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Production and characterization of a monoclonal antibody (BBH5) directed to ganglioside lactone

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The occurrence of lactones in various ganglioside preparations has been clearly demonstrated, yet the natural occurrence of ganglioside lactones in cells and tissues has been the subject of long debate, since lactones can be formed readily during preparation of gangliosides. We now report the generation of a monoclonal antibody (BBH5) that reacts specifically with lactones of disialogangliosides having the NeuAc2-8NeuAc2-3Gal sequence, but does not crossreact with the parent ganglioside. The specificity of the antibody resides on the first lactone ring between two sialic acid residues but not on the second lactone ring between sialic acid and galactose, as evidenced by reactivity with lactonized GD_{1b} having the first lactone ring (L1), and by reactivity with lactonized polysialic acid homo-oligomers ([NeuAcα2-8]$_n$NeuAc). The sialic acid carboxyl involved in the lactone ring was unequivocally determined after ammonolysis followed by methylation and fast atom bombardment mass spectrometry. The antibody BBH5 thus provides a novel tool for studies of the natural occurrence of lactones in cells and tissues.

Keywords: Lactone, ganglioside, monoclonal antibody, sialic acid

Abbreviations: BSA, bovine serum albumin; CM, chloroform–methanol; CMW, chloroform–methanol–water; FAB-MS, fast atom bombardment mass spectrometry; IHW, isopropanol–hexane–water; MAb, monoclonal antibody; PBS, phosphate-buffered saline; TLC, thin layer chromatography.

Ganglioside lactones have been detected in ganglioside preparations as base-sensitive, alkaline borohydride reducible components [1, 2]. On TLC, they are usually detected as faster-migrating bands relative to parent gangliosides [1]. More recently, their structures have been determined by mass spectrometry [2] and NMR spectroscopy [3, 4]. On the other hand, the presence of GM_3 lactone in B16 melanoma has been suggested by detection of ^3H labelled GM_3 ganglioside (reduced) after alkaline borohydride treatment of native B16 melanoma [5]. The lactone could be a melanoma-associated immunogen, as suggested by the finding that MAb M2590, which was originally directed to B16 melanoma, showed a higher affinity with GM_3 lactone than with GM_3, although the antibody M2590 reacted with both GM_3 lactone and GM_3 [5, 6]. Similarly, various other MAbs (A1-201, A1-287, and A1-410), which react with GD_2 ganglioside, also react with GD_2 lactones [7, 8]. The IgG_3 MAb DH2, which was established after immunization with GM_3 lactone and showed a remarkable inhibitory effect on B16 melanoma growth *in vitro* as well as *in vivo*, showed an equal reactivity with GM_3 lactone and the parent GM_3 [9].

Although lactones have been detected in isolated ganglioside fractions by chemical analysis, as described previously [1, 2], it has been difficult to determine whether they are natural components of cells and tissues, as opposed to artifacts introduced after extraction from cells and tissues during preparation procedures. A claim for the detectability of ^3H labelled ganglioside (reduced) after direct treatment of native cells or tissues with ^3H labelled borohydride [1, 5] does not exclude other possibilities, e.g., methyl or ethyl esterification of gangliosides, or intermolecular esterification through sialic acid carboxyl rather than inner ester lactonization of gangliosides. Thus, the debate on the natural occurrence of ganglioside lactones has not been concluded. In the present report we describe the isolation and characterization of a MAb, specific for the lactone form of gangliosides, which should provide a useful tool for *in situ* detection of lactones in cells and tissues.

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Materials and methods

Preparation of glycolipid antigens and their lactones

Frog brain was kindly donated by Dr. Jacques Portoukalian of Inserm U218, Lyon, France, and the ganglioside fraction was prepared from the upper phase of Folch partition [10]. Three major ganglioside bands were detected. The major component showing fastest migration on TLC was identified as $GD_{1\alpha}$, as previously described [11]. Other slower-migrating components were also detected as analogues of $GD_{1\alpha}$, e.g., $GT_{1\alpha}$ (see Table 1). Gangliosides GM_1, GD_{1a}, GD_{1b} and GT_{1b} were extracted from human brain and purified from the upper phase of Folch extract [10]. Individual gangliosides were separated by HPLC in an IHW system through an Iatrobeads 6RS-8010 column [12, 13]. Gangliosides GM_3 and GD_3 were prepared from human melanoma tissue and purified by HPLC as described above.

Table 1. Structures of glycosphingolipids used.

GM_3	NeuAcα2-3Galβ1-4Glcβ1-1Cer
GM_2	GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuAcα2
GM_1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuAcα2
GD_3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer
$GD_{1\alpha}$	NeuAcα2 | 6 Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuAcα2
GD_{1a}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 3 | | NeuAcα2 NeuAcα2
GD_{1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | NeuAcα2 8 | NeuAcα2
$GT_{1\alpha}$	(NeuAcα2-8)[a]NeuAcα2 | 6 Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 | (NeuAcα2-8)[a]NeuAcα2
GT_{1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer 3 3 | | NeuAcα2 NeuAcα2 8 | NeuAcα2

[a] The outer NeuAcα2-8 residue may be linked to either of the inner *N*-acetylneuraminic acid residues [11].

Lactonization of gangliosides was performed in CM–12 N HCl, 60:30:4.5 by vol, for 18–24 h at 4°C followed by treatment with DEAE-Sepharose as described by Nores *et al.* [5]. Lactonization was also carried out in glacial acetic acid as described by Yu *et al.* [3]. Briefly, 50 μg ganglioside was dissolved in 100 μl glacial acetic acid, and allowed to stand for 48–72 h. The solvent was lyophilized, the residue dissolved in CMW, 30:60:8 by vol, and lactonized components were separated on a DEAE-Sephadex A-25 acetate column [14].

Establishment of MAb BBH5

Gangliosides were purified from frog brain, and the fraction corresponding to di- and tri-sialo components was isolated by chromatography on DEAE-Sephadex. Following treatment in CM–12 N HCl, 60:35:4.5 by vol, about 80% of the compounds were lactonized. 20 μg of this mixture in 100 μl IHW was then mixed with 2 ml of an aqueous suspension of 500 μg of acid-treated *Salmonella minnesotae*, incubated for 2 h at 37°C, and the mixture was lyophilized and resuspended in 2 ml water. 200 μl aliquots were injected intravenously according to the schedule as previously described [15, 16]. Two days after the last injection, spleen cells of immunized mice were harvested and fused with NS/1 myeloma cells. Hybridomas were screened for reactivity with frog brain gangliosides and human brain gangliosides. Positive wells were expanded, recloned, and the hybrid BBH5 was established.

Preparation of polysialic acid oligomers

Polysialic acid oligomers were prepared from colominic acid of the culture filtrate of *E. coli* K1 strains (Sigma Chemical Co., St. Louis, MO, USA), according to previously published methods using anion exchange chromatography (Mono-Q HR 5/5; Pharmacia, Uppsala, Sweden) [17]. Briefly, 50 mg colominic acid in 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl was loaded onto the Mono-Q column equilibrated in the same buffer. The column was eluted with a linear gradient of 20 mM Tris-HCl, pH 7.5, 50–400 mM NaCl for 20 min with a flow rate of 1 ml min^{-1}. Fractions were collected every 60 s. Lactonization of the fraction was performed in glacial acetic acid [3].

Immunochemical analysis

Reactivities of gangliosides or their lactones with MAbs were determined by solid phase radioimmunoassay on 96-well flexible plastic plates (Falcon 3912, Becton Dickinson, Oxnard, CA, USA) as described previously [18]. Briefly, the glycolipid was dissolved in ethanol and adsorbed onto the bottom of each well by drying at 37°C. On antigen dilution test, 50 μl ganglioside solution (containing 100 ng) was serially diluted to 0.05 ng per well and dried at 37°C. Wells were incubated with 3% BSA in PBS to block nonspecific antibody binding, washed, and then incubated with culture supernatant containing ≈10–20 μg ml^{-1} IgM at 4°C

overnight. Next, 1:1000 diluted rabbit anti-mouse IgM antibody was added to each well and allowed to react at room temperature for 2 h, followed by incubation with ^{125}I labelled protein A (10^7 counts min^{-1} ml^{-1}). Radioactivity of each well was counted in a gamma counter.

Reactivity of individual gangliosides separated on TLC with MAbs was checked by immunoblotting, as originally described by Magnani *et al.* [19]. Briefly, gangliosides were separated on Whatman TLC plates, developed in CMW, 50:40:10 by vol containing 0.05% $CaCl_2$, dipped in 0.5% poly(isobutylmethylacrylate) in ethyl ether for 1 min, and dried. The plate was then immersed in 5% BSA in PBS, washed in PBS, and further incubated with culture supernatant overnight at 4°C. The plate was washed again in PBS and incubated with 1:1000 diluted rabbit anti-mouse IgM antibodies (purchased from ICN Immunobiologicals, Lisle, IL, USA) in 1% BSA in PBS, followed by reaction with ^{125}I labelled protein A (10^6 counts min^{-1} ml^{-1}). TLC plates were extensively washed, air-dried, and tested by autoradiography on Kodak X-OMAT AR film. After development of autoradiograms, plates were sprayed with 0.5% orcinol in 10% sulfuric acid for detection of ganglioside bands.

The reactivity of a lactone with its MAb was confirmed by its sensitivity with mild alkali. Briefly, glycolipids were dissolved in 100 µl CM (2:1 by vol), with addition of 20 µl 0.5% sodium methoxide in methanol. After 30 min at room temperature, the reaction mixture was subjected to TLC followed by immunostaining.

Inhibition assay

Inhibition of antibody binding to GD_{1b} lactones was performed in solid phase. For this, 25 µl of a solution containing 5, 2.5, 1.25, or 0.6 µg of lactonized samples or 2.5 µg of untreated samples was mixed with the same volume of diluted MAb BBH5 (1:20). After 40 min at room temperature, the binding of BBH5 antibody to wells coated with 2 µg of GD_{1b} lactones was assayed by solid phase radioimmunoassay.

Detection of ganglioside lactones on freshly-extracted lipids by TLC immunostaining

In order to eliminate the possibility of lactone formation during extraction and preparation of gangliosides from tissues, fresh mouse brain or freshly harvested cells were extracted and rapidly processed for TLC immunostaining. Tissues and cells were homogenized in IHW (55:25:20 by vol) [20] in a Sonifier II sonicator (Branson Ultrasonics Corp., Danbury, CT, USA), followed by centrifugation. The extract was dried under vacuum, submitted to Folch partition, and the upper phase was evaporated and subjected to TLC immunostaining. The entire process was completed in less than 3 h.

Characterization by FAB-MS

Negative ion FAB-MS of native and lactonized gangliosides was performed using a Jeol HX-110 mass spectrometer/DA-5000 data system (Jeol Ltd., Tokyo, Japan). Samples (≈20 µg) were suspended on the target with triethanolamine matrix containing 15-crown-5 [21] (Aldrich) and bombarded with a 6 kV xenon beam, while data were acquired in the accumulation mode (3 scans). Sodium iodide in glycerol was used as the calibration standard. Scan range was 100–2000 units, scan slope 3 min, acceleration voltage 10 kV, and resolution 3000.

For positive ion FAB-MS, samples were subjected to a previously published procedure designed to distinguish internally esterified sialic acid residues from those with a free carboxylate group [22]. Aliquots of each sample (≈100 µg) were treated with ammonia in chloroform–isopropanol as described by Sonnino *et al.* [23]. Following removal of NH_3 and solvent under a N_2 stream, the samples were desiccated *in vacuo* over P_2O_5 and then permethylated by the method of Ciucanu and Kerek [24], as modified by Larson *et al.* [25], except that equal volumes of MeI and DMSO were used (200 µl each). The reaction time was 30 min and MeI was removed by flushing with N_2 for 15 min at 37°C prior to partitioning between $CHCl_3$ and H_2O. After drying the $CHCl_3$ under N_2, portions of the permethylated samples were subjected to positive ion FAB-MS. Aliquots of permethylated samples (≈20 µg) were transferred in MeOH to a FAB target and suspended in 3-nitrobenzyl alcohol matrix [26, 27] containing 15-crown-5 [21]. Scan range was 100–2500 units, scan slope 2 min, acceleration voltage 10 kV, and resolution 3000. Three scans were accumulated for each spectrum. KI/CsI was used as calibration standard.

Results

Generation and reactivity of MAb BBH5

Mouse hybridoma BBH5 secreting IgM was established after immunization of BALB/c mice with lactonized total polysialoganglioside of frog brain. The antibody did not react with purified major gangliosides GM_3, GM_1, GD_3, fucosyl-GM_1, GD_{1a}, GD_{1b}, and GT_{1b}, but did react with components present in whole brain gangliosides (Fig. 1, lane HB). This reactivity was abolished by sodium methoxide treatment, as shown in Fig. 2. MAb BBH5 was observed to react strongly, in HPTLC immunostaining experiments, with components of a crude lactone mixture formed by treatment of GD_{1b} with CM–12N HCl. This staining was also abolished by treatment of the lactone mixture with sodium methoxide (data not shown).

Our original intention was to obtain MAb specific to the novel ganglioside series $GD_{1\alpha}/GT_{1\alpha}$, found in frog brain [11]. Lactonization was used in order to enhance the immunogenicity of gangliosides [9]. MAbs with the

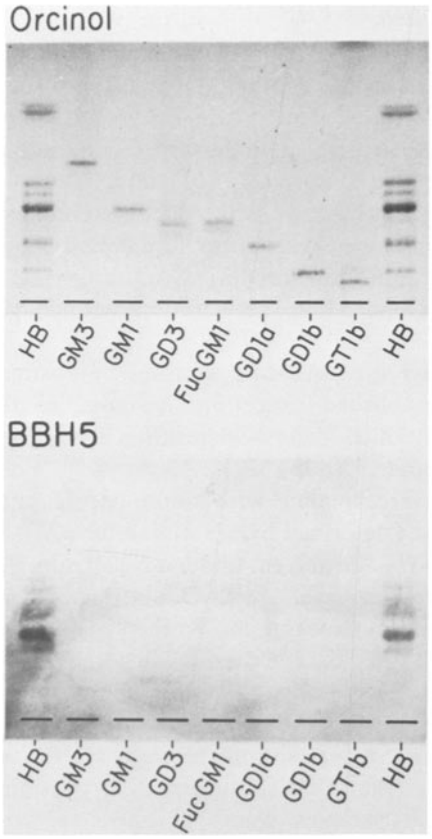

Figure 1. Reactivity of MAb BBH5 with various gangliosides. See the Materials and methods section for immunostaining conditions. HB, Ganglioside fraction extracted from human brain.

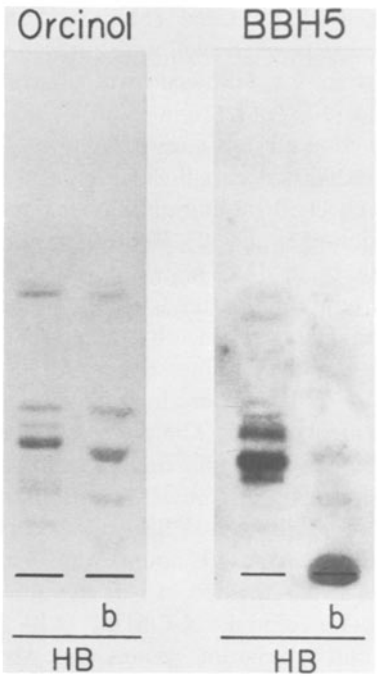

Figure 2. Sensitivity to sodium methoxide treatment. Human brain ganglioside mixture (HB) was subjected to base treatment (lane b) using sodium methoxide, as described in the Materials and methods section. A strong immunostaining band at the starting point in the base-treated sample is nonspecific staining due to the presence of salt.

intended specificity, i.e., directed to $GD_{1\alpha}$ and $GT_{1\alpha}$, have not been established so far.

Specificity of MAb BBH5 with various ganglioside lactones

The specificity of MAb BBH5 was explored further using lactones derived from different gangliosides. It reacted strongly with two types each (L1, L2) of GD_{1b} and GD_3 lactones, and also reacted with three types (L1, L2, L3) of GT_{1b} lactone, but did not react with GM_1 lactone, GM_3 lactone, or GD_{1a} lactone on TLC immunostaining (Fig. 3). The specific reactivity with both lactone types of GD_3 and GD_{1b} was further confirmed by solid-phase antibody-binding assay. Again, L1 and L2 of both GD_3 and GD_{1b}, but not lactones of GM_3 and GM_1, were reactive (Fig. 4). As shown in the following section, L1 has a lactone ring between the terminal and subterminal sialic acid, while L2 contains two lactone rings, one the same as that in L1 and the other between the internal sialic acid and the galactose residue. In view of the equal reactivity of L1 and L2, and the lack of reactivity of BBH5 with GM_1 and GD_{1a} lactones, it is likely that MAb BBH5 recognized the structure at the terminal sialic acid. This was further investigated by studies of polysialic acid homo-oligomers ([NeuAcα2-8]$_n$NeuAc) isolated from colominic acid (see Materials and methods). As shown in Table 2, lactones of larger polysialic acid oligomers inhibited BBH5 binding to GD_{1b} lactone, whereas no or little inhibition of native oligomers was observed. Based on elution of Mono-Q HR 5/5 as described previously [17], the sizes of inhibiting polysialic acid oligomers were from 10–12 up to >20 (Table 2).

The observation of additional reactive bands for lactones of GD_3, GD_{1b}, and GT_{1b} in Fig. 3 calls for some explanation, since these are obviously in excess of those which could be attributed to the known lactonization products. These may be due to a number of factors, including (i) acid-catalysed degradation (loss of terminal galactose and *N*-acetyl-galactosamine residues from GD_{1b} and GT_{1b} lactones); (ii) acid-catalysed formation of methyl esters of sialic acid carboxyl groups which are not part of the BBH5 epitope; or (iii) lactonization of nonessential sialic acid residues at alternate sites. Formation of such products may be associated with the strong reaction conditions used for preparing lactones in these experiments (CM–12 N HCl). In any case, they may be undetectable by chemical methods, and yet display strong immunostaining on a heavily loaded TLC plate. An additional source of smearing may be the equilibrium lactone ring opening and closing during and after TLC.

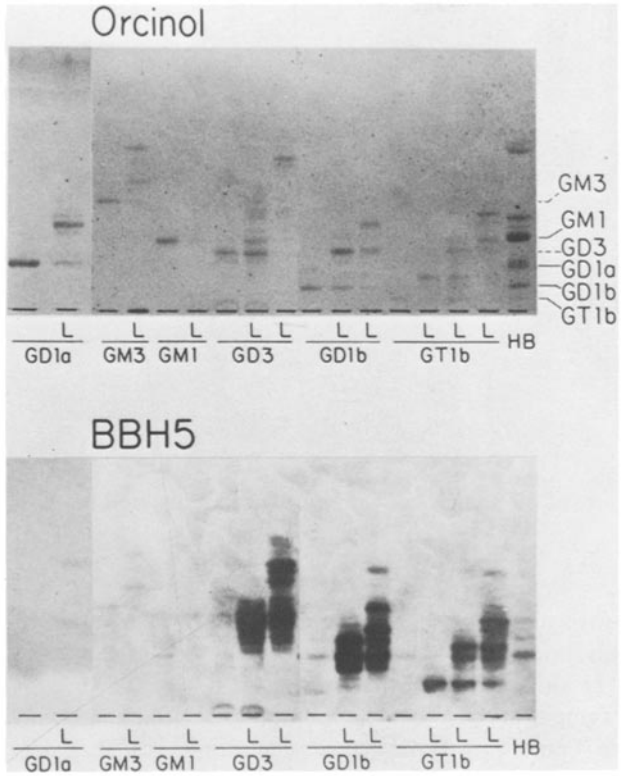

Figure 3. TLC immunostaining of MAb BBH5 with various gangliosides and their lactones (L). Lactonization was carried out by incubating each ganglioside for various durations in CM–12 N HCl (60:30:4.5 by vol) [5] (GT_{1b}: 15, 48 and 72 h (show, respectively, as three L lanes, left to right); GD_3, GD_{1b}: 15 and 48 h (shown respectively as two L lanes, left to right); GD_{1a}, GM_3, GM_1: 72 h).

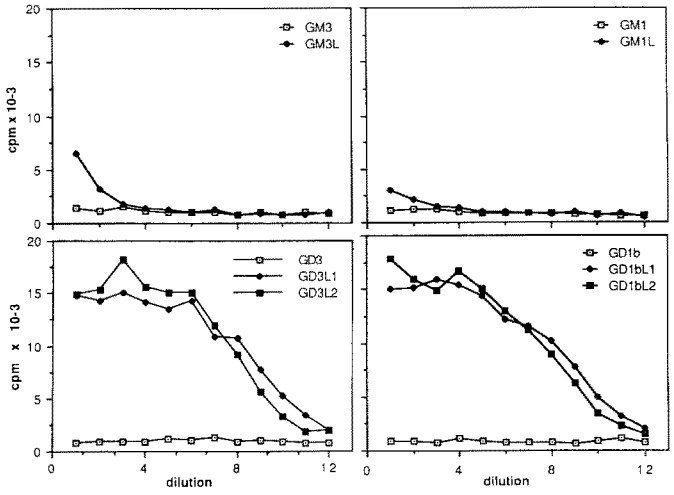

Figure 4. Binding activity of MAb BBH5 with various gangliosides and their lactones using solid-phase radioimmunoassay. Initial concentration of gangliosides: 100 ng. The assay was performed as described in the Materials and methods section. L1 and L2 were lactones with low and high TLC mobility, respectively. L1 was formed initially and was subsequently converted to L2 during the process of lactonization (see the Results section "Specificity of MAb BBH5 with various ganglioside lactones").

Table 2. Inhibition of BBH5 reactivity with GD_{1b} lactones by different colominic acid fractions after lactonization.[a]

Elution fraction number	*Approx. number of sialyl units*[b]	*% Inhibition*				
		Lactonized				*Untreated*
		5 μg	2.5 μg	1.25 μg	0.6 μg	2.5 μg
7	3	0	0	0	0	0
10	5	0	0	0	0	0
12	6	54	36	30	19	4
14	8	52	50	38	14	8
16	10	71	61	56	44	0
18	12	93	93	83	72	9
19	14	99	96	84	70	6
GD_{1b}		100	100	100	100	0

[a] 25 μl of the solution containing the component to be tested was mixed with the same volume of diluted MAb BBH5 (1:20). After 40 min at room temperature, the binding of BBH5 to wells containing 2 ng of GD_{1b} lactone was assayed as described in the Materials and methods section. Results are expressed as per cent inhibition compared to samples incubated without inhibitor.

[b] Based on Hallenbeck *et al.* [17], in which reduced sialyl oligomers were separated.

Characterization of two lactones (L1 and L2) from GD_{1b}

GD_{1b} L1 represents a slow-migrating component formed at the early stage of the lactonization reaction. GD_{1b} L2 is formed subsequently and increases later (Fig. 5). These two bands were separated on DEAE-Sephadex chromatography and characterized further by FAB-MS. In the negative ion mode, FAB spectra of L1 and L2 displayed pseudomolecular ions which were, respectively, 18 and 36 units; less than those for the parent ganglioside: for GD_{1b}, $[M-H]^-$ at *m/z* 1835 and 1863 for ceramide containing 18:0 fatty acid with d18:1 and d20:1 sphingosines, respectively [28]; for L1, $[M-H]^-$ at *m/z* 1817 and 1845; and for L2, $[M-H]^-$ at *m/z* 1799 and 1827 (data not shown). These values are consistent with formation of one lactone ring in L1 and two in L2. However, other modes of water loss could not be ruled out by these data alone. This was considered particularly pertinent since it was difficult to interpret the differences in glycosyl fragmentation patterns in a consistent, straightforward manner which would give clear indications of the structural differences between GD_{1b}, L1, and L2. Therefore, L1 and L2 were subjected to a sequence of ammonolysis followed by permethylation, and the resulting derivatives analysed by FAB in the positive ion mode, a procedure developed and used previously for analysis of GD_3 lactones [22]. As expected for permethylated glycosphingolipids analysed in this mode, mass spectra displayed clear pseudomolecular ion clusters accompanied by fragments produced by cleavages at glycosyl linkages, preferentially at *N*-acetylhexosaminyl and *N*-acetylneuraminosyl residues, with charge retention on the nonreducing portions

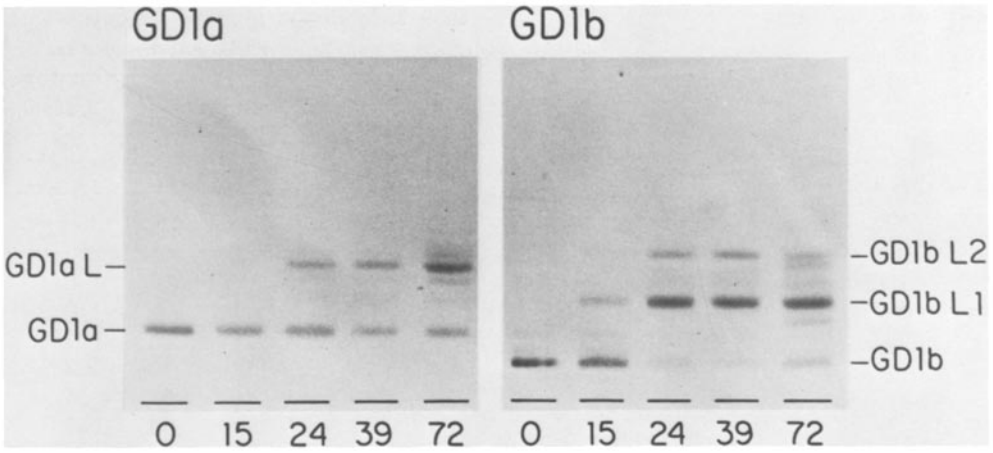

Figure 5. Preparation of ganglioside lactones. TLC of the reaction mixture of the gangliosides after incubation in glacial acetic acid at room temperature for 0, 15, 24, 39, and 72 h.

[29–31]. However, those fragments containing *N*-acetylneuraminic acid residues which were involved in ester linkages prior to derivatization were found at masses which reflected their different fates during these chemical procedures [22].

In the mass spectrum of the derivative of L1 (Fig. 6), an abundant fragment can be found at *m/z* 389, which corresponds to a terminal *N*-acetylneuraminic acid residue which has been transformed to an amide by the ammonolysis reaction, as expected for an internally esterified sialic acid [2, 23, 32, 33], and then converted to a 4,7,8,9-tetra-*O*-methyl-*N*-methylacetamido-*N'*,*N'*-dimethylamide as a result of the permethylation reaction. The ion containing both *N*-acetylneuraminic acid residues is found at *m/z* 750, corresponding to an internal sialic acid which is in the carboxymethyl ester form, which means that it was not in an ester form prior to ammonia treatment. These two ions are accompanied by ions at *m/z* 357 (389 − 32) and 718 (750 − 32), from neutral loss of MeOH, and at *m/z* 677 (750 − 73), most likely from neutral loss of $HCONMe_2$ (DMF). Other structurally significant fragments are found at *m/z* 464 and 228, corresponding to the ganglio-core Hex-*O*-3HexNAc-fragment [30]. Ceramide ions are found at *m/z* 576 and 604. Consistent with the conversion of one *N*-acetylneuraminic acid residue from COOMe to $CONMe_2$ form, the major pseudomolecular ions are found 13 units higher than those for permethylated GD_{1b}, at *m/z* 2200 and 2228. Interestingly, these are accompanied by two major ions 73 units lower in mass (*m/z* 2127 and 2155). These would correspond to loss of neutral $HCONMe_2$, which must be considerably more facile than the corresponding losses of HCOOMe or MeOH from permethylated gangliosides. Finally, the presence of unesterified terminal *N*-acetylneuraminic acid is indicated by ions at *m/z* 376 and 344 (376 − 32), with the corresponding $NeuAc_2$ ion found at *m/z* 737. These could be expected from GD_{1b} either not converted to lactone form, or partially hydrolysed during handling.

In the mass spectrum of the L2 derivative (Fig. 7), the fragment at *m/z* 389 is again found in abundance, while the ion containing both *N*-acetylneuraminic acid residues is found at *m/z* 763, consistent with both sialic acids being linked in internal esters in the parent compound. Accompanying secondary fragments are found at *m/z* 731 (763 − 32) and at *m/z* 690 (763 − 73). Consistent with these fragments, the pseudomolecular ions are now found at *m/z* 2213 and 2241, accompanied by even more abundant ions for loss of 73 units, at *m/z* 2140 and 2168. Impurity fragments are found at *m/z* 376, 344, and 750. Structures of the lactonized forms of ganglioside GD_{1b} are shown in Fig. 8.

Reactivity of MAb BBH5 with freshly prepared brain gangliosides

In order to assess the natural existence of lactones, fresh gangliosides were prepared rapidly from mouse brain. The ganglioside-containing upper phase fraction of the Folch partition was processed by immunoblotting with MAb BBH5, which revealed the presence of clear bands, indicating the presence of lactones, in both forebrain and hindbrain (Fig. 9). In contrast to human brain, in which lactones of GD_{1b} appear to be the major BBH5-detectable components [2], the predominant BBH5-detectable components of mouse brain appear to be lactones of GD_3. This is in agreement with the results obtained by Gross *et al.* [1]. There were no apparent compositional differences between forebrain and hindbrain of mouse.

Discussion

The occurrence of base-sensitive gangliosides was reported originally by Kuhn and Müldner [34]. The base-sensitive structure in ganglioside fractions was postulated by

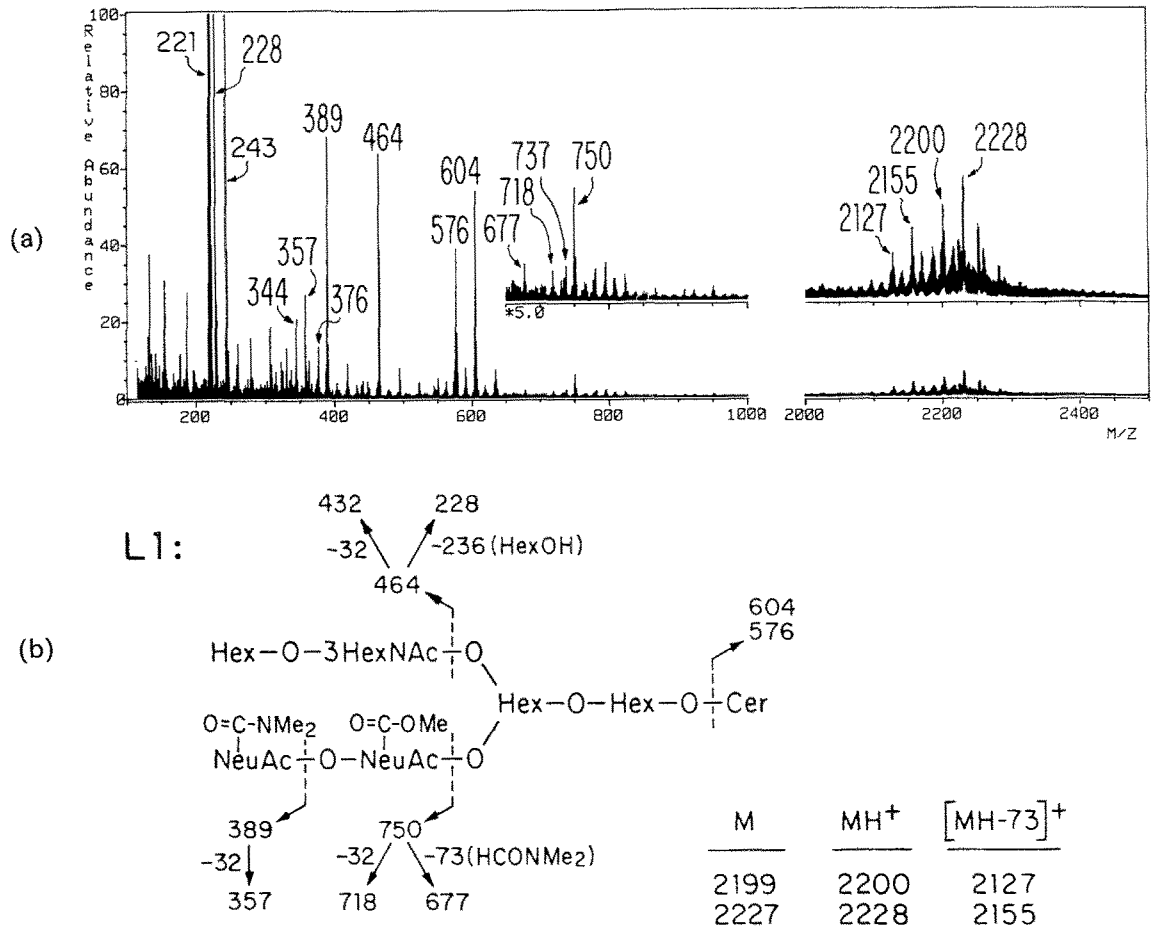

Figure 6. Positive ion FAB-MS of GD_{1b} lactone L1 following treatment with ammonia and permethylation. Structurally significant ions, corresponding to those in the proposed fragmentation scheme below, are labelled with nominal, monoisotopic mass assignments. The matrix was 3-nitrobenzyl alcohol plus 15-crown-5. The ions marked at *m/z* 221 and 243 are from the respective protonated and sodiated pseudomolecular ions of 15-crown-5. The source of other ions is discussed in the text.

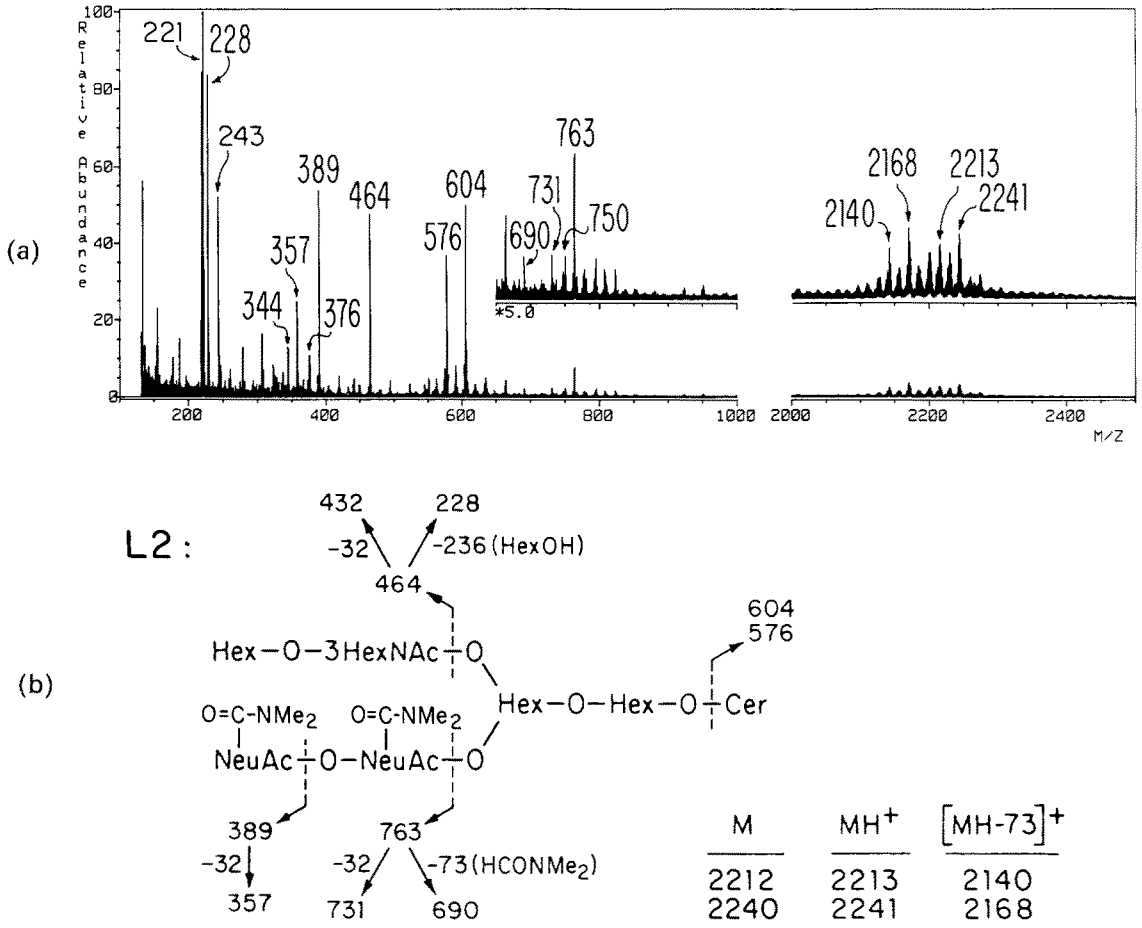

Figure 7. Positive ion FAB-MS of GD_{1b} lactone L2 following treatment with ammonia and permethylation. Further details as for Figure 6.

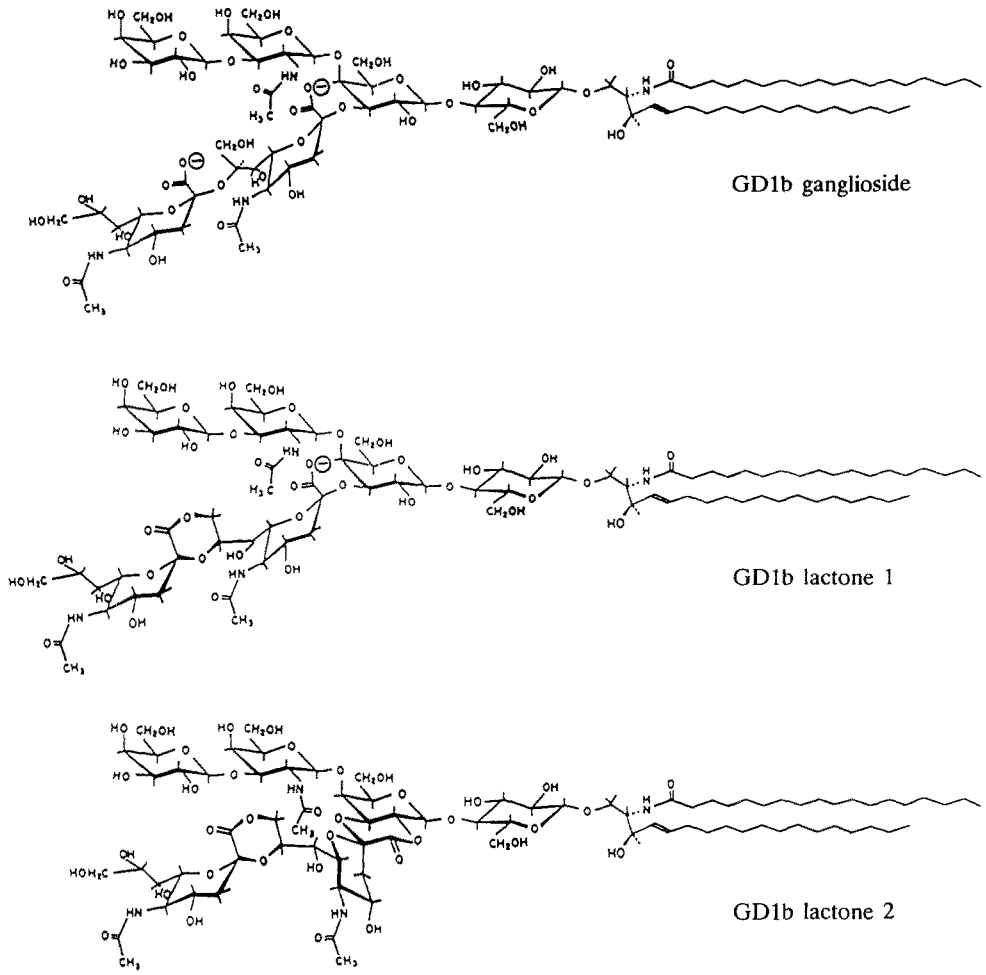

Figure 8. Modification of ganglioside GD_{1b} by lactonization.

Wiegandt [35] to consist of lactones in which the sialic acid carboxyl group could be internally esterified. In fact, McCluer and associates [1] demonstrated the presence of fast-migrating GM_3 gangliosides from adrenal tissue, and tentatively identified them as GM_3 lactones. On the other hand, gangliosides containing *O*-acetylsialic acid (e.g., 4-*O*- or 9-*O*-acetylsialic acid) also showed base-sensitive, fast-migrating properties similar to those of lactones [36, 37]. Characterizations of GD_{1b} lactone by mass spectrometry [2] and ^{1}H-NMR [32], GD_{1a} lactone [33], GM_1 lactone [23], GM_3 lactone [3], and most recently GD_3 lactone by ^{1}H-NMR spectroscopy [4] have been described. Through these studies, the presence of lactones in ganglioside preparations from various tissues, and their structural features, have been demonstrated. More recently, ^{14}C labelled GD_{1b} was intracysternally injected and ^{14}C labelled GD_{1b} lactone subsequently found in brain extract [38]. Because the carboxyl group of sialic acid is readily lactonized during preparation or storage of gangliosides, these studies do not provide direct evidence for the natural occurrence of lactones *per se*.

MAb M2590, established after syngeneic immunization of C57/BL mice with B16 melanoma [39], defines a melanoma-specific antigen. Surprisingly, this antigen was identified as GM_3 with high-density organization at the cell surface, although the real immunogen in melanoma was assumed to be GM_3 lactone [5]. In order to confirm the role of GM_3 lactone as an immunogen, and its natural occurrence in B16 melanoma, a MAb specific to the lactone but not reactive with parent GM_3 is obviously desirable. Attempts to produce GM_3 lactone-specific MAbs have resulted in the generation of antibodies reacting with both the lactone and the parent GM_3 [9]. Similarly, a variety of MAbs directed to GD_2 ganglioside were also reactive with GD_2 lactone [7, 8]. From our studies of GM_3 lactones, it has become apparent, however, that lactonization greatly increases the immunogenicity, at least in mice, of these generally lesser immunogenic gangliosides [9].

In the present study, MAb BBH5, reacting specifically with forms of GD_{1b} and GD_3 gangliosides containing the lactone ring formed between the NeuAcα2-8NeuAc residues (L1), was established. During the process of lactonization

Figure 9. Reactivity of MAb BBH5 with ganglioside fractions rapidly prepared from fresh mouse brain and human brain extracts. F, mouse forebrain; H, mouse hindbrain; HB, human brain.

of GD_{1b} and GD_3, the compound (L1) containing the first lactone ring structure between the terminal and internal *N*-acetylneuraminic acid is formed rapidly, and subsequently the compound (L2) containing the second lactone structure between the internal *N*-acetylneuraminic acid and galactose, in addition to the first lactone ring, is formed [4]. Although MAb BBH5 reacted with both L1 and L2 compounds, its true reactivity was assumed to be associated with the first lactone ring structure, as demonstrated on TLC immunostaining and on solid-phase antibody-binding assay. Thus, compound L1 (which has the first but not the second lactone ring) shows the same reactivity as compound L2 (which has both lactone rings). Independence of BBH5 reactivity from the second lactone structure was further confirmed by the absence of reactivity with GM_1 lactone, GD_{1a} lactone and GM_3 lactone, which share the same lactone ring structure between (2-3)-linked *N*-acetylneuraminic acid and galactose. The specificity of BBH5 toward the lactone structure between NeuAcα2-8NeuAc was further confirmed by inhibition with lactonized polysialic acid oligomers.

Using the lactone-specific MAb, the presence of lactone in freshly prepared ganglioside from mouse brain immediately *post mortem* was demonstrated. However, in preliminary immunohistological studies of fresh frozen mouse brain sections, no reactivity was observed. More extensive studies of immunohistological sample preparation and staining methods with BBH5 are needed.

Polyclonal and monoclonal antibodies directed to α(2-8)-polysialic acid homo-oligomers have been reported [40, 41]. Interestingly, these antibodies showed requirements for polysialic acid longer than 7–10 residues, which has been interpreted as the need for a conformational epitope only induced in longer chains [42, 43]. A similar requirement for longer chains appears to be necessary for a bacteriophage endosialidase [44]. The MAb BBH5 exhibited the same requirement for polysialic acid chains of more than 6–8 residues (Table 2), in the lactonized state. The studies of specificity of gangliosides, however, showed that a minimum requirement of the disaccharide NeuAcα2-8NeuAc-α2-R was sufficient.

The GD_{1b} lactones synthesized for this study were characterized using positive and negative ion FAB-MS. Because normal methylation techniques cannot be applied to the analysis of ganglioside lactones, due to their lability in alkaline media, use was made of their well-documented reaction with ammonia to produce sialic acid amides [2, 23, 32, 33]. Permethylation then produced derivatives bearing an *N'*,*N'*-dimethylamide "flag," differing by 13 units from the normally produced methoxycarbonyl group, for each neuraminic acid involved in an ester linkage [22]. As expected under positive ion FAB-MS conditions, these derivatives produced abundant A_1-type fragments [29–31], allowing the unambiguous sequencing of esterified *N*-acetylneuraminic acid residues from the nonreducing end. One disadvantage of using this technique alone, which is shared by all methods employing ammonolysis, is that *any* type of neuraminic acid ester can be converted to its amide. In this case, the results of negative ion FAB-MS of the native substances show that the ester linkages must be internal [2, 23, 32] by the observation of molecular ions 18 and 36 units less than that of the parent compound, for L1 and L2, respectively. A second, more difficult, problem is that the sugar hydroxyl groups which are esterified cannot be located by this or any other mass spectrometric technique thus far employed. This difficulty has recently been overcome by the introduction of a method in which all free hydroxyl groups are protected by methoxyethoxymethyl (MEM) groups [45, 46], which are stable in alkaline media. MEM groups survive permethylation procedures, which introduce a methyl ether at all acylated sites, including lactonized hydroxyl groups, but are removed by the acid conditions used for subsequent depolymerization. GC/MS analysis of partially *O*-methylated sugars after hydrolysis unequivocally determines the site(s) of esterification, as shown by application of the method to lactones of GM_3 and GD_3 [47, 48]. This method, together with ammonolysis and permethylation, has enabled us to determine ganglioside lactone structures unambiguously [22, 47, 48], and application of these procedures to further analysis of compounds L1 and L2 is underway.

Prior to the development of these chemical procedures, the most convincing evidence for the location of esterification sites came from 1- and 2-D ^{1}H-NMR studies. Based on the observation of characteristic downfield "lactonization shifts" for protons on carbon atoms acylated by neuraminic acid, the best indications are that *N*-acetylneuraminic acid residues attached to O-3 of galactose are lactonized to the C-2 hydroxyl of that sugar [3, 4, 23, 33], while *N*-acetylneuraminic acid residues attached to O-8 of another *N*-acetylneuraminic acid are lactonized to the C-9 hydroxyl of that sugar [4, 32]. These proposals have recently been confirmed unambiguously [47, 48]. The primary structures of L1 and L2 are proposed (Fig. 8) in accordance with these conclusions. The structure of L1 is in agreement with that previously proposed for a monolactone synthesized from GD_{1b} and isolated from human brain [32]. The structure of dilactone L2 has not been reported previously, but is analogous to the dilactone synthesized from GD_3 [4].

MAb BBH5 will be a valuable tool for studies of biological functions of lactonization. The demonstration of reactivity with lactonized polysialic acid oligomers is intriguing. The significance of identical polysialic chains bound to important cell adhesion molecules such as N-CAM [49, 50] and, more recently, integrins [51] is well established. The cell adhesion mediated by N-CAM is believed to be modulated by the length of these polysialic acid chains, presumably as a result of the charge effect or through conformational changes. The possibility that active lactonization is utilized as a more subtle way of decreasing charge or controlling conformation is intriguing.

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