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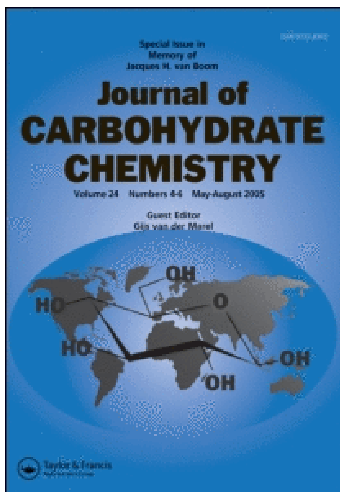

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
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On the Specificity of Anti-Sulfoglucuronosyl Glycolipid Antibodies

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To cite this Article Tokuda, Akira , Ariga, Toshio , Isogai, Yukihiro , Komba, Shiro , Kiso, Makoto , Hasegawa, Akira , Tai, Tadashi and Yu, Robert K.(1998) 'On the Specificity of Anti-Sulfoglucuronosyl Glycolipid Antibodies', Journal of Carbohydrate Chemistry, 17: 4, 535 — 546

To link to this Article: DOI: 10.1080/07328309808002335

URL: http://dx.doi.org/10.1080/07328309808002335

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J. CARBOHYDRATE CHEMISTRY, 17(4&5), 535-546 (1998)

ON THE SPECIFICITY OF ANTI-SULFOGLUCURONOSYL GLYCOLIPID ANTIBODIES[1]

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ABSTRACT

The mouse monoclonal anti-human HNK-1 antibody (also variously known as L2, Leu-7, CD57, VC1.1), monoclonal anti-sulfoglucuronosyl glycosphingolipids (SGGLs) antibody (mAb NGR50), and human sera from patients with demyelinating neuropathy and IgM paraproteinemia, are known to react with not only SGGLs, including sulfoglucuronosyl paragloboside (SGPG) and sulfoglucuronosyl lactosaminyl paragloboside (SGLPG), but also glycoproteins, such as myelin-associated glycoprotein (MAG), P0, PMP22, and certain adhesion molecules. These antigens are known to possess the so-called HNK-1 epitope (3-sulfoglucuronic acid, SGA) moiety. To further define the precise structural requirement of this carbohydrate epitope, we chemically synthesized 14 SGGLs, and their nonsulfated derivatives with defined carbohydrate chain lengths and aglycone structures. The various aglycones include ceramide, 2-(tetradecyl)hexadecyl (B30), and 2-(trimethylsilyl)ethyl (SE). These synthetic SGGLs were tested for their immunoreactivity with the above antibodies by high-performance thin-layer chromatography (HPTLC)-immunoblotting and ELISA. The anti-HNK-1 antibody (VC1.1) reacted with SGGL analogues containing a minimum of two sugars (SGA-Gal-Cer), but not with non-sulfated derivatives of SGGLs nor with SGGLs having a modified ceramide structure. mAb NGR50, on the other hand, reacted with only SGPG and SGLPG. A human patient serum (LT) reacted with all synthetic SGGLs except those with an SE aglycone structure. On the other hand, another human patient serum (YT),

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like the anti-HNK-1 antibody, VC1.1, reacted with SGPG, SGLPG, and SGGL analogues containing a minimum of two sugars (SGA-Gal-Cer). All antibodies reacted more strongly with synthetic SGGLs with longer carbohydrate chains. These results indicate that anti-SGGL antibodies recognize a minimum of two sugars bearing the following structure (3-sulfoglucuronosyl β1-3 galactosyl, SGA-Gal-) and that the aglycone ("ceramide") structure appears to play an important role for antibody-antigen interaction.

INTRODUCTION

Glycosphingolipids (GSLs) are important constituents of virtually all tissues and body fluids and are particularly abundant in the nervous system. In addition to their putative functions as signal modulators and cell recognition and adhesion molecules, recent studies have implied that they serve as important antigens and may underlie the pathogenesis of a number of immune-mediated diseases.[2] For example, antibodies activities against specific GSLs have been reported in patients with certain neurological disorders, including demyelinating peripheral neuropathy, acute or chronic inflammatory polyneuropathy (AIDP or CIDP), and motor neuron disease (MND).[2] In recent years, there is mounting evidence suggesting that SGGLs, including SGPG, and SGLPG are target antigens for demyelinating neuropathies. Sera from these patients have been shown to react with SGGLs,[3] which possess common L2/HNK-1 or SGA carbohydrate epitope as myelin-associated glycoprotein (MAG),[4] P0 protein,[5] PMP-22,[6] Schwann cell membrane protein,[7] several low molecular weight glycoproteins in the peripheral nerve,[8] and certain adhesion molecules.[9] The so-called anti-HNK-1 antibody was raised originally to a membrane antigen on human T cells, HSB-2,[10] and then several antibodies including L2,[8] 4F4,[11] NC-1,[12] VC1.1,[13] Elec-39,[14] and NGR50[15] as well as human patient monoclonal antibodies[2] have all been reported to react with the HNK-1 epitope. However, the precise structural requirement of this carbohydrate epitope reacting with the various antibodies, which are known to exhibit subtle differences in antigenic specificity, have never been clearly defined. Since these antibodies are known to be responsible for the clinical manifestation of neuropathies,[3] and they appear to recognize different aspects of the HNK-1 epitope, further studies on the fine structural features are warranted.

In this investigation we have determined the specificity of several anti-HNK-1 antibodies against synthetic SGGLs and their non-sulfated derivatives with modified aglycone and carbohydrate structures (Scheme 1) by high-performance thin-layer

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Compound	
189	GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (lignoceric acid)
190	HSO_3-3GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (lignoceric acid)
191	GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (Stearic acid)
192	HSO_3-3GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer (Stearic acid)
205	GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-SE
206	HSO_3-3GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-SE
207	GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-B30
208	HSO_3-3GlcUAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-B30
209	GlcUAβ1-3Galβ1-1'Cer (lignoceric acid)
210	HSO_3-3GlcUAβ1-3Galβ1-1'Cer (lignoceric acid)
216	GlcUAβ1-3Gal[β1-4GlcNAcβ1-3Gal]$_2$β1-4Glcβ1-1'Cer (lignoceric acid)
217	HSO_3-3GlcUAβ1-3Gal[β1-4GlcNAcβ1-3Gal]$_2$β1-4Glcβ1-1'Cer (lignoceric acid)
218	GlcUAβ1-3Gal[β1-4GlcNAcβ1-3Gal]$_2$β1-4Glcβ1-1'Cer (stearic acid)
219	HSO_3-3GlcUAβ1-3Gal[β1-4GlcNAcβ1-3Gal]$_2$β1-4Glcβ1-1'Cer (stearic acid)

Scheme 1

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chromatography (HPTLC)-immunooverlay and enzyme-linked immunosorbent assay (ELISA). We found each of the antibodies tested exhibited different reactivity to the various synthetic SGGLs, and that the ceramide structure and carbohydrate chain length were important determinants for the interaction between the antibody and the target antigen.

RESULTS

Chromatographic Behavior of Synthetic SGGLs and Their Reactivities with Human Sera

Synthetic SGGLs were developed with solvent A using HPTLC plates and immunostained with LT serum or YT serum as shown in Figs. 1 and 2C, respectively. All synthetic SGGLs except compounds **205** and **206** comigrated with the corresponding authentic samples of SGGLs isolated from bovine cauda equina. The nonsulfated

derivatives of SGGLS migrated slightly higher than SGGLs, consistent with the fact that they lack a sulfate group. SGGLs containing lignoceric acid in the ceramide portion migrated slightly higher than those containing stearic acid. SGGLs having longer carbohydrate chains migrated at lower positions as compared with those with shorter chains. However, SGGLs having a 2-(trimethylsilyl)ethyl (SE) moiety migrated near the origin.

With respect to its immunoreactivities, LT serum had a broad antigenic specificity, reacting with most SGGLs and nonsulfated SGGLs except those having the aglycone SE (compounds **205** and **206**). The reactivities were stronger with SGLPG than SGPG, indicating that the length of the carbohydrate chain plays an important role in immunoreactivity. In comparing the effect of the aglycone structure, LT serum reacted only weakly with SGGLs having B30 as compared with those having ceramide as well as the nonsulfated derivatives of SGGLs (Fig. 1). YT serum, on the other hand, reacted strongly with SGGLs, compounds **192** and **219**, but not the nonsulfated derivatives of SGGLs nor SGGLs with B30 or SE. YT serum also reacted strongly with compound **210** which has only 2 sugar units in the carbohydrate chain (Fig. 2C).

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Reactivities with Anti-HNK-1 Antibody (Leu-7; CD57; VC1.1) and Anti-SGGL Antibody (mAb NGR50)

Both VC1.1 and mAb NGR50 reacted with SGPG **192** and SGLPG **219**, but not with nonsulfated derivatives of SGGLs, nor those with SE or B30 (compounds **206, 208**) (Figs. 2A, and 2B). VC1.1 also reacted with compound **210** which has only two terminal sugars, [3-sulfated glucuronosyl β1-3-galactosyl (SGA-Gal)] (Fig. 2A). These monoclonal antibodies also reacted more strongly with SGLPG than with SGPG.

ELISA

The immunoreactivity profiles obtained from ELISA were nearly the same as those from HPTLC-immunoblotting (Figs. 3 and 4). The anti-HNK-1 antibody VC1.1 and human sera, LT and YT, reacted more weakly with SGGL having only the terminal two sugars (compound **210**) as compared to SGPG (compound **192**). No difference was observed for all antibodies when comparing SGGLs having a ceramide structure containing lignoceric acid and stearic acid. LT serum reacted more weakly with the nonsulfated derivatives of SGGLs than the corresponding sulfated counterparts.

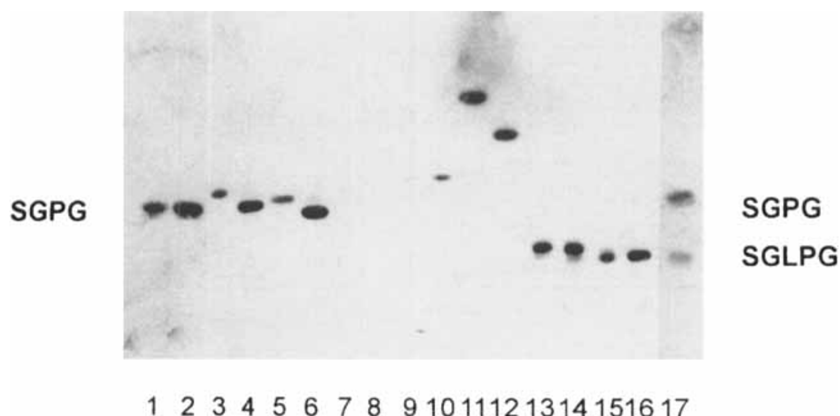

Figure 1. Binding of LT serum to synthetic SGGLs on HPTLC plate. Synthetic SGGLs and an authentic SGPG standard were applied on HPTLC plates. Lanes 1 and 2, authentic SGPG standard, 30 and 50 ng, respectively; Lane 3, 189, 300 ng; Lane 4, 190, 300 ng; Lane 5, 191, 300 ng; Lane 6, 192, 300 ng; Lane 7, 205, 1.8 ug; Lane 8, 206, 1 ug; Lane 9, 207, 300 ng; Lane 10, 208, 300 ng; Lane 11, 209, 1.3 ug; Lane 12, 210, 300 ng; Lane 13, 216, 80 ng; Lane 14, 217, 30 ng; Lane 15, 218, 50 ng; Lane 16, 219, 30 ng; Lane 17, authentic SGPG and SGLPG. Bands were immunoblotted with LT serum, diluted 1:100. The plate was developed in solvent A. HPTLC-overlay method was carried out as described in EXPERIMENTAL.

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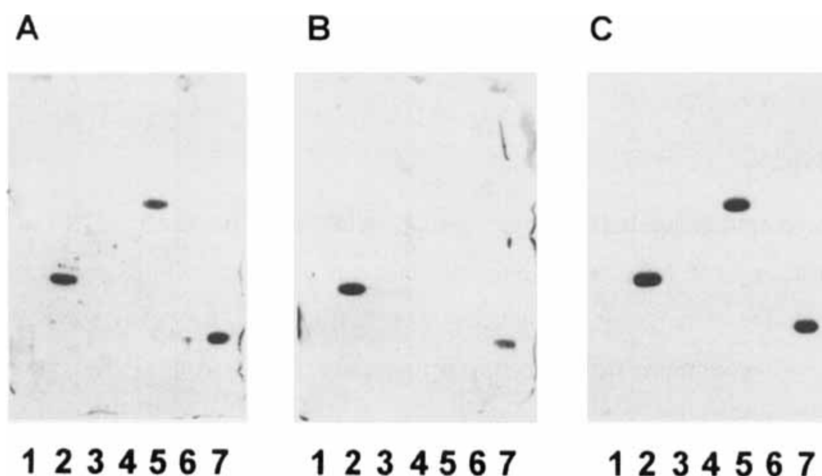

Figure 2. Binding of anti-SGGLs antibodies to synthetic SGGLs on HPTLC plates. Synthetic SGGLs were applied on HPTLC plates. Lane 1, 191, 300 ng; Lane 2, 192, 300 ng; Lane 3, 206, 1 ug; Lane 4, 208, 300 ng; Lane 5, 210, 300 ng; Lane 6, 218, 50 ng; Lane 7, 219, 30 ng. A: Bands were immunoblotted with mAb VC1.1, diluted 1:100. B: Bands were immunoblotted with mAb NGR50, diluted 1:100. C: Bands were immunoblotted with YT serum, diluted 1:100. Each plate was developed in solvent A. HPTLC overlay method was carried out as described in EXPERIMENTAL.

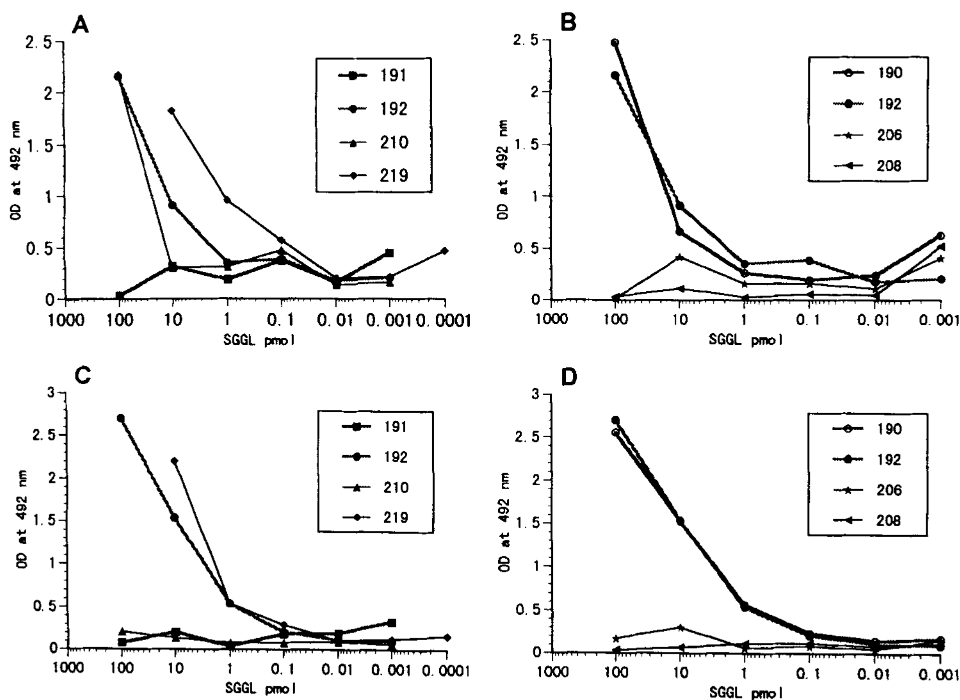

Figure 3. Sensitivity test of mAb VC1.1 and NGR50 toward synthetic SGGLs by ELISA. Microtiter wells were coated with different amounts of synthetic SGGLs (from 0.1 pmol-100 pmol), followed by addition of mAb VC1.1, (A and B), NGR50, (C and D). ELISA was carried out as described in EXPERIMENTAL.

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DISCUSSION

Glycosphingolipids (SGLs) and glycoproteins which possess the HNK-1 epitope are known to have important biological functions such as cell-cell interaction and adhesion during nervous system development. In addition, they may serve as target antigens for autoimmune demyelinating neuropathies.[2,3,9,14,16] Anti HNK-1 monoclonal antibody raised originally to antigenic marker of natural killer cells[10] have been used widely to investigate the development of nervous systems. However, the precise structural requirements for these antibodies have not been clearly delineated. They have been reported to recognize the terminal 3-sulfoglucuronosyl β1-3 galactosyl residue (SGA-Gal).[3,16]. Schmitz et al.[17] further reported that both the sulfated glucuronic acid and the neolactosyl core structures in synthetic SGGLs are essential for recognition of the

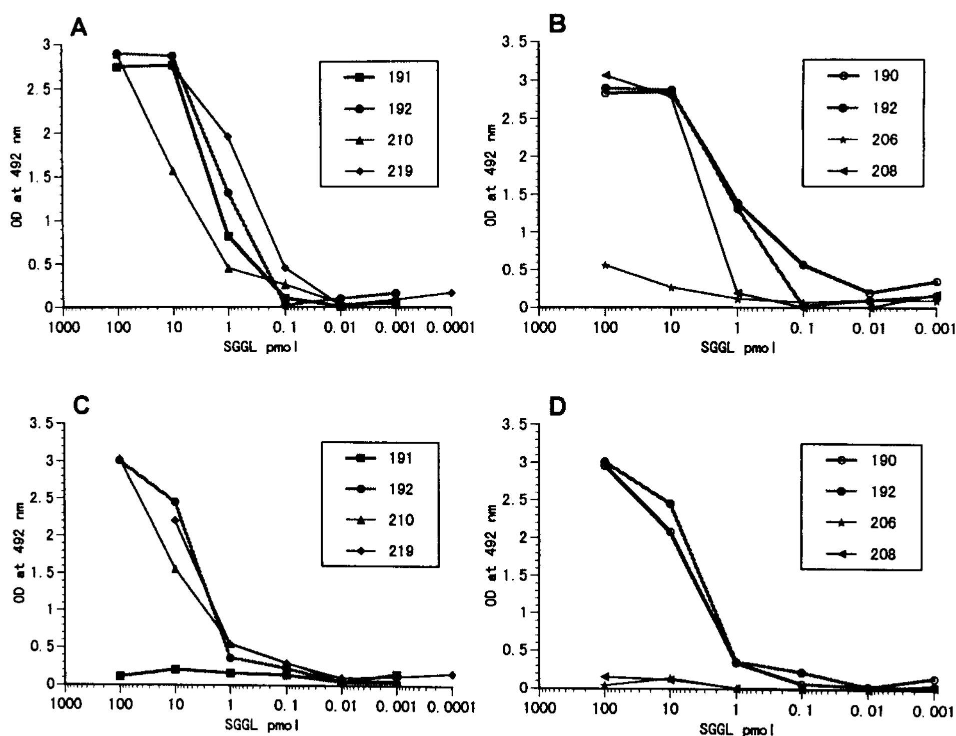

Figure 4. Sensitivity of human patients' sera LT and YT toward synthetic SGGLs by ELISA. Microtiter wells were coated with different amounts of synthetic SGGLs (from 0.1 pmol-100 pmol), followed by addition of LT serum, (A and B), YT serum, (C and D). ELISA was carried out as described in EXPERIMENTAL.

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L2/HNK-1 antibody and these structural features may be responsible for the biological function of these glycoconjugates. A GSL with the sulfated glucuronic acid as the only carbohydrate unit was inactive for the L2/HNK-1 antibody,[17] suggesting the requirement of additional structural features for the antigenic determinant. Thus, in binding studies for the anti HNK-1 antibody the carboxyl group but not the sulfate group in the terminal glucuronic acid was shown to be important.[3] Because the desulfated derivative of SGPG could interact with the serum from a patient (LT) with demyelinating neuropathy, whereas the desulfated lactone form could not, it suggests that the carboxyl residue in glucuronic acid is absolutely essential for binding to the anti-HNK-1 antibody.[3]

In this study we have extended the previous investigation using a series of synthetic SGGLs to further define the structural requirement for several antibodies

purported to have similar immunoreactivity. These antibodies include the anti-HNK-1 (Leu-7, CD57, VC1.1, or L2), mAb NGR50, and sera from two representative patients (LT and YT) suffering from demyelinating neuropathies and IgM gammopathy. LT serum showed the widest immunoreactivity profile by reacting with both SGGLs and the desulfated forms of SGGLs, as well as SGGLs with an artificial lipoidal anchor, B30. It also interacted with an SGGL and its nonsulfated derivative with only two terminal carbohydrate units. In contrast, YT serum had a much stricter structural requirement than LT in reacting only with SGPG and SGLPG. Such a difference in immunoreactivity may very well underlie the difference in clinical manifestation among patients, as demonstrated previously by Kusunoki et al.[3] Clearly this is an area that requires further investigation.

Concerning the anti-HNK-1, we found that VC1.1 (CD57) reacted with all SGGLs, but not their nonsulfated derivatives. Thus, this antibody requires the terminal sulfated glucuronic acid residue for reactivity. Since this antibody reacted also with compound **210**, HSO_3-3GlcUAβ1-3Galβ1-1'Cer but not with one with only the sulfated glucuronic acid residue,[17] it suggests that this antibody recognizes a minimum of two sugars (SGA-Gal-) instead of the entire neolactosyl core structure present in naturally occurring SGGLs.

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We have previously reported that mAb NGR50 reacts with SGPG and SGLPG, but not with other GSLs nor the nonsulfated derivatives of SGPG and SGLPG.[15] It reacted only weakly with several glycoproteins bearing the "HNK-1 carbohydrate epitope."[15] In the present study we have confirmed the previous observation. Interestingly, it did not react with compound **210** which has only two carbohydrate units, SGA-Gal-. Since this monoclonal antibody was generated using the natural occurring SGPG as the antigen, this strict antigenic requirement may suggest that it recognizes additional structural features such as the neolactosyl core. This is in contrast to anti-HNK-1 antibody (CD57;VC1.1) which was generated using a membrane fraction of the HNK-1 cells as the antigen. Thus, the difference in antigenic presentation may account for the difference in antibody specificity.

All antibodies studied here showed stronger immunoreactivity toward SGGLs with longer carbohydrate chain lengths: SGLPG > SGPG > SGA-Gal-Cer. Since SGLPG has an additional lactosamine unit (GlcNAc-Gal) than SGPG, the higher immunoreactivity may reflect the more exposed epitope which renders it more accessible to antibody

recognition on the solid surface.[18] With respect to the influence of the aglycone structure, all antibodies had a high reactivity for SGGLs with a ceramide structure, but there was no significant difference on reactivities for ceramides having a stearic acid or a lignoceric acid moiety. Anti-HNK-1 antibody, mAb NGR50, and human patient serum (YT) did not react with SGGLs with an artificial lipid anchor, B30. B30 is a branched long-chain alkane, but does not have an amide group at the branched position. This suggests that either the amide functional group may participate in antibody recognition or B30 may alter the antigen configuration on the solid surface, thus rendering the antigen unrecognizable by these antibodies. We favor the former explanation because human serum (LT) reacted with SGGLs having B30, although its reactivity was much weaker than that of SGGLs with a ceramide structure. Interestingly none of the antibodies tested interacted with SGGLs having a 2-(trimethylsilyl)ethyl (SE) residue (e.g. compound **206**). A plausible explanation for this observation could be that the absence of a "lipid-like" anchor in the aglycone structure renders the test antigens unable to firmly attach to the solid phase for effecting antibody-antigen interaction.

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Antibodies to SGGLs have been reported to be associated with the pathogenesis of demyelinating neuropathies because therapeutic reduction of these antibodies, for example, by plasmapheresis or administration of immunosuppressants, brings about improvement of clinical symptoms with a concomitant decrease in antibody titer.[2] However factors contributing to the heterogeneity in clinical manifestation, e.g., sensory or motor dominancy, in patients associated with anti-SGGL antibodies are only poorly understood.[3] In this study we have shown that sera from patients with demyelinating diseases possess different specificities for the various SGGL derivatives. For example, YT serum which resembles WW serum reported earlier[19] requires both the sulfate moiety in glucuronic acid and a neolactotetraose core structure. It thus resembles anti-HNK-1 antibody VC1.1 or Leu-7 with respect to the antigen structural requirement. In contrast, LT serum is considerably more promiscuous, reacting with the sulfated and unsulfated derivatives with as little as only two sugars, GlcUA-Gal, for reactivity. An understanding of the precise antigenic requirement should help design suitable diagnostic tests for these disorders as well as unravel the biological functions of the glycoconjugates bearing this interesting carbohydrate epitope.

EXPERIMENTAL

Preparation of SGGLs

Naturally occurring SGGLs (SGPG and SGLPG) were isolated and purified from bovine cauda equina according to the method described previously.[3,20] The purity of the SGGLs was examined by high-performance thin-layer chromatography (HPTLC) using at least two different solvent systems: chloroform-methanol-0.22% calcium chloride in water (50:45:10, v/v; solvent A) and chloroform-methanol-2.5N ammonium hydroxide-0.4% calcium chloride in water (50:45:5:5, v/v; solvent B).

Synthetic SGGLs (compounds **189**, **190**, **191**, **192**, **205**, **206**, **207**, **208**, **209**, **210**, **216**, **217**, **218**, **219**) were chemically synthesized in our laboratory (Scheme 1).[21] They were divided into three categories: SGGLs with modified carbohydrate chain lengths, consisting of 2, 5, or 7 sugar units with the SGA moiety at the non-reducing terminus; SGGLs with a modified aglycone, consisting of ceramide (containing lignoceric acid or stearic acid), SE, or B30; non-sulfated SGGLs with varying carbohydrate chain lengths and aglycone structures.

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Anti-HNK-1 antibodies

Sera from patients (LT, YT) with demyelinating neuropathy and IgM paraproteinemia were kindly provided by Dr. A. Pachner (LT; Georgetown University School of Medicine, Washington, DC) and Dr. M. Kuriyama (YT; Fukui Medical School, Fukui, Japan), respectively. Both LT and YT have been reported to interact strongly with MAG and SGGLs.[3,18] The mouse monoclonal antibody NGR50 was generated in our laboratory,[15] and monoclonal anti-HNK-1 antibody (Leu-7, CD57, or VC1.1) was purchased from Sigma Chemicals Co. (St. Louis, MO).

High-performance thin-later chromatography (HPTLC) and HPTLC-immunooverlay method

HPTLC-immunoblotting was performed as previously described.[15,22] Synthetic and naturally occurring SGGLs were chromatographed on a HPTLC plate (Merck, Darmstadt, Germany) with solvent A. After drying, the plate was dipped in a 0.2% poly(isobutyl methacrylate) (Aldrich Chemical Co., Milwaukee, WI) solution in *n*-hexane. Human serum, mAb NGR50, or HNK-1 antibody diluted 1:100 in a dilution buffer [0.3 % gelatin in phosphate-buffered saline (PBS), pH 7.3] was applied to the plate and incubated at room temperature for 1 h. After washing three times with PBS, the plate was incubated

with peroxidase-conjugated goat anti-human IgM or anti-mouse IgG or IgM (Cappel, West Chester, PA) diluted 1:1000 in dilution buffer for 1 h at room temperature. After washing with PBS, the plate was then incubated with cyclic diacyl hydrazine solution (ECL detection reagent, Amersham, Arlington Heights, IL) for 1 min and exposed to an X-ray film. To visualize the glycolipids, the plate was sprayed with the orcinol-sulfuric acid reagent followed by heating at 120 °C.[3]

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed by the method described previously.[15,19] Each of the microtiter wells (96-well microtiter plate, Nunc-Immunoplate, Polysorp, Denmark) was coated with various amounts of synthetic SGGLs. After blocking nonspecific sites with dilution buffer for 30 min at room temperature, the wells were incubated with various antibodies diluted 1:100 in a dilution buffer at room temperature for 1 h. After washing three times with PBS, a peroxidase-conjugated goat anti-human IgM solution or anti-mouse IgG or IgM diluted 1:1000 in a dilution buffer was added, and incubation was carried out at room temperature for 1 h. Coloring reaction was generated by SIGMA FAST OPD tablet (Sigma Chemical Co. St. Louis, MO), and Optical density (OD) at 492 nm was determined with an ELISA reader.

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ACKNOWLEDGMENTS

This work was supported by USPHS grant NS-26994-10, a grant from the National Multiple Sclerosis Society (RG2690A6/2) (to RKY), a grant from The Toa Boshoku Co. Ltd., and in part by Grant-in-Aid for Scientific Research on Priority Areas (05274102 and 07273226), Japan.

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