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SHORT COMMUNICATION

The detergent-insoluble microdomains, rafts, can be used as an effective immunogen

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Abstract Detergent-insoluble microdomains, or rafts, act as a platform to transduce signals from the extracellular space into the cytoplasm. In the process of developing monoclonal antibodies against raft molecules for the purpose of studying the molecular mechanism of raft-mediated signaling, we observed the uniqueness and certain advantages of immunization with rafts. Simple subcutaneous injection of mice with a phosphate-buffered saline (PBS) suspension of rafts without mixing with Freund's adjuvant made it possible to increase the titer of antiserum reacting with raft components. Interestingly, injection of rafts prepared from certain specific cell lines induced monoglycolipid-specific antibodies. Furthermore, antibodies were produced by raft-immunization of even syngeneic mice. Our findings suggest that this phenomenon does not represent a breakdown of immunological self-tolerance, but typical immune reactions accompanying the class switch from IgM antibodies to IgG antibodies.

Keywords Raft · Antibody · Immune Response · Monoglycolipid-specific · Syngeneic antigen

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Introduction

There is evidence that detergent-insoluble microdomains, or rafts, are important in signal transduction, because a variety of signaling molecules, such as Src-family kinases, heterotrimeric G proteins, and GPI-anchored proteins, are concentrated in rafts. We have previously shown that the binding of Shiga-toxin (Stx) to the globotriaosylceramide (Gb3[1]) in rafts temporally activates the Src-family kinase Yes in human renal cancer cell line ACHN [1]. In order to study the downstream signaling mechanism after Stx binding to Gb3, we attempted to develop monoclonal antibodies against components of rafts prepared from ACHN cells and established several clones [2].

In the process we observed the uniqueness of immunization using raft suspensions. Before immunizing animals in an attempt to induce antibody production, antigen solutions or cell suspensions are generally mixed with Freund's adjuvant to obtain an oil emulsion, whereas we succeeded in raising antibody titer by the raft immunization method without mixing them with adjuvants. Interestingly, two thirds of the clones obtained reacted with lipid components of the raft, and further analysis showed that all of the lipid-reactive clones recognized monosialosylgalactosylgloboside (sialylGb5).

To ascertain whether raft immunization always induces monospecific antibodies that recognize a certain glycolipid, we immunized mice with rafts prepared from several cell lines and examined the glycolipid antigens recognized by the antibodies induced. In this paper we report that injection with

[1] Glycosphingolipids are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature. http://www.chem.qmul.ac.uk/iupac/misc/glylp.html.

rafts prepared from certain specific cell lines can induce the production of monoglycolipid-specific antibodies and that raft immunization can induce antibody production even in syngeneic mice.

Materials and methods

Cell culture and antibodies Human renal cancer cell line ACHN, human T-cell leukemia cell lines Jurkat and MOLT-4, and mouse myeloma cell line P3U1 were purchased from the American Type Culture Collection, and the African green monkey kidney cell line Vero was a gift of Dr. T. Takeda of the Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Anaplastic large cell lymphoma Karpas 299 cells [3] were gifted by Dr. K. Kikuchi of Sapporo Medical University, School of Medicine, Sapporo, Japan. Human pre-B ALL cell line NALM-6, mouse T lymphoma cell line EL4, mouse melanoma cell line B16F1, and mouse leukemia cell line RL2 were obtained from the Institute of Development, Aging and Cancer of Tohoku University, Sendai, Japan. The ACHN cells, Vero cells, and B16F1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., CCT, Canada). All other cell lines were cultured in RPMI 1640 supplemented with 10% FBS. The NZB/WF1 serum was a kind gift of Dr. S. Kon of the Institute of Genetic Medicine, Hokkaido University, Sapporo.

Raft preparation Rafts were prepared as described previously [2]. Briefly, packed cells were homogenized in 1% Triton lysis buffer (1% Triton X-100, 25 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl) by 20 strokes with a hand-driven Teflon glass homogenizer. Cell lysates, sucrose concentration of which was adjusted to 40% with 85% sucrose solution, were placed on the bottom of an ultracentrifuge tube, and a 5/30% discontinuous sucrose gradient was formed over the sample. After centrifugation at 39,000 rpm for 18 h at 4°C in a Beckman SW 40Ti rotor, rafts were recovered as visible bands at the interface between 5 and 30% sucrose solution. After several washes with PBS, raft suspensions in PBS were stored at −30°C until used.

Immunization of mice Rafts prepared from 1.2×10^6~1.5×10^8 cells or 10^7 cells irradiated at 10 Gy were suspended in 100 μl of PBS. They were subcutaneously injected into mice in triplicate, followed by three booster shots at 1-week intervals. Five days after the final injection, a peripheral blood specimen was collected from the mice, and the level of antibodies against rafts was evaluated.

TLC immunostaining Lipids were prepared from packed cells as previously described [4] and separated on a Silica gel 60-precoated HPTLC aluminium sheet (Merck, Darmstadt, Germany) with a solvent system consisting of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, *v*/*v*). After drying, the TLC plates were coated with 0.1% polyisobutylmethacrylate (Sigma-Aldrich, Milwaukee, WI) in cyclohexane and blocked with 1% bovine serum albumin (BSA) in PBS. The plates were probed with anti-sera (diluted to 1:500 in 1% BSA in PBS) for 1 h at room temperature. After three washes with PBS for 5 min each, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins G+M (DAKO, A/S, Denmark) at a 1:2,000 dilution ratio were used as the second antibody. The antibodies that bound to the plates were visualized with enhanced chemiluminescence reagent Super Signal (Pierce, Rockford, IL) and detected with a luminescent imaging analyzer, LAS-1000 (Fuji Film, Tokyo, Japan). To compare the amounts of antibodies in the sera, the intensity of chemiluminescence was measured with Image Gauge analysis software equipped to LAS-1000 and shown as Photo Stimulated Luminescence (PSL).

Dot-blot immunostaining assay The ACHN rafts were dot-blotted on a PVDF membrane (Millipore Corp., Bedford, MA) and immunostained as described previously [2] with a slight modification. The dots were probed with antisera (diluted to 1 in 500 with 1% BSA in PBS) for 1 h at room temperature. After four washes with PBS containing 0.025% Tween 20 (PBS-Tween), the membranes were treated with HRP-conjugated rabbit anti-mouse IgG antibodies specific to Fcγ fragment and HRP-conjugated goat anti-mouse IgM antibodies specific to μ chain (Jackson Immuno Research Laboratories, West Grove, PA) to detect IgG and IgM, respectively. The antibodies that bound to the membrane were visualized with enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech. UK Ltd., Buckinghamshire) and detected by a luminescent imaging analyzer as mentioned above.

Flow cytometry Cells were harvested from culture plates, and after incubating with the antisera (diluted to 1:100 in RPMI medium containing 5% FBS and 0.1% NaN_3) for 1 h on ice, they were treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immuno Research Laboratories) at a 1:50 dilution ratio and analyzed by flow cytometry (EPICS-XL, Beckman-Coulter, Fullerton, CA).

Measurement of anti-ss and -dsDNA antibodies in sera by ELISA The ELISA was performed as described by Iizuka *et al.* [5] with a slight modification by using calf thymus ssDNA (Sigma) and salmon sperm dsDNA (Sigma). For the substrate solution, 120 μl of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (Dojindo, Kumamoto, Japan) solution in *N*,*N*,dimethylformamide and 1.3 μl of 30% H_2O_2 was mixed with 7.88 ml of 0.1 M sodium acetate buffer, pH 5.5, immediately before use. A 0.5 μg amount of ssDNA or dsDNA dissolved in 50 μl of PBS were allowed to dry in a flat 96-well NUNC-IMMUNO Plate (Nunc, Roskilde, Denmark). Wells were blocked with 3% BSA in PBS and washed with PBS-Tween. A 50 μl of the serum (diluted to 1:100) was added to a well in triplicate, and allowed to stand at room temperature for 2 h. After five washes with PBS-Tween, HRP-conjugated donkey anti-mouse μ chain antibodies or rabbit anti-mouse γ chain antibodies (Jackson Laboratory; diluted to 1:2,000) in 1% BSA in PBS was added to each well and incubated for 1 h at room temperature. After three washes with PBS-Tween, 50 μl of substrate solution was added, and the plates were incubated at room temperature until the solution turned yellow. The reaction was stopped by adding 50 μl of 2 M H_2SO_4, and absorbance at 450 nm was measured with a microplate reader (Model 550 Bio-Rad, Richmond, CA).

Results and discussion

The antisera obtained from Balb/c mice in response to subcutaneous injection of rafts prepared from a variety of cell lines were examined by TLC-immunostaining to analyze the reactivity of the antibodies against glycolipids. Both ACHN cells and Vero cells are derived from kidney and express globoseries glycosphingolipids, whereas Karpas cells predominantly express LacCer, and EL4 cells mainly express GM2 and GD2 (Fig. 1a). The antisera obtained by injection with ACHN rafts and Vero rafts were found to uniquely bound to sialylGb5, suggesting the development of mono-specific antibodies against sialylGb5 (Fig. 1b). As we previously showed, ACHN cells contain comparable amounts of Gb3, Gb4, Gb5, and sialyGb5, suggesting that the sialylGb5 of ACHN cells is strongly immunogenic [2]. The antisera obtained by injection with Karpas rafts were also found to specifically bind to a single glycolipid that has not yet been identified. Since the glycolipid was stained with resorcinol and not bound by choleratoxin even after digestion with *Clostridium perfringens* sialidase (data not shown), it is suggested that this antigen is a sialylated non-ganglioseries glycolipid. The observation that the anti-Karpas rafts antisera did not bind to any glycolipid extracted from mouse brains in which various kinds of gangliosides are abundantly contained (data not shown) should support this idea. The antisera obtained by injection with EL4 rafts were found to uniquely react with GD2. However, when antisera obtained by injection of rafts prepared from the other cell lines, *i.e.*, B16F1, P3U1, RL-2, Molt 4, Jurkat, or NALM-6, were tested, no such monoglycolipid-specific reactivity was observed (data not shown). These findings indicate that immunization with rafts prepared from some specific cell lines can induce the development of monoglycolipid-specific antibodies. Since we obtained identical results in a similar experiment in C57BL/6 mice (Fig. 1c), the development of monoglycolipid-specific antibodies is a common feature of immunization of these cell lines with rafts and not a phenomenon specific to a certain strain of mice.

Immunization of mice with a suspension of whole cells is one of the ways that is often used to obtain monoclonal antibodies against cell surface molecules [6, 7]. We therefore investigated whether whole-cell immunization is capable of inducing the development of monoglycolipid-specific antibodies in mice, the same as raft immunization does. The antisera obtained by injection with Vero, Karpas, and EL4 cell suspensions did not react with certain specific glycolipids, and only the antisera obtained by immunization with ACHN cell suspensions yielded a mono-specific reaction with sialylGb5 (Fig. 2). This suggests that immunization with suspensions of whole cells does not usually induce the development of monoglycolipid-specific antibodies and that the rafts on the cell surface of ACHN cells assemble in a manner that is favorable for inducing immune reactions against sialylGb5.

Next, we examined the quantitative and qualitative kinetics of the production of the specific antibodies in sera by immunization with rafts derived from ACHN cells. The antisera obtained after each immunization were examined by dot-blot immunostaining and TLC immunostaining (Fig. 3a). The relative amounts of antibodies that bound to rafts dot-blotted on a PVDF membrane or lipids separated on a TLC plate were shown as PSL (Fig. 3b). Production of IgM class anti-raft antibodies was detected after the second immunization, and it peaked after the third immunization, and then decreased. Production of IgG class anti-raft antibodies was also detected after the second immunization, but at a low level, and it continued to increase even after the fourth immunization. The specificity of the secondary antibodies used for typing the immunoglobulin class of anti-raft antibodies was confirmed in advance (data not shown). No anti-sialylGb5 antibodies were detected not after the first immunization (data not shown). They were faintly detected after the second immunization, and then increased in an immunization time-dependent manner. These results indicate that the production of anti-raft antibodies in mice is a typical immune response accompanying the class switch from IgM antibodies to IgG antibodies. Interestingly, the #3 antisera of the third immunization gave strong reactivity with the lower band glycolipid, while that of the fourth

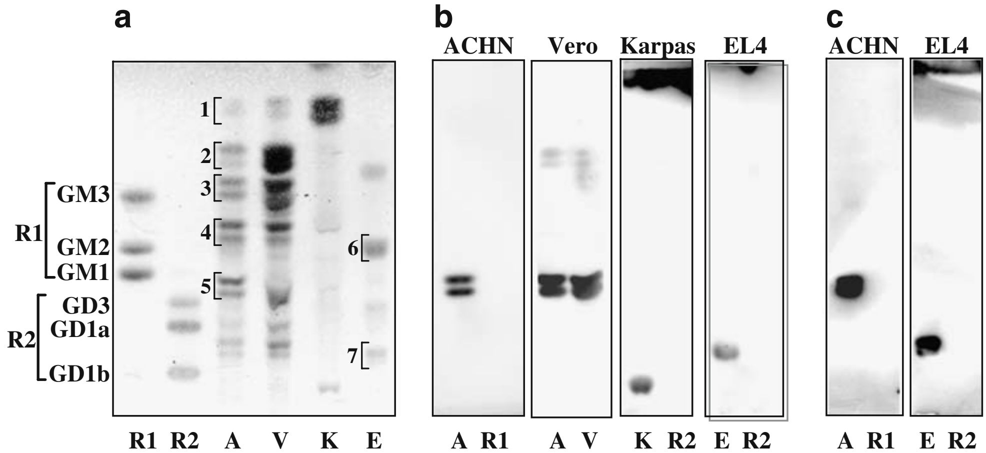

Fig. 1 TLC immunostaining with antisera against rafts components. The lipids extracted from ACHN (*A*), Vero (*V*), Karpas (*K*), EL4 (*E*) cells and the reference glycolipids (*R1* GM3, GM2, GM1; *R2* GD3, GD1a, GD1b) were separated by TLC and chemically stained with Orcinol reagent (**a**) or immunostained with antisera of Balb/c mice (**b**) and C57BL/6 mice (**c**) that had been immunized with rafts prepared from ACHN cells, Vero cells, Karpas cells and EL4 cells. Lipids extracted from 5×10^6 cells and 1×10^6 cells of each cell line are subjected to TLC for Orcinol staining and immunostaining, respectively. *1* LacCer; *2* Gb3; *3* Gb4; *4* Gb5; *5* sialylGb5; *6* GM2; *7* GD2

immunization reacted strongly with the upper band glycolipid (Fig. 3a). The result may indicate that ceramide structure is also involved in antigen presentation of glycolipid in rafts.

Next, we examined the correlation between the amounts of rafts injected and anti-raft antibody production. To do so, we immunized C57BL/6 mice with rafts prepared from various numbers of EL4 cells and evaluated the subsequent production of anti-EL4 raft antibodies by flow cytometry and TLC immunostaining. As shown in Fig. 4a, the amounts of anti-EL4 raft antibody increased with the amounts of EL4 rafts injected. Injection with the rafts prepared from 0.12×10^7 EL4 cells induced a slight elevation of reactivity, and the rafts prepared from 3×10^7 EL4 cells were sufficient to induce maximum reactivity. Rafts prepared from more than 0.6×10^7 EL4 cells appeared to be needed to obtain a significant level of anti-GD2 antibodies, (Fig. 4b).

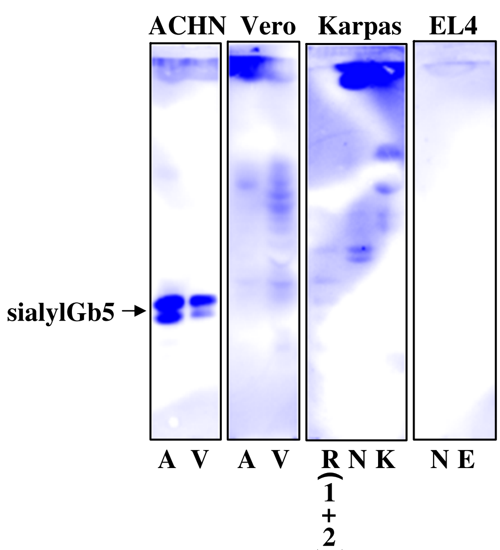

Fig. 2 TLC immunostaining with antisera against the cell suspension. The lipids were extracted from the cells as shown in the legends to Fig. 1 and from NALM-6 cells (*N*), and separated by TLC. The plates were immunostained with antisera from Balb/c mice immunized with the suspension of irradiated ACHN cells, Vero cells, Karpas cells, and EL4 cells

Since EL4 cells are derived from C57BL/6 mice, no immune responses to EL4 cells or EL4 cell components should be usually induced in syngeneic C57BL/6 mice. However, the injection of C57BL/6 mice with the EL4 rafts resulted in production of anti-raft antibodies in syngeneic mice as shown above. We therefore tried using flow cytometry to corroborate that injection of raft suspensions can induce anti-raft antibody production in syngeneic mice. The results showed that the antisera of C57BL/6 mice injected with rafts of syngeneic melanoma cell line B16F1 bound to B16F1 cells (Fig. 5a). Both mouse myeloma cell line P3U1 and lymphoma cell line RL-2 are derived from Balb/c mice, and antisera from Balb/c mice injected with rafts of these syngeneic P3U1 (Fig. 5b) and RL-2 rafts (Fig. 5c) were also confirmed to bind to P3U1 cells and RL-2 cells, respectively. Injection of mice with a PBS suspension of irradiated syngeneic cells did not result in the production of antisera that bound to syngeneic cells (data not shown).

Since repeated immunization of self- or syngeneic antigens is thought to induce autoimmune diseases, we repeated injection of C57BL/6 mice with EL4 rafts or Balb/c mice with the P3U1 rafts and investigated whether the mice produced anti-DNA antibodies by ELISA. The average A_{450} of anti-ssDNA IgM in the sera of the mice injected with PBS and the syngeneic rafts was 0.247±0.027

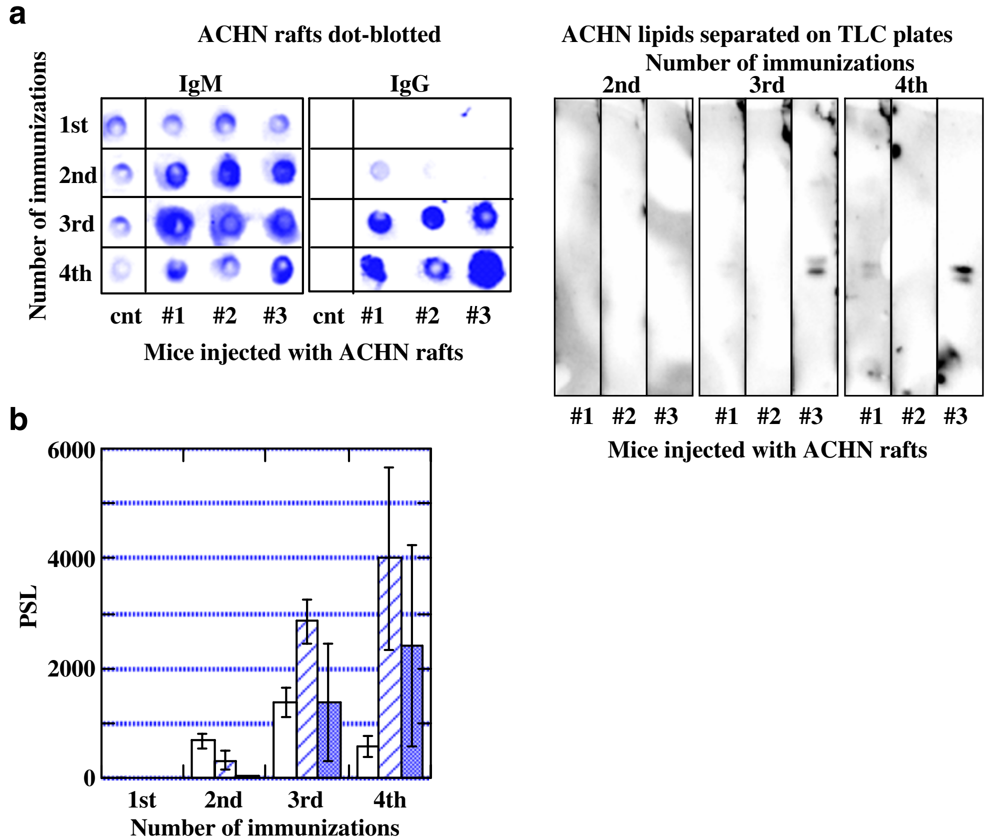

Fig. 3 Kinetics of production of antibody against ACHN rafts. Balb/c mice were injected with an ACHN raft suspension in triplicate (*#1*, *#2*, *#3*) or PBS (*cnt*) four times at 7 day intervals, and the sera were obtained 5 days after each immunization. The rafts dot-blotted on PVDF membranes were probed with each antiserum, and then probed with the HRP-conjugated anti-mouse IgM μ chain-specific antibodies or IgG γ chain-specific antibodies as secondary antibody. The lipids separated on the TLC plate were probed with each antiserum, and then with the HRP-conjugated anti-mouse IgG+M antibodies. **a** The images of dot-blot immunostaining of ACHN rafts (*left*) and TLC immunostaining of ACHN lipids (*right*) with the antisera. **b** Measurement of anti-raft IgM antibodies (*open column*), the anti-raft IgG antibodies (*striped column*), and anti-sialylGb5 antibodies (*shaded column*)

(column 1 in Fig. 6) and 0.240±0.043 (column 2 in Fig. 6), respectively, and the difference between the two groups was not significant. The A_{450} for anti-ssDNA IgM in the serum of NZB/WF1, which are well known to spontaneously develop autoimmune disease, was 0.325. No elevation of IgG class anti-DNA antibodies or anti-dsDNA antibodies was observed in the sera of either the immunized mice or NZB/WF1 mice (data not shown). No anti-DNA antibody production or other diagnostic signs of autoimmune disease were observed in these mice. These results show that the development of antibodies against syngeneic rafts components by the mice was not due to the development of an autoimmune disease.

The results of this study show that subcutaneous injection of mice with rafts prepared from specific cell lines induces production of antibodies that recognize single glycolipids, namely monoglycolipid-specific antibodies. For example, rafts prepared from ACHN cells and Vero

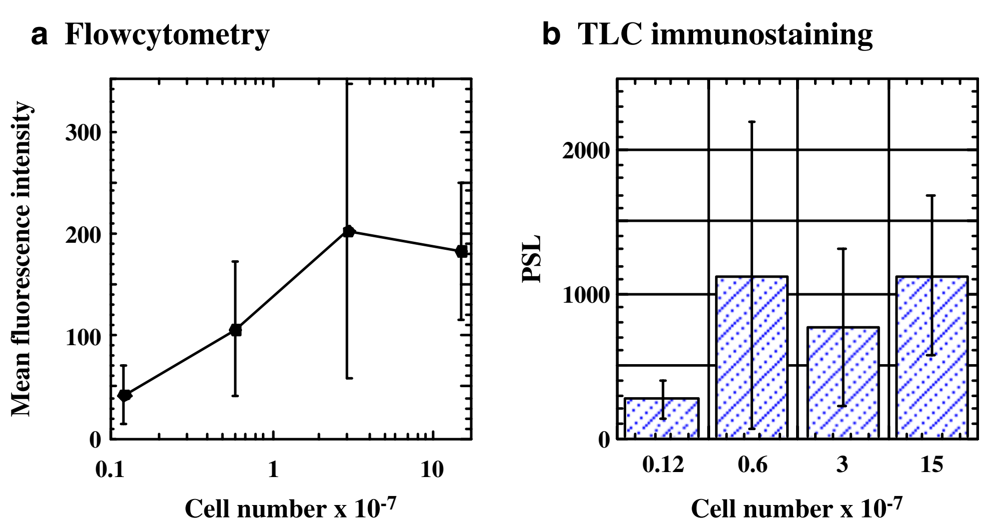

Fig. 4 Reactivity of mouse sera after immunization with the rafts prepared from various numbers of EL4 cells. The sera were obtained from C57BL/6 mice immunized with rafts prepared from 0.12, 0.6, 3 and 15×10^{7} EL4 cells. The experiments were performed in triplicate. **a** Evaluation of antibody reactivity to EL4 cells by flowcytometry. **b** Evaluation of antibody reactivity to GD2 by TLC immunostaining

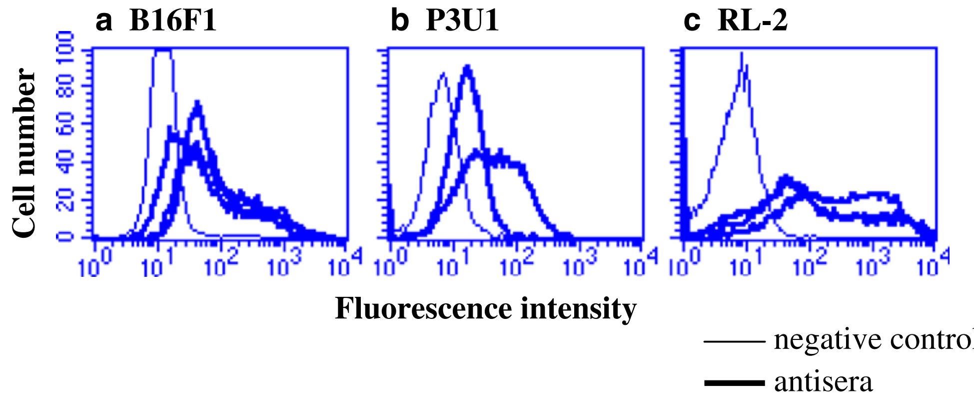

Fig. 5 Flow cytometric analysis of the antisera of mice immunized with syngeneic rafts. Cells were stained with the antisera of C57BL/6 mice immunized with B16F1 rafts (**a**), Balb/c mice immunized with P3U1 rafts (**b**) and Balb/c mice immunized with RL-2 rafts (**c**), and analyzed by flow cytometry (*bold line*). The sera of each mouse injected with PBS were used as a negative control (*thin line*)

cells strongly induced the production of mono-specific antibodies against sialyGb5. However, since sialyGb5 is not the quantitatively predominant glycolipid in ACHN cells or Vero cells, quantitative lipid dominance may not be necessary for monospecific antibody production. Since all four glycolipids to which specific antibodies were produced shown in Fig. 1b were sialylated, sialylation is thought to be the most important factor for inducing monospecific antibody production. However, the rafts from other cell lines gave no production of such antibodies. For example, although B16 melanoma cells are known to highly express GM3 [8], injection of the B16 melanoma rafts did not induce monoglycolipid-specfic antibody. Since Kawashima *et al.* [9] reported that when they intravenously injected ten strains of inbred mice with 100 μg of gangliosides adsorbed to *Salmonella minnesota*, gangliosides such as GD3, GD2, GD1b, GT1a, and GQ1b that have a trisaccharide sequence of NeuAcα2,8NeuAα2,3Gal induced high-titer antibody responses, whereas gangliosides such as GM4, GM3, GM2, GM1, GD1a, and GT1b that have a disaccharide sequence of NeuAcα2,3Gal induced low-titer antibody responses, the diversity of immunogenicity among the glycolipids should be present. Since SSEA-4, an epitope carried by sialyGb5 has been well known highly immunogenic, a saccharide sequence of sailylGb5 can be thought to induce high-titer antibody production. Therefore, if the cells contain highly immunogenic glycolipids such as sialyGb5 and GD2 in lipid rafts, these glycolipids may be effectively presented as immunological targets for antibody production, whereas the rafts containing only low immunogenic glycolipids may be insufficient for antigen presentation to produce anti-glycolipids antibodies. Yamazaki Y. *et al.* [10] obtained several monoclonal antibodies by injecting mice with HL60 cell lipid rafts. One of the antibodies reacted with both GM1a and GD1b, and another reacted with phosphatidylglucoside. HL60 cells, however, mainly express glycolipids of the neolactoseries, not the ganglioseries [11], suggesting that raft immunization enables antibody production against such an extremely minor glycolipid. In order to induce effective

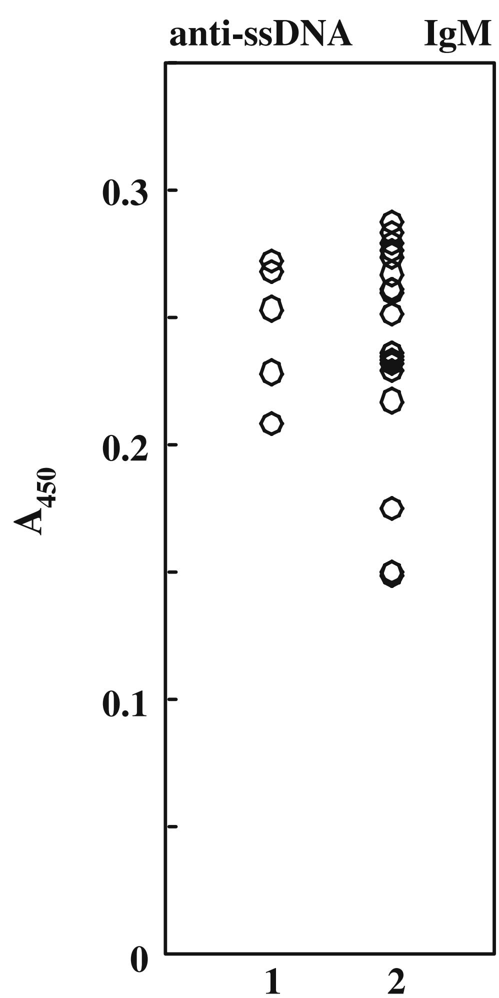

Fig. 6 ELISA of anti-ssDNA antibodies in the sera of mice immunized with syngeneic rafts. Calf thymus ss-DNA was coated and probed with the serum of C57BL/6 or Balb/c mice injected with PBS (*column 1*) and C57BL/6 mice injected with EL4 rafts or Balb/c mice injected with P3U1 rafts (*column 2*). The mean values of triplicate experiments are shown

immune responses against glycolipids in mice, a large amount of purified antigen usually must be immobilized by adsorbing it to the cell walls of bacteria, such as *Salmonella minnesota*, or by incorporating it into liposomes [12], whereas rafts themselves are insoluble and do not need to be immobilized. Furthermore, without mixing with Freund's adjuvant, rafts may retain adjuvant effects and be capable of inducing an immune response even in syngeneic mice.

It still remains unclear how monoglycolipid-specific antibodies are produced, which cells should be used for raft preparation, and to which glycolipids antibodies are predominantly produced. Although further experiments are certainly needed to answer these questions, raft immunization can be used as an effective method of producing monoclonal antibodies against glycolipids and can be applied as new approach in many fields.

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