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Production of a Recombinant Single-Chain Variable-Fragment (scFv) Antibody against Sulfoglycolipid

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Mammalian sulfoglycolipids are comprised of two major classes of compounds, sulfatide (SO_3-3Gal-ceramide) and seminolipid (SO_3-3Gal-alkylacylglycerol). Sulfatide is present in relatively high levels in myelin, and seminolipid is present in testis. The sulfation of these sulfoglycolipids is catalyzed by a common enzyme, cerebroside sulfotransferase (CST). Disruption of the *Cst* gene in mice revealed that sulfatide and seminolipid are essential for, respectively, myelin formation and spermatogenesis. The present study describes the generation of a recombinant single-chain variable fragment (scFv) antibody against sulfoglycolipid, for use in the functional analysis of sulfoglycolipids in living cells. A positive hybridoma producing anti-sulfoglycolipid IgG3, referred to as DI8, was initially obtained by immunizing CST-null mice with an isolated sulfatide. The DI8 monoclonal antibody was found to bind specifically to sulfoglycolipids with the terminal 3-*O*–sulfated galactose structure, as evidenced by ELISA and thin-layer chromatogram-immunostaining. The antibody stained seminolipid on the cell surface of spermatogenic cells of wild-type testis, but it did not react with any cells in the seminiferous tubules of CST-null testis. Total RNA was extracted from this hybridoma, and cDNAs that encode the variable regions of the heavy and light chains of IgG3 were obtained by RT-PCR. These DNA fragments were linked through a DNA linker coding $(Gly_4Ser)_3$, and the recombinant scFv fragment was then inserted into a phagemid vector pCANTAB 5E. The scFv antibody that was displayed at the tip of the M13 phage in the form of a g3p fusion protein bound to sulfatide. Furthermore, a soluble form of the scFv antibody was also found to bind to the sulfoglycolipids in ELISA.

Key words: cerebroside sulfotransferase, monochronal antibody, phage display, seminolipid, fulfatide.

Abbreviations: CST, cerebroside sulfotransferase; ELISA, enzyme-linked immunosorbent assay; HRP, horse radish peroxidase; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RT, room temperature; RT-PCR, reverse transcription–polymerase chain reaction; scFv, single-chain variable fragment; VH, variable region of the antibody heavy chain; VL, variable region of the antibody light chain.

Two major sulfoglycolipids are produced by mammals: sulfatide, which is a sphingolipid, and seminolipid, which is an ether glycerolipid (*1*). Sulfatide is present in relatively high levels in the myelin sheath, and seminolipid is expressed by spermatogenic cells. The sulfation of these sulfoglycolipids is catalyzed by a common enzyme, cerebroside sulfotransferase (CST, EC 2.8.2.11) (*2*, *3*). CST-deficient mice, which completely lack the ability to produce sulfatide and seminolipid, were generated by gene targeting (*3*, *4*). CST-null mice manifest some neurological disorders due to myelin dysfunction (*3*, *5*), an aberrant enhancement in oligodendrocyte terminal differentiation (*6*), and an arrest of spermatogenesis (*3*), indicating that the sulfation of glycolipids is essential for both myelin formation and spermatogenesis. However, the role of sulfoglycolipids in this process is currently unknown.

Glycosphingolipids self-associate in cellular membranes to form a microdomain, referred to as lipid raft (*7*). Glycosylphosphatidylinositol-anchor proteins and plasmalogens, which contain an ether glycerolipid similar to seminolipid, are also present in unusually high levels in this microdomain (*8*). In a preliminary study, we demonstrated that sulfatide and seminolipid are recovered in detergent-insoluble floating membrane fractions (unpublished results by Honke *et al.*), suggesting that sulfoglycolipids are also present in the lipid raft. Originally, these microdomains were thought to serve as a platform for intracellular lipid transport (*7*). In order to develop a better understanding of the molecular mechanisms associated with sulfoglycolipids, a powerful tool is needed to follow the movement of sulfoglycolipids in living cells.

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In the past decades, several antibodies against sulfatide have been produced and are in widespread use (*1*). However, the production of scFv antibodies against sulfatide has never been attempted. An scFv antibody would have many advantages as compared to natural antibodies that are secreted from lymphocytes or hybridomas. Since the scFv antibody is a single-chain peptide with a smaller size, it is easier to manipulate its gene. For example, an scFv antibody library constructed in a phage display system is used for screening of humanized monoclonal antibody (*9*). Furthermore, this small antibody exhibits better pharmacokinetics in terms of tissue penetration, a desirable characteristic for therapy and imaging (*10*). However, the scFv antibody has a disadvantage, in that it is monovalent and often exhibits a fast off-rate and poor retention time on the target. In this study, we describe the production of an anti–sulfoglycolipid scFv antibody, in an attempt to develop a probe for sulfoglycolipids in living cells.

MATERIALS AND METHODS

Materials—Sulfatide and GalCer were prepared from bovine brain, seminolipid was isolated from bovine testis, and lactosylsulfatide SM3 (SO_3-3Galβ1-4Glc-ceramide), SM2 [GalNAcβ1-4(SO_3-3)Galβ1-4Glc-ceramide], LacCer, gangliosides GM3 and GD3 were isolated from human kidney (*2*). Heparin, biglycan, decorin, cholesterol sulfate and mouse monoclonal antibody isotyping antibodies were purchased from Sigma.

Immunization of Mice—Six- to 8-week-old female mice from $Cst^{-/-}$ mice with the background of C57BL/6 (*3*) and wild-type C57BL/6 mice were immunized with 50 μg/ml of sulfatide adsorbed to 0.25 mg/ml of acid-treated *Salmonella minnesota* (*11*) in PBS. A dose of 200 μl/time of the immunogen was inoculated through the tail vein at day 0, 3, 7, 12, 21, 29 and 36. At day 21 and 38, sera from the immunized mice were examined for anti-sulfatide activity by ELISA. All experiments were performed in strict compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH, 1985). Specific protocols were approved by the Animal Care and Use Committee of Osaka University Medical School.

Hybridoma Formation—At day 40, 3 days before fusion, the mouse was given a booster of 100 μg/ml of pure sulfatide solution. At day 43, spleen cells were isolated and fused with PAI cells, a myeloma line (*12*), with polyethyleneglycol 4000 (Sigma) according to a previously described method (*11*). The resulting fused cells were distributed into nine 96-well microtiter plates and cultured in HAT medium (Sigma). Twelve days after the initiation of the culture, the supernatant from each well was tested for anti-sulfatide activity by ELISA. The monoclonal hybridoma was then isolated by limiting dilution.

ELISA—Each well of a 96-well polystyrene plate (Immulon 1B, Thermo Labsystems, Franklin, MA) was coated with 50 μl of 100 μg/ml sulfatide solution in methanol/0.2%$CaCl_2$/H_2O (1/0.7/4, v/v). After blocking with 1.5% human serum albumin/PBS, 50 μl of diluted serum or culture supernatant was added, followed by incubation at RT for 1 h. After washing with PBS containing 0.05% Tween 20, 50 μl of HRP-conjugated sheep anti–mouse IgG antibody (Sigma) was added, followed by incubation at RT for 1 h. After washing, peroxidase reaction was carried out using *o*-phenylenediamine and hydrogen peroxide as a substrate at RT for 20 min. The reaction was terminated by adding 5 N HCl, and the absorbance at 490 nm was measured with a microplate reader (NJ-2001, Intermed).

When the phage-displayed recombinant antibody was assayed, the phage was incubated with the sulfatide-coated wells after blocking. Peroxidase-conjugated sheep anti-M13 antibody, which binds to the phage coat protein, was used as a second antibody.

Isolation of the VH and VL Genes—The VH and VL genes of the DI8 antibody were isolated according to a previously described protocol (*13*). Total RNA was extracted from the DI8 hybridoma with the Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed with the MuIgG3For primer: 5′-CTGGACAGGGCTCCATAGTTCCA-3′. The resulting cDNA was subjected to PCR using various combinations of the primer set originally described by Amersdorfer *et al.* (*14*). PCR was carried out with 35 cycles under conditions of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. When the primer set of MuJH1For: 5′-TGAGGAGACGGTGACCGTGGTCCC-3′ and MuVH1-Back: 5′-GAGGTGCAGCTTCAGGAGTCAGG-3′ was used, amplification of the VH gene was successful. Similarly, the primer set of MuJK1For: 5′-TTTGATTTCCAGCTTGGTGCCTCC-3′ and MuVK2Back: GATGTTCTGATGACCCAAACTCC-3′ enabled the VL gene to be amplified.

Assembly of Antibody scFv DNA Fragment—The VH and VL genes of the DI8 antibody were connected by inserting a DNA linker coding $(Gly_4Ser)_3$ between them, in the direction of 5′-VH-linker-VL-3′, and the recombinant scFv fragment was then inserted into a phagemid vector pCANTAB 5E using a Recombinant Phage Antibody System kit (Amersham Biosciences). The resulting phagemid is hereafter referred to as pCANTAB-DI8.

Phage Display of scFv Antibody—All procedures for phage expression were performed using a Recombinant Phage Antibody System kit (Amersham Biosciences). The pCANTAB-DI8 phagemid was transfected into *Escherichia coli* TG1 cells. The transformed *E. coli* cells were grown at 30°C in 2YT medium containing 2% glucose and 100 μg/ml of ampicillin. Ampicillin-resistant cells were infected with 3×10^{10} pfu of M13KO7 helper phage and grown in a glucose-deficient medium containing 100 μg/ml of ampicillin and 50 μg/ml of kanamaycin to yield a recombinant phage which displays the antibody scFv-g3p fusion protein. A complete phage particle was released into the supernatant.

The scFv antibody–displaying phage was enriched by panning. A 25 cm^2 tissue culture flask was coated with 0.5 mg of sulfatide at 4°C overnight, blocked with 2% BSA for 1 h, and incubated with the phage-containing culture supernatant at RT for 4 h RT. After washing with 0.05% Tween-20 in PBS, the attached phage was incubated with 5 ml of log-phase *E. coli* TG1 cells, and the infected TG1 cells were plated on SOBAG medium. The phage rescued from individual colonies was then assayed for anti-sulfatide activity by ELISA.

Production of a Soluble scFv Antibody in E. coli HB2151—A phage clone that gave a positive signal in the ELISA experiment was selected for infecting *E. coli*

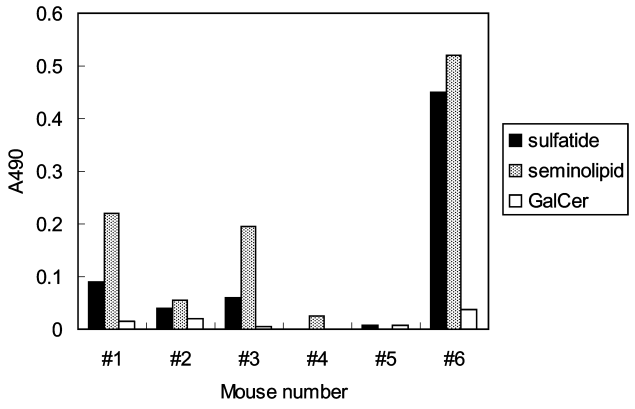

Fig. 1. **Anti-sulfoglycolipid activity in sera from sulfatide-immunized CST-null mice.** Six CST-null mice were inoculated with sulfatide as described in "MATERIALS AND METHODS." Three weeks after immunization, the anti-sufoglycolipid activity of 1:250 diluted sera from the immunized CST-null mice was examined by ELISA. Sulfatide, seminolipid, and GalCer were coated on the wells of microtiter plates. The values indicate the absorbance after the subtraction of background absorbance (none coated).

HB2151 cells. The infected HB2151 cells were cultured in 2YT medium containing 100 μg/ml of ampicillin and 1 mM isopropyl-thio-β-D-galactopyranoside at RT for 3 to 24 h. The supernatant or the periplasmic extract of the HB2151 cells was collected and subjected to SDS-PAGE (12% acrylamide gel) under non-reducing conditions, and transferred to a nitrocellulose membrane (Schleicher & Schuell). After blocking with 4% skim milk in PBS, the soluble scFv antibody was detected with an HRP-conjugated anti–E-Tag antibody.

Immunohistochemistry—Seminolipid expression in murine testis was examined by immunofluorescence staining using the DI8 antibody. Testes from wild-type and CST-null mice (*3*) were fixed with 4% paraformaldehyde at 4°C overnight, incubated in 10% sucrose/PBS at 4°C for 12–24 h, embedded in OCT compound, and cut into 6-μm sections. The sections were sequentially reacted with the Sulph-1 antibody, biotin-conjugated goat anti–mouse IgG $F(ab')_2$ (DakoCytomation, Carpinteria, CA, USA), and FITC-conjugated streptoavidin (DakoCytomation). Nuclear staining was achieved by the use of 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan).

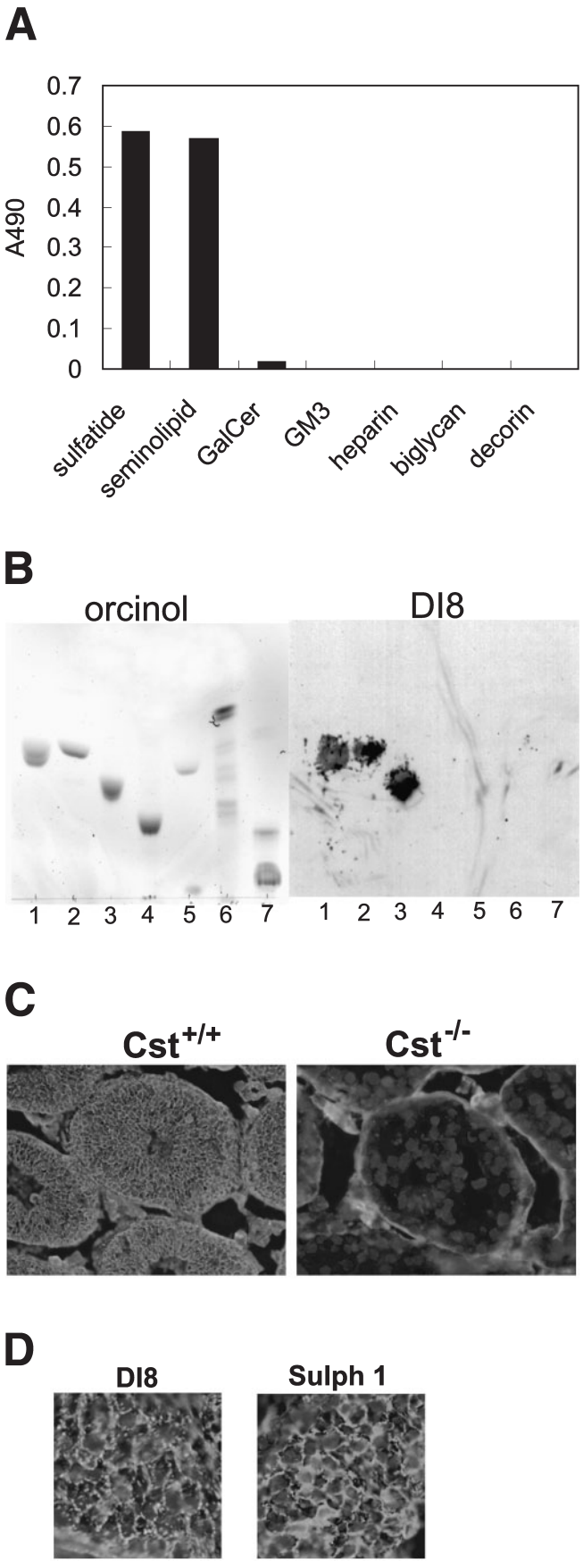

Fig. 2. **Reactivity of the DI8 antibody to sulfoglycolipids.** Various glycolipids and sulfated glycoconjugates were reacted with DI8 and examined by ELISA (A) and thin-layer chromatogram immunostaining (B). (A) The values indicate the absorbance after the subtraction of background absorbance (none coated). (B) Thin-layer chromatogram immunostaining was performed according to a previously described method (*21*). Left, orcinol-sulfuric acid staining; right, immunostaining with DI8. Lane 1, sulfatide; lane 2, seminolipid; lane 3, lactosylsulfatide SM3; lane 4, SM2; lane 5, cholesterol sulfate; lane 6, GalCer, LacCer, globotriosylceramide and globotetraosylceramide; lane 7, gangliosides GM3 and GD3. (C) Immunohistochemical staining of wild-type (left) and CST-null (right) testes was performed using the DI8 antibody (green). Nuclear staining was achieved with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (D) A high magnification of immunostaining of wild-type testis using the DI8 or Sulph 1 antibody (green). Nuclear staining was achieved with DAPI (blue).

VH gene

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  1  GAGGTGCAGCTTCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCATATGTACTGTCTCTGGGTTCTCATTAACC  90
     E  V  Q  L  Q  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I  I  C  T  V  S  G  F  S  L  T

 91  AACTATGCTATAACCTGGGTTCGCCAGCCACCAGGACAGGGTCTGGAGTGGCTTGGGGTAATCTGGACTGGTGGAGGCACAAATTATAAT  180
     N  Y  A  I  T  W  V  R  Q  P  P  G  Q  G  L  E  W  L  G  V  I  W  T  G  G  G  T  N  Y  N
        CDR1                                                                    CDR2
181  TCAGGTCTCAAATCCAGACTGAGCATCAGTAAAGACAACTCCAAGAGTCAAGTTTTCTTAATAATGACCAGTCTGCAAACTGATGACACA  270
     S  G  L  K  S  R  L  S  I  S  K  D  N  S  K  S  Q  V  F  L  I  M  T  S  L  Q  T  D  D  T

271  GCCAGGTACTACTGTGCCAGAAACAATTACGGTAGAAGTTACGAAAGGTACTTCGATGTCTGGGGCACAGGGACCACGGTCACCGTCTCC  360
     A  R  Y  Y  C  A  R  N  N  Y  G  R  S  Y  E  R  Y  F  D  V  W  G  T  G  T  T  V  T  V  S
                    CDR3
361  TCA  363
     S
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VL gene

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  1  GATGTTGTGATGACCCAAACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCCATCTCTTGCAAGTCAGGTCAGAGCCTCTTA  90
     D  V  V  M  T  Q  T  P  L  T  L  S  V  T  I  G  Q  P  A  S  I  S  C  K  S  G  Q  S  L  L
                                                                              CDR1
 91  CATAATGATGGAAAGCCATTTGTGAATTGGTTGTTTCAGAGGCCAGGCCAGTCTCCAAAGCGCCTACTCTATCTGCTGTCTAAACTGGAC  180
     H  N  D  G  K  P  F  V  N  W  L  F  Q  R  P  G  Q  S  P  K  R  L  L  Y  L  L  S  K  L  D
                                                                  CDR2
181  TCTGGAGTCCCTGACAGGTTCACTGGCAGTCGATCAGGGACAGATTTCACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTT  270
     S  G  V  P  D  R  F  T  G  S  R  S  G  T  D  F  T  L  K  I  S  R  V  E  A  E  D  L  G  V

271  TATTATTGCGGACAAGGTACACATTTTCCGTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAA  336
     Y  Y  C  G  Q  G  T  H  F  P  W  T  F  G  G  G  T  K  L  E  I  K
        CDR3
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Fig. 3. **cDNA and deduced amino acid sequences of the VH and VL of the DI8 antibody.** The complementarity determining regions (CDR 1–3) are indicated.

RESULTS

Production of an Anti-Sulfoglycolipid Monoclonal Antibody Using CST-Null Mice—Since CST-null mice completely lack the ability to produce sulfoglycolipids (*3*, *4*), they represent a suitable system for generating an antibody against this molecule. Six CST-null mice were immunized with purified sulfatide adsorbed to acid-treated *S. minnesota*. Three weeks after immunization, anti-sulfatide activity was found in the sera of three of the mice (Fig. 1). All of the anti-sera samples reacted with seminolipid as well. Since the reactivity was strongest in mouse No. 6, it was chosen for the subsequent experiments. Although six wild-type mice were immunized following the same procedure at the same time, none raised an anti-sulfatide antibody (data not shown).

Spleen cells were prepared from mouse No. 6 and fused with a myeloma line, PAI cells. Culture media of the hybridoma cells were screened for reactivity to sulfatide, and the cells that gave a positive signal were cloned by limiting dilution. Finally a hybridoma clone, referred to hereafter as DI8, was isolated. The DI8 antibody secreted from this hybridoma was identified as IgG3 by isotyping analysis. The DI8 antibody bound to sulfatide as well as seminolipid, but did not bind to galactosylceramide, ganglioside GM3, heparin, biglycan and decorin, as evidenced by ELISA (Fig. 2A). The DI8 antibody also specifically bound to sulfatide, seminolipid and lactosylsulfatide SM3 in a thin-layer chromatogram-immunostaining analysis (Fig. 2B). However, DI8 did not bind to SM2, in which a sulfate group is attached to the internal galactose. In addition, DI8 did not bind to 3-*O*–sulfated β-galactose biotinylated polymeric-probe (Seikagaku Co.) immobilized to avidin-coated wells in ELISA (data not sown). These findings suggest that the DI8 antibody recognizes the terminal 3-*O*–sulfated galactose structure attached to the lipid molecule. A similar property was observed for other anti-sulfatide monoclonal antibodies, such as Sulph 1 (*15*). When testes from wild-type and CST-null mice (*3*) were tested with the DI8 antibody, the antibody clearly stained the seminolipid on the cell surface of spermatogenic cells of wild-type testis, whereas it did not react with any cells in the seminiferous tubules of CST-null testis (Fig. 2C). The overall staining pattern was similar to that for the Sulph 1 antibody, but DI8 gave a punctate pattern that was slightly more detailed (Fig. 2D).

DNA Sequences of the VH and VL Genes of the DI8 Anti-Sulfoglycolipid Antibody—Total RNA was extracted from the DI8 hybridoma, and the VH and VL genes that encode the IgG3 antibody were cloned by RT-PCR. The VH gene (333 bp) showed 84% identity to the mouse Q52 IgVH a-allotype "VOx-1" germline gene, and the VL gene (336 bp) showed 89% identity to the mouse IgVκ bd2 germline gene (Fig. 3).

Generation of scFv Antibody against Sulfoglycolipids—A recombinant scFv gene was constructed by connecting the VH and VL genes through a DNA linker coding

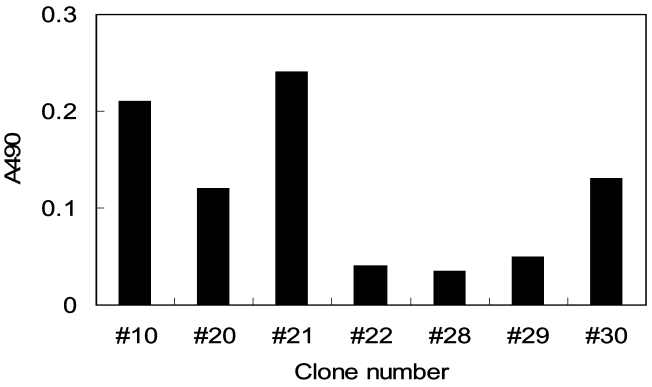

Fig. 4. **Screening of phage clones for reactivity to sulfatide.** Anti-sulfatide activity was examined by ELISA as described in "MATERIALS AND METHODS." The values indicate the absorbance after the subtraction of background absorbance (none coated).

$(Gly_4Ser)_3$. This scFv gene was then inserted into a phagemid vector pCANTAB 5E, and the resulting phagemid was referred to as pCANTAB-DI8. After the transfection of pGANTAB-DI8 into *E. coli* TG1 cells, complete phage particles were rescued by use of a helper phage, M13KO7. The phage clones were then screened for reactivity to sulfatide (Fig. 4). All of the positive phage clones included the inserted scFv gene (data not shown), indicating that the phage display system functioned well. These findings indicate that the scFv antibody displayed at the tip of the M13 phage as a g3p fusion protein was able to bind to sulfatide.

To produce a soluble form of scFv antibody, *E. coli* HB2151 cells were infected with the phage in the supernatant from the positive clones. DNA extracted from the infected HB2151 cells contained the scFv insert (Fig. 5A). A Western blotting analysis showed that a soluble scFv protein with a size of 30 kDa was mainly secreted into the periplasmic fraction of the infected HB2151 clones (Fig. 5B). The reactivity of the soluble scFv antibody to various glycolipids and sulfated glycoconjugates was then examined by ELISA. As a result, the soluble scFv antibody retained the ability to bind to sulfoglycolipids in a similar way to the intact DI8 antibody (Fig. 5C). The scFv antibody did not bind to galactosylceramide, ganglioside GM3, heparin, biglycan and decorin. To demonstrate affinity and capacity of the soluble scFv antibody, titration curves of the native DI8 antibody, Sulph-1 and the scFv antibody toward sulfatide were drawn (Fig. 6). The binding affinity of the native DI8 antibody was slightly higher than that of the Sulph-1 antibody, while their binding capacities were almost equal. On the other hand, the affinity and binding capacity of the scFv antibody were much lower than that of the native DI8 antibody.

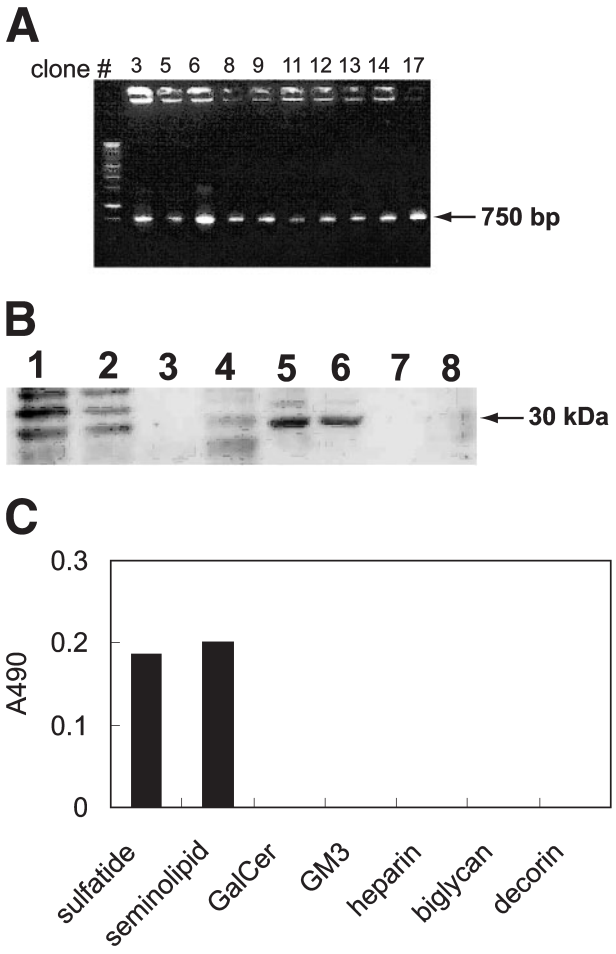

Fig. 5. **Generation of a soluble scFv antibody against sulfoglycolipids.** A soluble scFv antibody was produced in *E. coli* HB2151 cells, a nonsuppressor strain, as described in "MATERIALS AND METHODS." (A) Detection of the scFv DNA (750 bp) by PCR. DNA was extracted from the infected HB2151 colonies and subjected to PCR using scFv-specific primers. (B) Detection of a soluble scFv protein by Western blotting. The soluble scFv protein (30 kDa) was stained with an anti–E-tag antibody. Supernatants (lanes 1–4) and periplasmic extracts (lanes 5–8) of the infected HB2151 clones: #13 (lanes 1 and 5), #14 (lanes 2 and 6) and mock (lanes 3 and 7) were subjected to SDS-PAGE. The levels of soluble scFv were significantly reduced when HB2151 clone #13 was cultured in the presence of 2% glucose, which turns off the *lac* promotor (lanes 4 and 8). (C) Reactivity of the soluble scFv antibody to various glycolipids and sulfated glycoconjugates in ELISA. The values indicate the absorbance after the subtraction of background absorbance (none coated).

DISCUSSION

The sulfate group of sulfoglycolipids is transferred from 3′-phosphoadenosine 5′-phosphosulfate by CST, a glycolipid-specific sulfotransferase (*2*). Since *Cst*$^{-/-}$ mice are inherently devoid of sulfoglycolipids (*3*, *4*), the normally auto-antigenic sulfatide acts as a xenobiotic in the mutant mice. Therefore, it would be expected that antibodies against sulfatide could be raised more easily in CST-null mice than wild-type mice. This was found to be case in this study. This success prompted us to attempt to produce an antibody capable of discriminating between sulfatide and seminolipid. However, all the antibodies cross-reacted with both sulfoglycolipids, even though they were raised against sulfatide or seminolipid separately (data not shown). There is no anti-sulfoglycolipid antibody with the capability to distinguish between the lipid moieties at this moment (*1*). The staining pattern of the testis with the DI8 antibody was roughly similar to that with the Sulph 1 antibody, but slightly different in the details. These findings suggest that DI8 and Sulph I

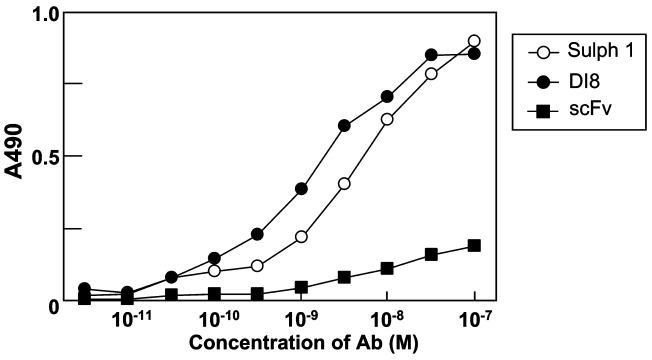

Fig. 6. **Titration curves of Sulph 1, the native DI8 and soluble scFv antibodies toward sulfatide.** Indicated concentrations of the antibodies were reacted with sulfatide by ELISA as described in "MATERIALS AND METHODS." HRP-conjugated anti-Etag antibody (Amersham Pharmacia Biotech) was used as a second antibody for the soluble scFv antibody.

are able to discern a subtle difference in a situation where seminolipid is expressed on the cell surface of germ cells.

The VH gene of the DI8 antibody was found to belong to the Q52 family. Moreover, highly homologous sequences were found in some anti-DNA antibodies (accession no. U55468, U55484 and AF072776) by a BLAST search. On the other hand, the DNA sequence of the DI8 Vκ gene belonging to the bd2 family showed similarity to those of an anti-dextran antibody (M17722) and an anti-DNA antibody (U28859). Considering that DNA and dextran are anionic molecules with a carbohydrate moiety like sulfoglycolipid, these findings suggest that a similar mechanism is involved in the recognition of these molecules.

It is generally thought that lipids are poorly immunogenic and, hence, it would be difficult to generate antibodies against them in comparison with proteins. In addition, it has been postulated theoretically and demonstrated experimentally that single-chain antibodies show a weaker binding affinity toward antigens than the corresponding intact antibodies, which are dimeric or oligomeric in the nature (*16*). These concerns raised the question of whether a single chain antibody can effectively bind to sulfoglycolipid. The present study shows that a monovalent scFv antibody retains binding ability toward sulfoglycolipid, although its binding affinity is weaker than that of the native antibody.

The scFv antibody is useful for investigating the biological functions of sulfoglycolipids. Sulfoglycolipids play important roles in development and differentiation during myelin formation and spermatogenesis (*3–6*). In addition to these physiological roles, sulfoglycolipids are present in increased levels in many human cancer cells including renal cancer cells (*17*), implying their pathological roles in tumor activities. Some such activities can be blocked by an anti-sulfoglycolipid antibody (*18*). The small size of scFv permits it to penetrate solid tumor tissues more effectively than natural immunoglobulins (*19*). Therefore, the scFv antibody could intervene and inhibit tumor growth or invasion more effectively. The generation of scFv antibodies could be very helpful, since these molecules could be further modified to produce chimeric molecules such as immunotoxin (*20*). If the scFv-green fluorescent protein chimera gene is introduced into sulfoglycolipid-expressing cancer cells, the locations of sulfoglycolipids in the living cells could be detected by fluorescent microscopy. In addition, the expression of the scFv gene within the cells could regulate the transport and biological function of sulfoglycolipids.

In the present study, an anti-sulfoglycolipid monoclonal antibody, referred to as DI8, was produced using CST-knockout mice. In addition, a recombinant scFv antibody against sulfoglycolipid was generated based on the VH and VL genes of the IgG3 antibody.

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REFERENCES

1. Ishizuka, I. (1997) Chemistry and functional distribution of sulfoglycolipids. *Prog. Lipid Res.* **36**, 245–319
2. Honke, K., Yamane, M., Ishii, A., Kobayashi, T., and Makita, A. (1996) Purification and characterization of 3′-phosphoadenosine-5′-phosphosulfate:GalCer sulfotransferase from human renal cancer cells. *J. Biochem.* **119**, 421–427
3. Honke, K., Hirahara, Y., Dupree, J., Suzuki, K., Popko, B., Fukushima, K., Fukushima, J., Nagasawa, T., Yoshida, N., Wada, Y., and Taniguchi, N. (2002) Paranodal junction formation and spermatogenesis require sulfoglycolipids. *Proc. Natl Acad. Sci. USA* **99**, 4227–4232
4. Honke, K., Zhang, Y., Cheng, X., Kotani, N., and Taniguchi, N. (2004) Biological roles of sulfoglycolipids and pathophysiology of their deficiency. *Glycoconj. J.* **21**, 59–62
5. Ishibashi, T., Dupree, J.L., Ikenaka, K., Hirahara, Y., Honke, K., Peles, E., Popko, B., Suzuki, K., Nishino, H., and Baba, H. (2002) A myelin galactolipid, sulfatide, is essential for maintenance of ion channels on myelinated axon but not essential for initial cluster formation. *J. Neurosci.* **22**, 6507–6514
6. Hirahara, Y., Bansal, R., Honke, K., Ikenaka, K., and Wada, Y. (2004) Sulfatide is a negative regulator of oligodendrocyte differentiation: Development in sulfatide-mull mice. *Glia* **45**, 269–277
7. Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569–572
8. Rodemer, C., Thai, T.-P., Brugger, B., Kaercher, T., Werner, H., Nave, K.-A., Wieland, F., Gorgas, K., and Just, W.W. (2003) Inactivation of ether lipid biosynthesis causes male infertility, defects in eye development and optic nerve hypoplasia in mice. *Hum. Mol. Genet.* **12**, 1881–1895
9. Rosok, M.J., Yelton, D.E., Harris, L.J., Bajorath, J., Hellstrom, I., Cruz, G.A., Kristensson, K., Lin, H., Huse, W.D., and Glaser, S.M. (1996) A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. *J. Biol. Chem.* **271**, 22611–22618
10. Colcher, D., Pavlinkova, G., Beresford, G., Booth, B.J., Choudhury, A., and Batra, S.K. (1998) Pharmacokinetics and biodistribution of genetically-engineered antibodies. *Q. J. Nucl. Med.* **42**, 225–241
11. Ozawa, H., Kotani, M., Kawashima, I., and Tai, T. (1992) Generation of one set of monoclonal antibodies specific for b-pathway ganglio-series gangliosides. *Biochim. Biophys. Acta* **1123**, 184–190
12. Stocker, J.W., Forster, H.K., Miggiano, M., Stahli, C., Staiger, G., Takacs, B., and Staehelin, T. (1982) Generation of 2 new mouse myeloma cell lines 'PAI' and 'PAI-0' for hybridoma production. *Res. Disclos.* **217**, 155–157

13. Leger, O.P. and Saldanha, J.W. (2000) Preparation of recombinant antibodies from immune rodent spleens and the design of the humanization by CDR grafting in *Monoclonal Antibodies* (Shepherd, P. and Dean, C., eds.) pp. 25–65, Oxford University Press, Oxford
14. Amersdorfer, P., Wong, C., Chen, S., Smith, T., Deshpande, S., Sheridan, R., Finnern, R., and Marks, J.D. (1997) Molecular characterization of murine humonal immune response to botulinum neurotoxin type A binding domain as assessed by using phage antibody libraries. *Infect. Immun.* **65**, 3743–3752
15. Fredman, P., Mattson, L., Andersson, K., Davidsson, P., Ishizuka, I., Jeansson, S., Mansson, J.-E., and Svennerholm, L. (1988) Characterization of the binding epitope of a monoclonal antibody to sulphatide. *Biochem. J.* **251**, 17–22
16. Goel, A., Colcher, D., Baranowska-Kortylewicz, J., Augustine, S., Booth, B.J., Pavlinkova, G., and Batra, S.K. (2000) Genetically engineered tetravalent single-chain Fv of the pancarcinoma monoclonal antibody CC49: Improved biodistribution and potential for therapeutic application. *Cancer Res.* **60**, 6964–6971
17. Honke, K., Tsuda, M., Hirahara, Y., Miyao, N., Tsukamoto, T., Satoh, M., and Wada, Y. (1998) Cancer-associated expression of glycolipid sulfotransferase gene in human renal cell carcinoma cells. *Cancer Res.* **58**, 3800–3805
18. Kobayashi, T., Honke, K., Kuramitsu, Y., Hosokawa, M., Miyazaki, T., Murata, J., Saiki, I., Ishizuka, I., and Makita, A. (1994) Cell-surface sulfoglycolipids are involved in the attachment of renal-cancer cells to laminin. *Int. J. Cancer* **56**, 281–285
19. Yokota, T., Milenic, D.E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* **52**, 3402–3408
20. Tur, M.K., Sasse, S., Stocker, M., Djabelkhir, K., Huhn, M., Matthey, B., Gottstein, C., Pfitzner, T., Engert, A., and Barth, S. (2001) An anti-GD2 single chain Fv selected by phage display and fused to Pseudomonas exotoxin A develops specific cytotoxic activity against neuroblastoma derived cell lines. *Int. J. Mol. Med.* **8**, 579–58421
21. Taki, T., Kasama, T., Handa, S., and Ishikawa, D. (1994) A simple and quantitative purification of glycospingolipids and phospholipids by thin-layer chromatography blotting. *Anal. Biochem.* **223**, 232–238