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THE OCCURRENCE OF POLYSIALOGANGLIOSIDES INCLUDING GANGLIO-N-TETRAOSE SERIES IN ADULT BOVINE NASAL CARTILAGE

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SUMMARY: We extracted glycolipids from adult bovine nasal cartilage and purified some glycolipids by DEAE-Sephadex A-25 and Iatrobeads column chromatography. Cartilage contained 20 nmol of lipid bound sialic acid per gram wet tissue. The relative content of mono, di, tri, and tetrasialo gangliosides were 14%, 40%, 28% and 18%, respectively, as sialic acid content. We characterized some by examining carbohydrate composition, methylation analysis, sialidase treatment and mild acid hydrolysis. The ganglio-N-tetraose series, including GD1a, GD1b, GT1a, GT1b and GQ1b, was identified as one of the major ganglioside groups of this cartilage.

INTRODUCTION: It has been suggested that some glycolipids interact with extracellular components such as fibrinonectin (1,2). These interactions of extracellular components with the glycolipid in the cell membrane are a very important aspect of mesenchymal cells which are surrounded by, and adhere to matrix components. Although there are many analyses of the glycolipid of cultured fibroblast (3,4), there have been very few reports of glycolipid analysis of connective tissue such as tendon, cartilage, and bone without using cell culture technique. Since a cell may often change its glycolipid pattern to adapt itself to its environmental condition, there must be some difference between the glycolipid pattern of intact tissues excised from the body and that of cultured cells. This investigation was intended to elucidate the *in vivo* glycolipid pattern which exists in these tissues.

As one step in characterizing and comparing the glycolipid spectrum which exists in various connective tissues, we examined the glycolipid of

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bovine nasal cartilage which has never before been reported. Our investigation showed that bovine nasal cartilage has a very complex ganglioside pattern, and contains many species of polysialoganglioside. In this paper we report preliminary characterization of gangliosides of a ganglio-N-tetraose series which is relatively abundant in this cartilage but has rarely been reported in extraneural tissue.

MATERIALS AND METHODS:

Tissue materials: Bovine nasal cartilage (from Holstein species) was removed from the body within 5 hrs after death at the slaughter house and preserved frozen at -20°C until used. Tissue collected from several hundred cattle was combined and used for extraction of lipid. Nasal cartilage was sliced by plane after removing the surrounding tissue.

Isolation of the glycolipids: Sliced cartilage was homogenized in about 10 volumes of solvent (chloroform-methanol-water = 2 : 1 : 0.1) with a Virtis Homogenizer. Homogenized samples were heated in a reflux for 3 hrs at 55°C - 60°C. Extracts were isolated from the residue by filtration. The residue was reextracted sequentially with boiling solvents (chloroform -methanol-water = 1 : 1 : 0.1 and 1 : 2 : 0.1). The combined extracts were evaporated to dryness, treated with acetone to remove cholesterol and fatty acid and subjected to mild alkaline hydrolysis in 0.5 N KOH in methanol -chloroform (1 : 1) for 8 hrs at 37°C. After being neutralized with 1 N HCl, the hydrolysate was dialyzed against water, and then evaporated to dryness. These crude glycolipids were applied to a DEAE-Sephadex column to separate the unbound neutral from the bound acidic ones (5). Acidic glycolipid fraction, eluted from the column by chloroform-methanol-0.8 M sodium acetate (30 : 60 : 8) was further fractionated on a DEAE-Sephadex column with a continuous gradient elution of 0 to 0.5 M ammonium acetate in methanol (5). For further purification of each species of ganglioside, each fraction was fractionated repeatedly on an silicic acid column packed with Iatrobeads 6RS 8060 (Iatron Laboratories, Inc.), according to Momoi et al.(6). The final separation of glycolipids into homogenous species was sometimes performed by preparative thin layer chromatography (TLC).

Analytical procedure: To determine lipid bound sialic acid in the tissue, we performed small scale experiment using 290 g of tissue. After partition of a crude glycolipid fraction (described above) in a Folch system with 0.9 % KCl solution and washing the lower phase two times, the combination of upper phase was analyzed for lipid bound sialic acid by the resorcinol-HCl method (7).

TLC was carried out on high performance TLC plates of silica gel 60 (Merk). The following solvent was used for development: chloroform-methanol-water containing 0.02% $CaCl_2$ (55 : 45 : 10). The bands were visualized with resorcinol-HCl reagent. Carbohydrate composition was determined by gas liquid chromatography (GLC) of trimethylsilyl derivatives of methyl-glycoside after methanolysis at 75°C for 16 hrs with 0.5 N methanolic-HCl. (8). GLC was carried out with a glass column packed with 3% OV-1 on chromosorb AW-DMCS. Sialic acid species were analyzed by GLC using 3% OV-101 column according to the method of Yu and Ledeen (9).

Methylation analysis was performed essentially according to Stellner et al. (10), and acetates of partially methylated alditol acetates were analyzed by GLC and GLC-mass spectrometry on a column of 3% OV-225 and 1% OV-17 GLC was performed on a Shimazu GC-7A, and GC-mass spectrometry was performed on a Shimazu LKB-900B or a Nihondenshi JEOL-D300.

Mild acid hydrolysis of ganglioside was done according to Ishizuka et al. (11). Enzymatic treatment of gangliosides by neuraminidase from Clostridium perfringens, type VI (Sigma) was performed by the method of Ando (12).

RESULTS: In the small scale experiment, 1.7 mg of lipid bound sialic acid was extracted from 290 g of wet tissue. Thus, bovine adult nasal cartilage contains 20 nmol lipid bound sialic acid per gram wet tissue. An elution profile of sialic acid from a DEAE-Sephadex column is shown in Fig. 1. Four peaks are obvious. Peaks 1 to 4 correspond to mono, di, tri, and tetrasialogangliosides, respectively. Peak 2 is further divided into peak 2A and peak 2B. The former contains a relatively high content of ganglio-N-tetraose series and the latter contains a relatively high content of lactoseries of ganglioside, although they can not be separated clearly. The respective percentages of lipid bound sialic acid in the peaks of mono, di, tri, and tetrasialogangliosides were 14%, 40%, 28% and 18% of the total.

Plate A of Fig. 2 shows a very complex pattern, in which the individual gangliosides of bovine nasal cartilage overlap each other on TLC. (lane 1). However, when each ganglioside fraction (P1 to P4) from a DEAE-Sephadex column was analyzed, as seen on lane 2 to lane 6 of plate A, individual ganglioside species separated well and several bands of ganglioside could be recognized on each fraction from P1 to P4. As shown in Plate B of Fig. 2, several species od ganglioside could be isolated from each fraction.
In this study we partially characterized some isolated gangliosides; P2A-1, P2A-2, P3-1, P3-2 and P4-1. From observation of Fig. 2, it is evident that

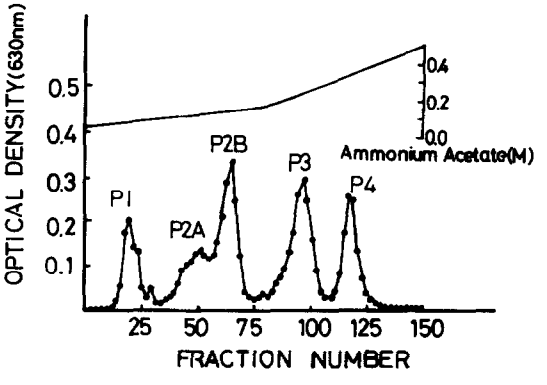

Fig. 1. Elution profile of ganglioside from DEAE-Sephadex column chromatograph. Ganglioside was eluted with a linear gradient with increasing concentration of ammonium acetate in methanol. Fractions of 20 ml of effluent from the column were collected and 0.1 ml of each fraction was used for determination of sialic acid content. P1 to P4 correspond to mono, di, tri, and tetrasialoganglioside fractions, respectively.

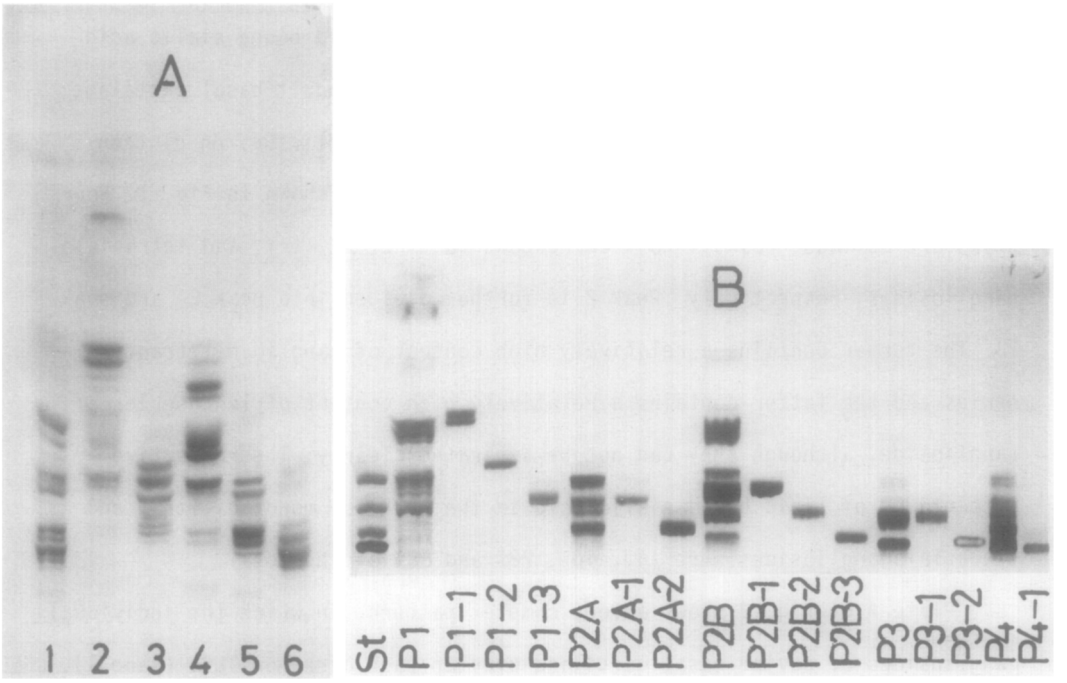

Fig. 2. Thin layer chromatograms of gangliosides from adult bovine nasal cartilage. Plate A: Lane 1 -- Total ganglioside of bovine nasal cartilage. Lane 2, 3, 4, 5, and 6 are gangliosides of P1, P2A, P2B, P3 and P4, respectively, from DEAE-Sephadex column chromatograph. Plate B: Gangliosides purified from each fraction (P1 to P4). St: standard mixture of GM1, GD1a, GD1b and GT1b from upper to bottom band, respectively. Both plates developed with chloroform-methanol-water containing 0.02% $CaCl_2$ (55 : 45 : 10). Bands visualized with resorcinol-HCl reagent.

P3-2 and P4-1 are major gangliosides contained in bovine nasal cartilage. As shown in Fig. 2, P2A-1, P2A-2 and P3-2 show almost the same respective mobilities as authentic brain GD_{1a}, GD_{1b} and GT_{1b} on TLC. The P3-1 band is between those of GD_{1a} and GD_{1b}, and the P4-1 band is below GT_{1b}.

The carbohydrate composition of isolated ganglioside is shown in Table 1. The neutral sugar moiety of the characterized gangliosides consisted of glucose, galactose and N-acetylgalactosamine in molar ratios of 1 : 2 : 1, which suggests a ganglio-N-tetraose series as their back bone structure. All of the sialic acid in the gangliosides which we examined was N-acetylneuraminic acid. In the methylation analysis (Table 2), P2A-1, P3-1, P3-2 and P4-1 each gave 2,3,6-tri-O-methylglucitol, 2,4,6-tri-O-methylgalactitol, 2,6-di-O-methylgalactitol and 4,6-di-O-methyl-1,3,5-tri-O-acetyl-2-deoxy-2-N-methylacetamidogalactitol. P2A-2 gave 2,3,4,6-tetra-O-methylgalactitol

Table 1 Carbohydrate compositions of gangliosides from bovine adult nasal cartilage

Gangliosides	Molar ratio			
	Glc	Gal	GalNAc	NeuAc
P2A-1	1.00	2.06	1.10	2.30
P2A-2	1.00	2.19	1.18	1.94
P3-1	1.00	1.89	0.97	3.15
P3-2	1.00	1.79	0.78	2.65
P4-1	1.00	1.74	1.08	3.98

instead of 2,4,6-tri-O-methylgalactitol, but the other methylated sugars were the same as for the other four gangliosides. Methylation analysis thus confirmed that these gangliosides are classified into the ganglio-N-tetraose group.

All of the characterized gangliosides were susceptible to neuraminidase from Clostridium perfringens and each gave a final product at the same Rf value as that of GM_1 from bovine brain (Fig. 3).

P4-1 was subjected to mild acid hydrolysis. The partially desialylated product gave bands corresponding to GT_{1b}, GD_{1b}, GT_{1a}, GD_{1a} and GM_1 as shown in Fig. 4. P4-1 was identified to be GQ_{1b}, judged from the appearance of the

Table 2 Permethylation analysis of gangliosides as partially methylated aldohexitol acetates.

Gangliosides	Methylated Hexitol and Hexosaminitol Acetates				
	2,3,6-Glc	2,4,6-Gal	2,3,4,6-Gal	2,6-Gal	4,6-GalNAc
P2A-1	+	+		+	+
P2A-2	+		+	+	+
P3-1	+	+		+	+
P3-2	+	+		+	+
P4-1	+	+		+	+

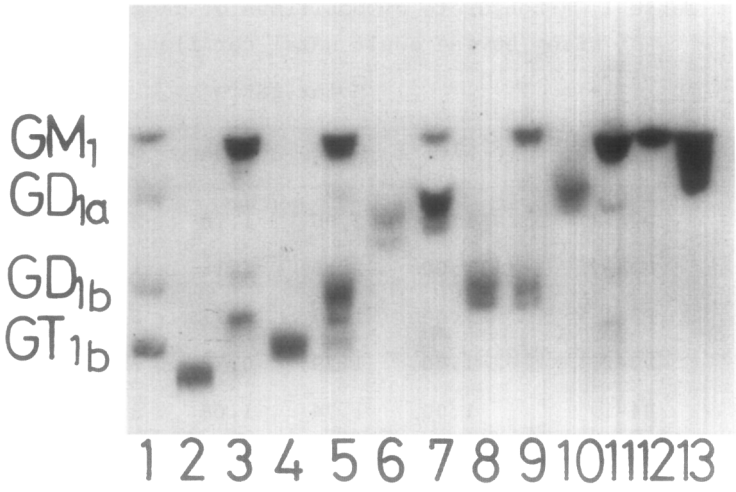

Fig. 3. Thin layer chromatogram of ganglioside product of hydrolysis by Clostridium perfringens neuraminidase. Lane 1 -- GM1, GD1a, GD1b and GT1b from upper to bottom band. Lane 2, 4, 6, 8 and 10 are P4-1, P3-2, P3-1, P2A-2 and P2A-1 before enzyme treatment, respectively. Lane 3, 5, 7, 9 and 11 are degradative products after enzyme treatment of P4-1, P3-2, P3-1, P2A-2 and P2A-1, respectively. Lane 12 and 13 are standard GM1 before and after enzyme treatment. Plate developed and bands visualized as in Fig. 2

band corresponding to GT_{1a}. This band is characteristic of the degradation of GQ_{1b} (12).

From densitometric determination of the gangliosides of this cartilage, GQ_{1b} and GT_{1b} each amounts to 15% of all gangliosides based on sialic acid

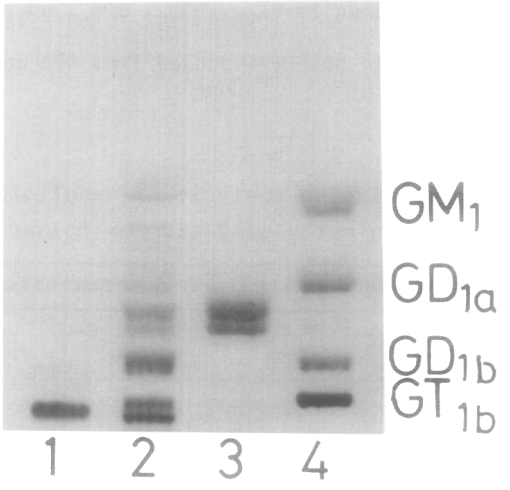

Fig. 4. Thin layer chromatogram of mild acid hydrolysis products of P4-1 by 5.6 mM formic acid at 80°C for 2 hr; Lane 1 -- P4-1 before treatment. Lane 2 -- P4-1 after treatment. Lane 3 -- P3-1 (GT1a). Lane 4 -- standard GM1, GD1a, GD1b and GT1b from bovine brain. Plate developed and bands visualized as in Fig. 2.

content, and the ganglio-N-tetraose group accounts for almost 50% of the total gangliosides as sialic acid content in this cartilage (Table not shown).

DISCUSSION: In this study it is shown that bovine adult nasal cartilage has a very complex ganglioside spectrum, and high proportion of polysialoganglioside content including the ganglio-N-tetraose series, GD_{1a}, GD_{1b}, GT_{1a}, GT_{1b} and GQ_{1b}. However, the absolute content of lipid bound sialic acid per gram wet tissue is very low compared with that of other organs reported (13). This is due to the existence of large amounts of matrix components such as collagen and proteoglycans in cartilage. Therefore we do not know the real content of ganglioside per cell compared with other organs.

The existence of the high proportion of GQ_{1b} found in this cartilage is very rare in extraneural tissue. There has been only one report: that of the Bewo cell, a human cell line of trophoblastic origin examined by Friedman et al. (14).

We do not know whether or not this unique ganglioside pattern of this adult bovine nasal cartilage is common to all the other cartilages localized in the different parts of the body. Moreover, we want to know the developmental change of ganglioside pattern of bovine nasal cartilage.

It is reported that GQ_{1b} appears in chicken brain in relatively large amounts in early stages of development and decreases later (15). Investigation of the change in the relative amount of these polysialoganglioside during development of this cartilage, as well as chicken brain, may suggest a possible role of these gangliosides in differentiation and development.

Analysis of the sphingosine and fatty acid of these gangliosides, in addition to that of the other glycosphingolipids in this cartilage, will be described in another report.

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