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GM2-1 pancreatic islet ganglioside: identification and characterization of a novel islet-specific molecule

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Summary Recent studies have indicated that GM2-1, a pancreatic islet monosialo-ganglioside, is an islet-specific component whose expression is metabolically regulable and represents one of the target antigens of cytoplasmic islet cell antibodies. In the present study we aimed to biochemically characterize this molecule using a panel of biochemical techniques including gas chromatography, thin layer chromatography, enzymatic digestion and mass spectrometry. GM2-1 ganglioside was extracted from human pancreas and purified by thin-layer chromatography. Fatty acids in the ceramide (the hydrophobic portion of the molecule), identified by gas chromatography ranged from C16:1 to C24:1. The oligosaccharide chain was enzymatically digested by the sequential application of various exoglycosidases (neuraminidase followed by β-galactosidase, followed by β-hexosaminidase) and characterized by gas chromatography identification of the liberated sugars. The following structure was deducted from enzymatic studies and confirmed by mass spectrometry analysis: N-acetyl neuraminic acid-galactose-galactosamine-galactosamine-glucose-*ceramide*. This is a novel ganglioside structure, not yet described, which shares characteristics with a neuronal glycolipid autoantigen: the LM1 ganglioside. Both GM2-1 and LM1 have a single sialic acid residue in the terminal position, the same migration position on thin layer chromatography and the same number of carbohydrate moieties. In conclusion, we have characterized a novel islet-specific ganglioside molecule with unusual characteristics, such as the terminal sialic acid and the galactosamine residues, which may facilitate both its antigenicity and its involvement in beta-cell autoimmunity. [Diabetologia (1995) 38: 1117–1121]

Key words Gangliosides, pancreatic islets, beta-cell autoimmunity, autoantigen.

Gangliosides are sialic acid-containing glycolipids that are components of the cell plasma membrane and of the cytoplasm; they are involved in cell-cell interaction and in the binding of hormones, toxins, viruses and autoantibodies to plasma membranes [1]. Gangliosides consist of a hydrophilic portion (the oligosaccharide chain) and a hydrophobic tail (the ceramide). The latter contains a sphingosine and a fatty acid, while the oligosaccharide chain can be of variable length with at least one sialic acid residue. Insulin-dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of insulin-producing cells in the pancreatic islets in genetically-predisposed individuals. For most patients, despite the acute clinical onset of the disease, there is a subclinical latency period lasting for years. Autoantibodies directed against islet antigens are detectable in the serum of individuals with, or at risk for, IDDM [2], thus representing important markers for the detection of anti-islet autoimmunity before the appearance of clinical symptoms. Cytoplasmic islet cell antibodies (ICA) are a heterogenous population of antibodies [3], which to date represent the most preva-

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; ICA, islet cell antibodies; GC, gas chromatography; TLC, thin layer chromatography; MS, mass spectrometry; amu, atomic mass units; NANA, N-acetyl-neuraminic acid; HPTLC, high performance thin layer chromatography.

lent marker of beta-cell autoimmunity in population studies. One of the ICA target antigens was recently demonstrated to be glutamic acid decarboxylase [4]; another appears to be a pancreatic monosialoganglioside migrating between GM2 and GM1 standards (GM2-1), since this molecule has been shown to be a potent inhibitor of ICA binding on pancreatic frozen sections of some ICA-positive sera [5]. In addition, biochemical studies have shown that GM2-1, which is not expressed in other endocrine or non-endocrine tissues of human or rodent origin including central and peripheral nervous system tissue, is the major ganglioside from isolated human, rat and mouse pancreatic islets [6, 7], where it is expressed at a concentration 100-times higher than in whole pancreas, suggesting that this glycolipid is confined to islets within the pancreas. It is worth noting that the GM2-1 molecule is hyperexpressed [7] in islets of an animal model of beta-cell autoimmunity, the nonobese diabetic mouse, where the amount of this ganglioside has been shown to decrease significantly with the progression of beta-cell destruction. The involvement of gangliosides as a target of autoantibodies in IDDM has also been suggested by the finding of circulating antibodies directed against the trisialo-ganglioside GT3 in a significant percentage of recent-onset IDDM patients [8]. Consequently, we aimed to biochemically characterize the GM2-1 ganglioside.

Materials and methods

The GM2-1 ganglioside was purified from human pancreas glycolipid extracts by ion exchange column chromatography and thin layer chromatography (TLC); the structure of the ceramide and the carbohydrate sequence of the sialo-oligosaccharide chain were determined employing chromatographic techniques and enzymatic digestions.

The GM2-1 ceramide: identification of the fatty acids. The fatty acids in the ceramide were identified by gas chromatography (GC). Briefly, the GM2-1 was purified by high performance TLC (HPTLC) from a mixture of acidic glycolipids extracted from human pancreas followed by TLC separation on silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) in chloroform:methanol:0.25 % aqueous KCl (5:4:1, v:v:v). After exposure to iodine vapours, the band migrating between GM2 and GM1 standards was scraped, eluted from silica with chloroform and methanol (2:1, v:v) and dried under a stream of nitrogen. The purity of the band was determined by two-dimensional HPTLC analysis followed by resorcinol staining. The types and percentages of fatty acids in the ceramide of purified GM2-1 were determined as methyl esters after vigorous methanolysis with 0.5 N methanolic anhydrous HCl at 80 °C for 18 h. After cooling, the solution was extracted 4 times with hexane. The hexane phase containing the fatty acid methyl esters was collected, dried under nitrogen and injected (in CH_2Cl_2) into an SPB 2380 fused silica capillary column (Supelchem, Bellefonte, Pa., USA) programmed at 5 °C/min from 170 to 230 °C, using a Perkin Elmer 8310 gas chromatograph (Perkin Elmer, Norwalk, Conn., USA), equipped with a flame ionization detector. Saturated and unsaturated fatty acids (Sigma, St. Louis, Mo., USA) were used as standards.

The GM2-1 oligosaccharide: determination of the sugar composition and sequence. The type of sialic acid was identified as follows: briefly, after methanolysis in 0.05 N methanolic-HCl of HPTLC purified GM2-1 ganglioside, the dried products were converted to trimethyl-silylester derivatives with 50 µl of trimethylsilylimidazole (Supelchem) at 70 °C for 20 min. Aliquots of the reaction mixture were injected into an SPB-5 fused silica capillary column (Supelchem) programmed at 3 °C/min from 220 to 280 °C. Standard N-acetyl-neuraminic acid (NANA), N-glycolyl-neuraminic acid, 7-O-acetyl-NANA and 9-O-Acetyl-NANA (BioCarb, Lund, Sweden) were used as controls. The monosaccharide moieties of the oligosaccharide chain were identified according to the following procedure: briefly, HPTLC purified GM2-1 was subjected to methanolysis with 3 N methanolic HCl at 80 °C for 90 min. After cooling, the solution was extracted 4 times with hexane; the methanolic phase containing the monosaccharides was dried under nitrogen and the dried products were converted to trimethylsilylester derivatives with 50 µl of trimethylsilylimidazole (Supelchem) at 70 °C for 20 min. Trimethylsilyl-methylglycosides were injected into an SPB-5 fused silica capillary column (Supelchem) programmed at 5 °C/min from 150 to 210 °C, maintained isothermally for 5 min and programmed at 5 °C/min from 210 to 280 °C. Standard glucose, galactose and fucose (Merck); galactosamine, glucosamine, N-acetyl-galactosamine and N-acetyl-glucosamine (Sigma) were used as controls. In order to establish the position of the sialic acid on the oligosaccharide, GM2-1 ganglioside has been subjected to digestion with exoneuraminidase from *Clostridium perfringens* and with neuraminidase from *Arthrobacter ureafaciens* (Boehringer). The sugar sequence of the GM2-1 oligosaccharide has been determined by sequential enzymatic digestions of HPTLC purified GM2-1 with exo-neuraminidase and with exoglycosidases (β-galactosidase from *Escherichia coli*, β-hexosaminidase from bovine kidney, Boehringer) as described by Macher and Klock [9], followed by HPTLC analysis of the digested products and by GC identification of the liberated monosaccharides. HPTLC analysis was performed on silica gel 60 HPTLC plates (Merck) in chloroform:methanol:0.25 % aqueous KCl (5:4:1, v:v:v) followed by visualization of carbohydrate-containing bands with α-naphtol. Liberated monosaccharides from each enzymatic digestion were identified by GC as previously described.

Mass spectrometric (MS) analysis of GM2-1 ganglioside. A Perkin Elmer Sciex API III tandem mass spectrometer, equipped with the articulated ion spray source, was employed. Mass calibration and resolution were checked with a polypropylene glycol solution. The MS experiments were run with a resolution better than 0.8 atomic mass units (amu); for the MS MS runs, resolution for both quadrupoles was set at 1 amu. All the instrumental operating parameters were standard except the orifice voltage operated at 50 V. MSMS gas collision fragmentation was run at a collision energy of 50 eV and with argon at a thickness of 280×10^{12} molecules/cm^2. Samples were dissolved in an aqueous solution of 50 % ethanol containing 0.1 % formic acid and 2 mmol/l ammonium acetate. Introduction was operated by a Harvard Infusion Pump (Harvard, Mass., USA) at a flow rate of 2 µl/min. MS and MSMS spectra were collected at a rate of 10 ms per amu with a step size of 0.1 amu for MS and 1 amu for MSMS experiments. Acquired data were processed by "MacSpec" software; "Hypermass" option was used to handle data concerning multiply-charged ions.

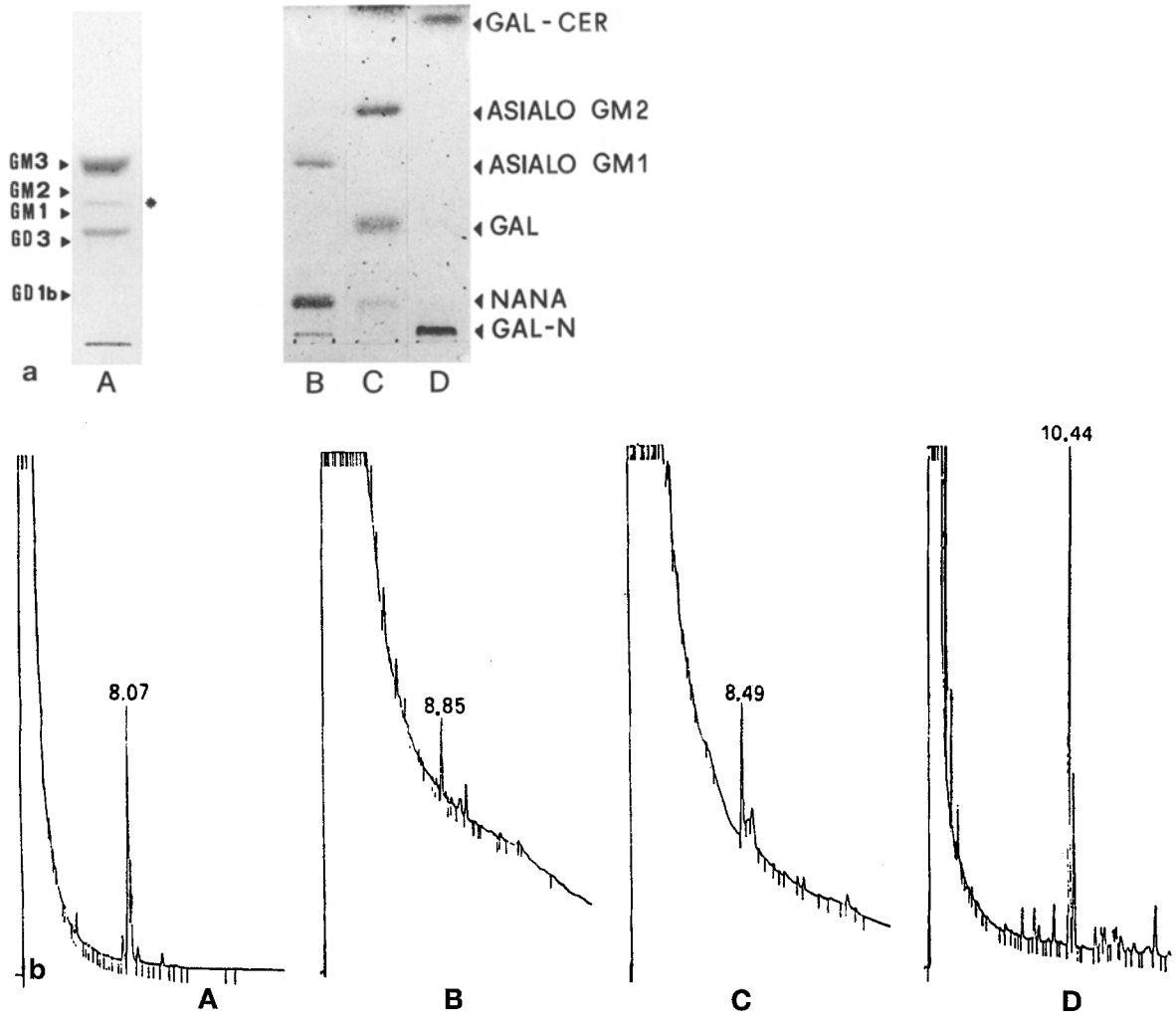

Fig. 1. (a,b) The GM2-1 oligosaccharide: determination of the sugar sequence. The sugar sequence of the GM2-1 oligosaccharide has been determined by sequential enzymatic digestions of HPTLC purified GM2-1 with exo-neuraminidase and with exoglycosidases (β-galactosidase, β-hexosaminidase) followed by HPTLC analysis (**a**) of the digested products and by GC identification (**b**) of the liberated monosaccharides. (**a**) HPTLC analysis was performed on silica gel 60 HPTLC plates followed by visualization of carbohydrate-containing bands with α-naphtol. *Lane A)* Pancreatic ganglioside mixture (GM3, GM2-1, GD3); the GM2-1 band (*) has been scraped and digested as follows: *Lane B)* After treatment with exoneuraminidase from *Clostridium perfringens,* hydrolyzed GM2-1 gives a band comigrating with standard NANA and a band comigrating with asialo-GM1. *Lane C)* The neutral glycosphingolipid obtained after neuraminidase treatment of GM2-1, has been treated with β-galactosidase. After the reaction, a band comigrating with standard galactose (GAL) and a band comigrating with asialo-GM2 were obtained. *Lane D)* The neutral glycosphingolipid product obtained after treatment with β-galactosidase has been hydrolized with β-hexosaminidase to a component with TLC properties of galactosyl-ceramide (GAL-CER) and to a band comigrating with standard galactosamine (GAL-N). (**b**) Liberated monosaccharides from each enzymatic digestion have been identified by GC. *Panel A)* GC analysis of the liberated sugar moiety after treatment with neuraminidase. The peak had a Retention Time (RT) corresponding to that of NANA. *Panel B)* GC analysis of the liberated sugar after treatment with β-galactosidase. The peak had a RT corresponding to that of standard galactose. *Panel C)* GC analysis of the liberated sugar after treatment with β-hexosaminidase. The peak had a RT corresponding to that of standard galactosamine. *Panel D)* GC analysis of the liberated sugar(s) after exhaustive acid hydrolysis of the neutral glycosphingolipid comigrating with galactosyl-ceramide obtained after treatment with β-hexosaminidase. The only component had a RT corresponding to that of standard glucose, indicating that this neutral glycolipid is glucosyl-ceramide

Results and discussion

The following fatty acid composition was found in the GM2-1 ceramide: C16:0 (29%), C16:1 (9%), C18:0 (20%), C18:0 (26%), C18:2 (9%), C22:1 (7%). The monosaccharides forming the oligosaccharide chain were identified as NANA, glucose, galactose and galactosamine at a ratio respectively of 1:1:1:2 by acid hydrolysis of purified GM2-1 followed by GC identification of the liberated sugars. The sensitivity

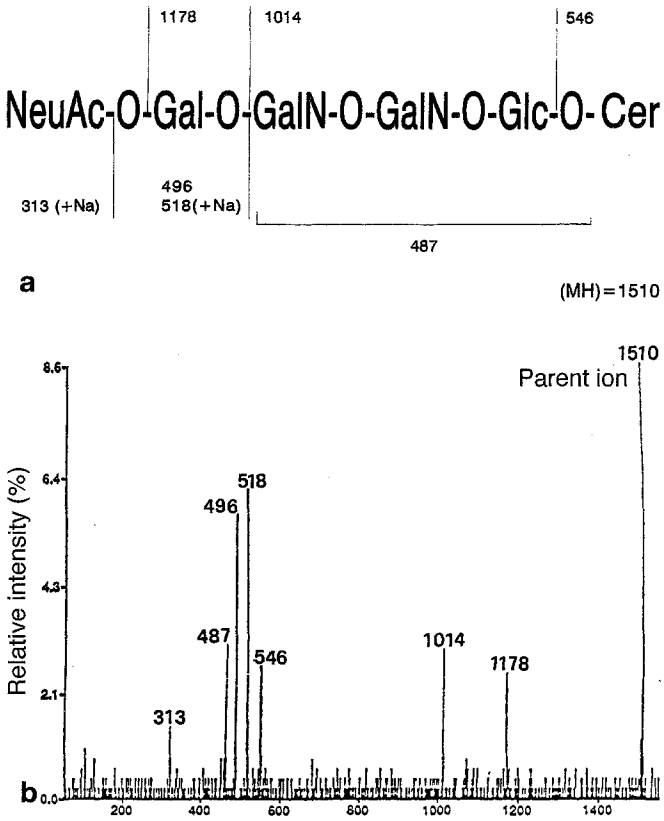

Fig. 2. **(a, b)** Mass spectrometric analysis of GM2-1 ganglioside. **(a)** Structure hypothesized according to GC and enzymatic studies confirmed by tandem mass spectrometry. **(b)** Intact ganglioside gave a molecular adduct at m/z 1510. Fragments corresponding to the sequential elimination of NANA and galactose residues clearly detected at m/z 1178 and 1014. Fragments corresponding to the trisaccharide galactosamine-galactosamine-glucose and to the ceramide detected respectively at m/z 487 and 546

of the sialic acid to the enzymatic digestion with both neurominidases used (Fig. 1) indicates its terminal location in the GM2-1 oligosaccharide chain. The sialic acid type was identified as NANA by GC (Fig. 1). In order to establish the carbohydrate sequence of GM2-1, the oligosaccharide chain was enzymatically degraded by sequential application of various exoglycosidases followed by identification of the liberated sugars and of the digestion products by GC and by TLC. The GM2-1 was digested by the sequential application of exoneuraminidase which liberated NANA; followed by β-galactosidase which liberated galactose; followed by β-hexosaminidase which liberated galactosamine. After this last digestion, the remaining glycolipid was identified as glucosyl-ceramide by GC and TLC (Fig. 1). Taking into account that GC studies showed the presence of two galactosamine residues, these data indicate the following carbohydrate sequence: NANA-galactose-galactosamine-galactosamine-glucose-*ceramide*. The hypothesized structure (Fig. 2 A) has been confirmed by tandem MS analysis (Fig. 2 B) where the intact ganglioside gave a molecular adduct at m/z 1510. The peaks showed that the molecule contained one NANA, four hexoses and a ceramide composed of a fatty acid of C18:1. Fragments corresponding to the sequential elimination of NANA and galactose residues were clearly detected at m/z 1178 and 1014. Fragments corresponding to the trisaccharide galactosamine-galactosamine-glucose and to the ceramide were detected at m/z 487 and 546, respectively.

The GM2-1 pancreatic islet ganglioside that we have biochemically characterized appears to have a novel structure that has a sialic acid moiety in the terminal position and two galactosamine residues in the oligosaccharide chain, in addition to a glucose and a galactose. The specificity of the bacterial neuraminidase used, suggests that the sialic acid residue is 2-linked with an α anomerity. Surprisingly, the galactosamine residues appear not to be acetylated, in contrast with other ganglioside structures so far described. In addition, unlike GM1 that contains N-acetyl-galactosamine, GM2-1 ganglioside gives a positive reaction when stained with ninhydrin, confirming the presence of at least one free amino group. The peculiarity of this novel glycolipid structure should be noted, with the sialic acid in terminal position and the galactosamine moieties containing a free amino group, which may confer on this ganglioside a particular conformation and reactivity, thus determining its antigenicity. Interestingly, GM2-1 shares some similarities with the monosialo-ganglioside LM1: they both have a terminal NANA residue, have the same number of sugar moieties and migrate between GM2 and GM1 standards on TLC plates. LM1 is a lactoseries acidic glycolipid that represents the major ganglioside in peripheral nerve myelin and has also been shown to be an important autoantigen in the demyelinating autoimmune disease Guillain Barre syndrome [10]. Furthermore, previous studies [8] have shown that about 30 % of newly-diagnosed IDDM patients express antibodies against the trisialo-ganglioside GT3, confirming that autoimmunity to this class of molecules is present in IDDM.

In conclusion, we have identified and biochemically characterized the GM2-1 islet ganglioside, finding it to be a novel and peculiar glycolipid structure. The identification, isolation and characterization of the GM2-1 islet ganglioside autoantigen and its availability in large quantities, will aid in pathogenetic studies examining the role of glycolipid autoantigens in modulating diabetes autoimmunity and in studies of humoral or cellular immune response towards this antigen in beta-cell autoimmunity.

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