C, Ito, A, Takahashi, T, Expression of globo-series gangliosides in human renal cell carcinoma, *Jpn J Cancer Res* **88**, 652–9 (1997).
19 The nomenclature of lipids. Recommendations (1976) 2,3 IUPAC-IUB Commission on Biochemical Nomenclature, *Lipids* **12**, 455–63 (1977).
20 Svennerholm, L, The gangliosides, *J Lipid Res* **5**, 145–55 (1964).

Received 24 May 2001; revised 19 September 2001; accepted 21 September 2001