Detection of antibody to sialyl-i, a possible antigen in patients with Meniere’s disease

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Received 9 September 1999; received in revised form 6 January 2000; accepted 28 January 2000

Abstract

An autoimmune hypothesis for the etiology of Meniere’s disease has been proposed. In this study, we focused on gangliosides as potential antigens for autoantibodies in Meniere’s disease patients. In an attempt to investigate ganglioside antigens which respond to the serum of patients with Meniere’s disease, we analyzed gangliosides of human acoustic neurinomas, and used them as antigens to broadly explore gangliosides that react to serum. All the acoustic neurinoma samples used in the present study showed a similar ganglioside profile on TLC (thin-layer chromatography). For the microscale ganglioside analysis, a newly developed TLC blotting/secondary ion mass spectrometry (SIMS) system together with TLC immunostaining method was employed. Most of the ganglioside bands could be analyzed, and they were identified as GM3, GM2, SPG, GM1a, GD3, S-i (sialyl-i ganglioside) and GD1a. GD1a was the predominant ganglioside and many neolactoseries gangliosides were recognized by immunological analysis. Next, the immune reactivity of serum samples, from patients with Meniere’s disease, with the acoustic neurinoma gangliosides was studied by TLC immunostaining. The result showed that five of 11 patients with Meniere’s disease and one of eight normal subjects reacted with a specific band, which was identified as S-i by the TLC blotting/SIMS system. The findings of the present study indicate that S-i ganglioside is an autoantigen and possibly involved in the pathogenesis of Meniere’s disease. © 2000 Elsevier Science B.V. All rights reserved.

Abbreviations: TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; SIMS, secondary ion mass spectrometry; PVDF, polyvinylidene difluoride; MAb, monoclonal antibody; BSA, bovine serum albumin; BBG, bovine brain ganglioside; C16:0, hexadecanoic acid; C18:0, octadecanoic acid; C24:0, tetracosanoic acid; d18:1, 2-amino-4-octadecene-1,3-diol; Hex, hexose; HexNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; Cer, ceramide; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylgalactosamine; SPG (sialosyl paragloboside), NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer; S-i (sialyl-i ganglioside), NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer; S-I (sialyl-I ganglioside), NeuAc α 2-3Gal β 1-4GlcNAc β 1-3 (NeuAc α 2-3Gal β 1-4GlcNAc β 1-6) Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer; nLc4Cer (paragloboside), Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer; SGPG (sulfoglucuronosyl paragloboside), SO3-3GlcA β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer; SGLPG (sulfoglucuronosyl lactosaminyl paragloboside), SO3-3GlcA β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-1Cer

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1. Introduction

Meniere’s disease is an inner ear disease characterized by episodic attacks of vertigo, fluctuating cochlear hearing loss, tinnitus, and a feeling of fullness in the affected ear. The condition underlying Meniere’s disease has been found to be endolymphatic hydrops [2]. However, the causes of endolymphatic hydrops remain unknown, although various hypotheses have been proposed. Recently, the autoimmune hypothesis has been proposed and actively discussed [3,4]. This hypothesis is based on clinical findings of increases in blood immune complex levels [5,6], the efficacy of steroids for hearing loss [3,5,7,8], and the high concentration of IgG in endolymph [9], as well as the basic findings that endolymphatic hydrops are observed in animals immunized with such antigens as inner ear tissues [10,11] or collagen [12,13], and that immune reaction in the endolymphatic sac causes fluctuating hearing loss [14]. Gangliosides are acidic glycosphingolipids with sialic acid, and are particularly abundant in the vertebral nervous system. We have elucidated the involvement of glycosphingolipids in the onset of Guillain-Barré syndrome, an autoimmune neurological disease [15]. The inner ear has peripheral sensory cells, and is thought to include large amounts of gangliosides. We planned to examine whether autoantibodies against ganglioside in the inner ear play a role in the etiology of Meniere’s disease. However, it is extremely difficult to collect samples of human inner ear tissues, and amounts are extremely small even if samples can be collected. Accordingly, we attempted to take advantage of the neoplastic growth of the 8th cranial nerve (vestibular nerve) in acoustic neurinoma [16]. Neurinoma is a benign tumor which originates from Schwann cells in the peripheral nerve. Despite the nerve origin of neurinomas, very few studies have been made on gangliosides in them. We analyzed gangliosides of human acoustic neurinomas, and used them as antigens to broadly explore gangliosides that react to the sera of patients with Meniere’s disease. We used TLC blotting/SIMS [17,18], as well as a combination of this technique and TLC immunostaining. The purpose of this study is to isolate and characterize the gangliosides of human acoustic neurinoma, and to examine the ganglioside antigens that respond to sera from patients with Meniere’s disease.

2. Materials and methods

2.1. Materials

Acoustic neurinoma samples were obtained from seven cases. Surgery was performed at the Department of Otolaryngology, Tokyo Medical and Dental University. All cases were diagnosed histopathologically as neurinoma (mixed type Antoni A+B).

2.2. Purification of gangliosides

Acoustic neurinoma samples were suspended in a mixture of chloroform:methanol (2:1, by vol). Lipids were extracted by stirring the mixture, and then filtering it through a glass wool-packed Pasteur pipette and evaporating the filtrate to dryness. Total lipid extracted from the tumor was applied to a DEAE Sephadex column. Neutral glycolipids and most of the phospholipids were eluted with a mixture of chloroform:methanol:water (30:60:8, v/v). Gangliosides were eluted with a mixture of chloroform:methanol:0.8 N CH3COONa (30:60:8, v/v). The ganglioside-containing fraction was evaporated to dryness under reduced pressure in a rotary evaporator, and then dissolved in distilled water. The ganglioside solution was applied to a Sep-Pak C18 (Waters Division of Millipore, Milford, MA, USA). Gangliosides were eluted with a mixture of chloroform:methanol:0.8 N CH3COONa (30:60:8, v/v). The ganglioside-containing fraction was evaporated to dryness under reduced pressure in a rotary evaporator, and then dissolved in distilled water. The ganglioside solution was applied to a Sep-Pak C18 (Waters Division of Millipore, Milford, MA, USA). Gangliosides were eluted with a mixture of chloroform:methanol:0.8 N CH3COONa (30:60:8, v/v). The ganglioside-containing fraction was evaporated to dryness under reduced pressure in a rotary evaporator, and then dissolved in distilled water. The ganglioside solution was applied to a Sep-Pak C18 (Waters Division of Millipore, Milford, MA, USA). Gangliosides were eluted with a mixture of chloroform:methanol:0.8 N CH3COONa (30:60:8, v/v). The ganglioside-containing fraction was evaporated to dryness under reduced pressure in a rotary evaporator, and then dissolved in distilled water. The ganglioside solution was applied to a Sep-Pak C18 (Waters Division of Millipore, Milford, MA, USA).
developing solvent system was a mixture of chloroform:methanol:0.2% aqueous CaCl$_2$ (55:45:10, v/v). Gangliosides were visualized by spraying the plate with resorcinol-HCl reagent. Standard gangliosides GM1a, GD1a, GD1b and GT1b were prepared from bovine brains. Standard gangliosides GM3, SPG, S-i and S-I were prepared from human placenta [19]. Standard glycosphingolipids SPG and SGLPG were gifts from Dr. T. Ariga (Virginia Commonwealth University, VA, USA) [20].

2.4. TLC blotting

TLC blotting was performed as described elsewhere [17,18]. Briefly, gangliosides were applied to an HPTLC plate and then developed. After development, primuline reagent was sprayed over the plate. The gangliosides on the plate were viewed under ultraviolet light at 365 nm. While the plate was illuminated, each ganglioside area was marked with a color drawing pencil. The HPTLC plate was then immersed for 20 s in a solvent composed of isopropyl alcohol:0.2% aqueous CaCl$_2$:methanol (40:20:7, v/v). It was then placed on a glass plate, after which a PVDF membrane sheet (Clear Blott Membrane-P, ATTO Co. Ltd., Tokyo, Japan) and then a glass microfilter sheet (GF/A, Whatman International Ltd., Maidstone, UK) were placed over the glass plate. The assemblage was pressed for 15 s with a 180‡C iron. The PVDF membrane was then separated from the plate and dried. This method has been referred to as the ‘Far-Eastern blot’ [18].

2.5. Mass spectrometric analysis of gangliosides

The ganglioside areas on the PVDF membrane were subjected to SIMS. Each marked ganglioside area was excised and trimmed to form a circle 2 mm in diameter that fit the probe tip of the MS equipment. One microliter of triethanolamine was placed on the PVDF membrane sheet (Clear Blott Membrane-P, ATTO Co. Ltd., Tokyo, Japan) and then a glass microfiber filter sheet (GF/A, Whatman International Ltd., Maidstone, UK) were placed over the glass plate. The assemblage was pressed for 15 s with a 180°C iron. The PVDF membrane was then separated from the plate and dried. This method has been referred to as the ‘Far-Eastern blot’ [18].

2.6. TLC immunostaining and antibodies

TLC immunostaining was performed as described elsewhere [21]. The SPS-20 monoclonal antibody specific for SPG and S-i was a gift from Dr. Y. Kushi (Tokyo Medical and Dental University) [22]. MAb specific for GM1a was a gift from Dr. T. Tai (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). MAb specific for GD3 was a gift from Dr. J. Portoukalian (INSERM, Lyon, France). The monoclonal antibody H11, directed to the Gal β 1-4GlcNAc β 1-3Gal of glycosphingolipids, was prepared as described elsewhere [23]. These MAbs were used as first antibodies. Horseradish peroxidase-conjugated goat anti-mouse IgM antibody, for use as a second antibody, was purchased from Cappel Lab (Westchester PA; 1:1000 dilution). A Konica Immunostain Kit (Konica Co., Inc., Tokyo, Japan) reagent was used as a staining reagent.

2.7. Sialidase assay on HPTLC plate

This assay was performed as reported elsewhere [24]. Clostridium perfringens sialidase (type VI) was purchased from Sigma (St. Louis, MO, USA). Gangliosides obtained from human acoustic neurinoma were developed on an HPTLC plate, and the entire plate was incubated with 50 mUnits of C. perfringens sialidase/ml of 0.1 M aceate buffer (pH 5.5) at 37°C for 2 h. After incubation, the plate was washed with phosphate-buffered saline (PBS), and the amount of nLc4Cer present was determined by immunostaining with the H11 monoclonal antibody.

2.8. Detection of serum antibodies to gangliosides

Sera from 11 patients with Meniere’s disease were used in this study. All patients met the diagnostic criteria of the Ministry of Health and Welfare of Japan (1974). Sera from eight individuals without any hearing-related complaints were used as normal controls.

The serum samples were diluted with 1% BSA containing PBS at 1:25, and mounted on HPTLC plates on which gangliosides from acoustic neurinomas were developed. The plates were incubated at 4°C overnight and were then washed with PBS. Peroxidase-conjugated anti-human γ-chain and μ-chain-spe-
cific antibody (Tago, Burlingame, CA, USA; 1:1000 dilution with 1% BSA containing PBS) was overlaid on the plates, and the plates were incubated at room temperature for 2 h. After washing the plates with PBS, staining was performed using a Konica Immuno-nostain Kit.

2.9. SGPG and SGLPG in human acoustic neurinomas

Serum with high antibody titer against SGPG and SGLPG was gift from Dr. T. Ariga (Virginia Commonwealth University, Virginia, USA). TLC immunostaining with serum antibody was performed as described above. Serum samples were diluted at 1:1000.

3. Results and discussion

3.1. Structural analysis of gangliosides in human acoustic neurinomas

TLC profiles of gangliosides from human acoustic neurinoma samples are shown in Fig. 1. Similar patterns were obtained in the TLC chromatograms of gangliosides extracted from seven subjects.

The ganglioside fraction of sample No. 7 in Fig. 1 was subjected to TLC blotting and then used for SIMS analysis. Seven bands were visualized with ultraviolet light after being sprayed with primuline reagent. The characteristic SIMS spectra of gangliosides from human acoustic neurinomas are shown in Fig. 2. The SIMS spectrum clearly showed that band 1 was GM3 ganglioside with C24:0 and C24:1 fatty acids and a d18:1 long chain base in its ceramide moiety. The spectrum of band 2 indicated that the ganglioside was GM2 with the same fatty acid constituents as GM3. The spectra of bands 3 and 4 revealed the same major peaks at m/z 1626 and 1628. The fragmentation profile of band 3 clearly indicated that the ganglioside has a NeuAc-Hex-HexNAc-Hex-Cer structure corresponding to SPG with the C24:0 and C24:1 fatty acids. While, the SIMS spectrum of band 4 showed the appearance of peaks at m/z 1464 and 1466 corresponding to GM2 fragment and GM3 fragment peak at m/z 1263, in addition to the fragments in band 3 spectrum. This fragmentation profile suggested that band 4 is GM1a with the C24:0 and C24:1 fatty acids as interpreted in Fig. 2, band 4. Band 3 was stained by anti-SPG MAb (SPS-20), and band 4 was stained by anti-GM1a MAb. Band 3 was thus identified as SPG, and band 4 as GM1a. The SIMS spectrum in Fig. 2 showed that band 5 contained two components, one corresponding to GM1 and the other to NeuAc(Hex)4(HexNAc)1Cer. The latter ganglioside appears to be a previously unidentified component. The ganglioside with a deprotonated molecule (1626, 1628) appeared to be GM1b with C24:0 or C24:1 fatty acid in the ceramide moiety, since its mobility was a little less than that of standard GM1a and the band was not immunostained with

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![Fig. 1. TLC profile of gangliosides from human acoustic neurinomas in seven subjects. Bands were visualized by spraying the plate with resorcinol reagent. Std.: standard ganglioside. Ganglioside nomenclature accords with Svennerholm [1].](image1)

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![Fig. 2. Characteristic SIMS spectra of the gangliosides in sample No. 7 in Fig. 1. Left upper part: Gangliosides from standard BBG and acoustic neurinoma (AN) were separated on an HPTLC plate. Bands were visualized by spraying the plate with resorcinol reagent. Arrows show the number of ganglioside bands visualized by ultraviolet light after being sprayed with primuline reagent, and correspond to those of the SIMS spectrum, respectively. SIMS spectra of (1) band 1, (2) band 2, (3) band 3, (4) band 4, (5) band 5, (6) band 6, and (7) band 7. The interpretation of fragment ions is shown.](image2)
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1. GM3 (d18:1 C24:0) (d18:1 C24:1)

2. GM2 (d18:1 C24:0) (d18:1 C24:1)

3. SPG (d18:1 C24:0) (d18:1 C24:1)

4. GM1a (d18:1 C24:0) (d18:1 C24:1)

5. 1708: NeuAc(Hex)3, HexNAc3, Cer (d18:1 C18:0)
1706: NeuAc(Hex)3, HexNAc3, Cer (d18:1 C18:1)
1628: Glc (d18:1 C24:0)
1626: Glc (d18:1 C24:1)
1544: Glc (d18:1 C18:0)
1516: Glc (d18:1 C18:1)

6. 1. GD3 NeuAc-Hex-Hex-Cer

7. GD1a (d18:1 C24:0) (d18:1 C24:1)

8. 1. NeuAc-Hex-Hex-Cer
     2. NeuAc-Hex-HexNAc-Hex-Hex-Cer

9. Hex-HexNAc-Hex-Hex-Cer
MAb against GM1a (Fig. 3). The SIMS spectrum suggested that band 6 was a mixture of S-i with the C24:0 and C24:1 fatty acid and GD3 with the C24:0 fatty acid. The peaks at $m/z$ 1993 and 1991 correspond to the deprotonated ion of S-I, and their fragment peaks are interpreted in the band 6 spectrum in Fig. 2. The peak at $m/z$ 1554 in band 6 spectrum corresponds to the deprotonated ion of GD3 and appearance of the peak at $m/z$ 1263 corresponding to GM3 fragment ion supports the presence of GD3 ganglioside in band 6. The MAb against GD3 was used for TLC immunostaining, and it was reconfirmed that band 6 contained GD3 (Fig. 3). Band 7 corresponded to GD1a with C24:0 and C24:1 fatty acid as the major component of the ceramide moiety. GD1a was the most abundant ganglioside in all the samples (Fig. 1). Band 7 was the most abundant ganglioside in all the samples (Fig. 1). The SIMS spectrum of band 7 shows a typical fragment profile of GD1a. Peaks at $m/z$ 1919 and 1917 show the deprotonated ion of GD1a with the C24:0 and C24:1 fatty acids in the ceramide moieties. All fragment ions required for GD1a are interpreted in the spectrum of band 7. The results are presented in Fig. 4. Seven gangliosides, GM3, GM2, SPG, GM1a, GD3, S-i and GD1a, were identified in human acoustic neurinomas. The presence of GM1b was also suggested. In these gangliosides, C24:0 or C24:1 fatty acid was a major component rather than C18:0, which is a major fatty acid of gangliosides in the central nervous system.

The ganglioside composition of the peripheral nerve differs from that in the central nervous system [25]. Since human acoustic neurinoma is a peripheral nerve tumor, we used neuraminidase treatment to examine the differences between ganglioside species. The results are shown in Fig. 5. On incubation with

![Fig. 3. Immunostaining of human acoustic neurinoma with monoclonal antibody specific for GM1a, SPG and GD3. Gangliosides from acoustic neurinoma (AN) and standards (BBG and SPG) were separated on an HPTLC plate. 1: Bands were visualized by spraying the plate with resorcinol reagent. 2: Immunostaining with monoclonal antibodies specific for GM1a (2A), SPG (2B) and GD3 (2C).](image-url)

![Fig. 4. Characteristic ganglioside pattern in human acoustic neurinoma. Gangliosides from standard and acoustic neurinoma (AN) were separated on an HPTLC plate. Bands were visualized by spraying the plate with resorcinol reagent. Sample numbers correspond to those of Fig. 1.](image-url)
this enzyme, many ganglioside bands corresponding to N-acetyllactosamine (Gal β 1-4GlcNAc β 1-3Gal epitope with MAb H11 after C. perfringens sialidase treatment. Gangliosides from standard and acoustic neurinoma (AN) were separated on an HPTLC plate. 1: Bands were visualized by spraying the plate with resorcinol reagent. 2: Immunostaining with H11 after sialidase treatment.

The presence of SGPG and SGLPG was also confirmed by TLC immunostaining (Fig. 6). These are also very minor components and very slightly detected by immunostaining.

The ganglioside pattern found in the present study differed in some respects from that in previous reports [26,27]. We found that neurinomas contained many types of neolactoseries gangliosides, and significantly higher proportions of GD1α ganglioside. Pfeiffer et al. [26] reported that the ganglioside composition of acoustic neurinoma was GM3-rich, and also included GM4, GM2, GM1, GD3 and GD1α. Shinoura et al. [27] reported that the ganglioside patterns of neurinoma were divisible into two groups: GD1α- and GM1-rich type, and GM3-rich type. Neither study mentioned neolactoseries gangliosides.

3.2. Detection of antibodies to ganglioside antigens in patients with Meniere’s disease

There have been several studies of the immunoreactivity of sera from patients with Meniere’s disease. Autoantibodies mentioned in these studies include an inner ear protein antigen with a molecular weight of 68 000 [28,29], SGLPG [30] and type II collagen [31]. However, none of these autoantibodies has been definitely confirmed. It has been reported that some of

![Fig. 5. Detection of gangliosides having Gal β 1-4GlcNAc β 1-3Gal epitope with MAb H11 after C. perfringens sialidase treatment.](image)

![Fig. 6. TLC immunostaining of the glycosphingolipids with serum from patients with high antibody titers against SGPG and SGLPG. SGPG, SGLPG and gangliosides from acoustic neurinoma (AN) were separated on HPTLC plates. The developing solvent system was a mixture of chloroform:methanol:0.2% CaCl₂ (55:45:10, v/v). 1: Bands were visualized by spraying the plate with orcinol reagent. 2: Bands were visualized by spraying the plate with resorcinol reagent. 3: TLC immunostaining of the glycosphingolipids with serum from patients with high antibody titers against SGPG and SGLPG.](image)
the autoimmune neurological diseases are related to the gangliosides. Acute [32,33] and chronic [34] pure motor neuropathy are often associated with the anti-GM1 antibody [32,34] or anti-GQ1b antibody [33], and acute [35] and chronic [36] sensory ataxic neuropathies are associated with the autoantibody against \( \text{b-series gangliosides.} \)

We examined the immunoreactivity of sera of patients with Meniere’s disease using gangliosides from acoustic neurinoma as antigens. TLC immunostaining revealed that five of 11 patients with Meniere’s disease and one of eight normal control subjects reacted with a specific band. The positive band which exhibited reactivity on TLC immunostaining was then subjected to TLC blotting/SIMS. The one spot yielded a major peak of \( m/z \) 1993, corresponding to the mass of S-i. S-i used as the standard also exhibited reactivity to patient sera on TLC immunostaining (Fig. 7). Yuki et al. reported the occurrence of anti-S-i antibody in Guillain–Barré syndrome and chronic inflammatory demyelinating neuropathy, but the frequently is not significant when the disease control and normal control were compared [37].

It was reported that the oligosaccharide of S-i is located on the tectorial membrane surface (e.g. Kimura’s membrane), sensory epithelia (outer and inner hair cells) of Corti’s organ, the stria vascularis, and Reissner’s membrane, in an immunohistological examination of guinea pig cochleas stained using monoclonal antibodies [38]. In immuno-fluorescence testing for identification of serum antibodies against human inner ear tissue, patients with Meniere’s disease or bilateral progressive sensorineural hearing loss frequently exhibit antibody binding (mainly IgG class) to parts of the stria vascularis and Reissner’s membrane [39]. In endolymphatic hydrops, a distention of Reissner’s membrane is consistently observed [2]. Detection of the antibody responding to gangliosides in patients with Meniere’s disease may provide evidence supporting the autoimmune hypothesis for the etiology of Meniere’s disease. Ours is the first study to report that Meniere’s disease patients have antibodies against gangliosides. The results of the present study suggest that S-i ganglioside is involved in the pathogenesis of Meniere’s disease.

References


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by monoclonal antibody directed to lactoneotetraosylecera-

