

Quantitative Determination of *N*-Glycolylneuraminic Acid Expression in Human Cancerous Tissues and Avian Lymphoma Cell Lines as a Tumor-associated Sialic Acid by Gas Chromatography-Mass Spectrometry¹

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ABSTRACT

N-Glycolylneuraminic acid (NeuGc) is distributed in most animals except humans and chickens. However, human and chicken cancerous tissues often synthesize this heterophilic sialic acid as a tumor-associated Hanganutziu-Deicher antigen [M. Naiki and H. Higashi, *Adv. Exp. Med. Biol.*, 152: 445–456, 1982; H. Higashi *et al.*, *Cancer Res.*, 45: 3796–3802, 1985]. In this paper, NeuGc in human cancerous tissues and chicken Marek's disease lymphoma cell lines was determined quantitatively with gas chromatography-mass spectrometry analysis using mass fragmentography. The detectable limit of NeuGc was 40 pg (0.12 pmol) in each injection using 5 ng of trideuteriomethyl ester trideuteriomethyl glycoside of the sialic acid as an internal standard sample when a pair of ions at *m/e* 386 and 389 was chosen for ion monitoring. NeuGc was detected in ganglioside-rich fractions of various human cancerous tissues from 5 of 8 patients examined but was not detected in glycosphingolipids of normal human tissues. The contents of NeuGc in these cancerous tissues ranged from 0.02 to 0.5% of the total sialic acid content. NeuGc was also detected in freeze-dried samples of 5 different cell lines from chicken Marek's disease lymphomas but was not detected in a cell line from chicken lymphoid leukemia lymphoma and normal chicken skeletal muscle tissue. The contents of NeuGc in the positive cell lines ranged from 0.03 to 0.11% of the total sialic acid content. These results indicate that NeuGc can be synthesized in both humans and chickens in some cancers.

INTRODUCTION

Sialic acids, NeuAc³ and NeuGc are widely distributed in all species of vertebrates and certain invertebrates as well. Most of them possess both sialic acids. But humans and chickens cannot synthesize NeuGc (1–3). Higashi *et al.* (4) purified NeuGc-containing gangliosides from equine and bovine erythrocytes as human heterophilic HD antigen whose antibody is occasionally elevated in sera of patients with various cancers (5–7). A similar antibody directed to the NeuGc residue as an antigenic epitope was also detected in avian antisera to a cell line (MSB1) derived from avian Marek's disease lymphoma (8). The HD antigens expressed in human cancerous tissues, avian Marek's disease lymphoma, and these cancer-derived cell lines were demonstrated by a membrane immunofluorescence technique (9–11), enzyme-linked immunosorbent assay (12), flow cytometry tech-

nique (13), and radioimmunoassay (14). The HD antigenic molecules were identified as 8 different GSL antigens (15–18) or glycoprotein antigens (19) in various human cancers by a thin layer chromatography-immunostaining technique or Western immunoblotting technique. In all the antigenic molecules, NeuGc was an essential immunodominant epitope, and in all immunological detection methods, hyperimmune chicken antisera to purified HD3 ganglioside were usually used. As described in this paper, we have succeeded in determining NeuGc content in some human cancerous tissues and different cell lines derived from avian Marek's disease lymphomas by GC-MS analysis with mass fragmentography (selected ion monitoring), one of the most sensitive physicochemical determination methods.

MATERIALS AND METHODS

Human Cancer Tissues. Human cancer tissues were obtained from patients who had operations at the hospital attached to the Research Institute for Microbial Diseases, Osaka University, or at Teikyo University Hospital. Normal human tissues were obtained from a man who died in a traffic accident and was autopsied at the Department of Legal Medicine, Faculty of Medicine, Hokkaido University. All tissues were stored at –80°C before lyophilization.

Avian Lymphoma Cell Lines. Cell lines used are as follows: MDCC-MSB1 (MSB1) derived from MD splenic lymphoma (20), MDCC-HP1 (HP1) from MD ovarian lymphoma (21), MDCC-HP2 (HP2) from MD lymphoma, MDCC-JP2 (MOGA2) from MD ovarian lymphoma (22), MDCC-RP1 (RP1) from MD transplant JMV (23), and LSCC-1104X5 (1104X5) from lymphoid leukemia lymphomatous bursa (24). They were cultured with RPMI-1640 medium supplemented with 10% chicken serum (Flow Laboratories Inc., Irvine, Scotland) at 41°C in a humidified atmosphere containing 5% CO₂ in air for more than 3 months after recovery from liquid N₂ stock. After the cell pellets were washed with phosphate-buffered saline, they were lyophilized. As a control tissue, chicken skeletal muscle was lyophilized and used.

Extraction and Preparation of GSL Fraction. In the cases of human cancerous tissues, GSLs were used for analyses. Ganglioside-rich GSL fraction was extracted from 0.5-g dry tissues with CHCl₃-CH₃OH-H₂O (4:8:3, v/v) and filtered through a filter paper. The extract was evaporated and dissolved in 0.2 N KOH/CH₃OH overnight at 37°C. The alkaline-treated sample was neutralized with Dowex 50 (H⁺ form) and evaporated after removal of the resin. GSL fraction was isolated from the crude fraction by phenylboronate agarose PBA 60 (Amicon Co., Danvers, MA) chromatography according to the method of Higashi *et al.* (12). The GSL fraction was dissolved in 3 ml of CHCl₃-CH₃OH (4:1, v/v) and applied to a column (0.9 x 1.6 cm) packed with Iatrobeads 6RS-8060 (Iatron Chemical Products, Tokyo, Japan) which had been equilibrated with the same solvent. Parts of neutral GSLs and sulfatide were eluted with 5 ml of the same solvent, and the ganglioside-rich GSLs were eluted with 20 ml of CHCl₃-CH₃OH-H₂O (5:5:1, v/v).

Deuterium-labeled Sialic Acid Preparation. GM₁ ganglioside (II³NeuAc-GgOse,Cer) was purified from bovine brain (25) and HD3 was purified from equine erythrocytes (4). Each of them (20 mg) was hydrolyzed in 1 ml of 0.05 N HCl-CD₃OD (99.99%, E. Merck, Darms-

Received 9/5/90; accepted 12/6/90.

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¹This work was supported by Grants-in-Aid for Scientific Research No. 63010023 and 63010027 from the Ministry of Education, Science and Culture of Japan.

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³The abbreviations used are: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; HD, Hanganutziu-Deicher; MD, Marek's disease; GSL, glycosphingolipid; GC-MS, gas chromatography-mass spectrometry; MF, mass fragmentography; m.u., mass units; TMS, trimethylsilyl; HD3, II³NeuGc-LacCer; HD5, IV³NeuGc-nLcOse,Cer; HD7, VI³NeuGc-nLcOse,Cer; other gangliosides follows the abbreviations recommended by Svennerholm (40).

tadt, West Germany) at 80°C for 1 h. After fatty acid methyl esters were removed by extraction with hexane, the deuteriomethanolic HCl solution was evaporated to dryness on ice by blowing N₂ gas. It was then incubated in a vacuum alkaline decicator for 15 min to remove HCl completely. The deuteriomethyl ester deuteriomethyl glycoside of each sialic acid produced was measured by the periodate-resorcinol method (26) and dissolved in C₂H₅OH at a constant concentration and stored in a -20°C freezer. The methyl ester methyl glycosides of NeuAc and NeuGc were prepared from the same gangliosides in the same manner by methanolysis using 0.5 N HCl-CH₃OH instead of 0.05 N HCl-CD₃OD.

GC-MS Analysis. Crude GSL fractions from human cancerous and normal tissues were methanolized in 1 ml of 0.05 N HCl-CH₃OH at 80°C for 1 h at a concentration of 0.1–0.5 mg/ml or 0.5–1.0 mg/ml for NeuAc or NeuGc assay, respectively. The methanolysate was extracted with hexane and the CH₃OH phase was mixed with 750 and 75 ng of deuterium-labeled internal standard of NeuAc and NeuGc, respectively. The mixture was dried and trimethylsilylated by incubation with 15 µl of TMS reagent, pyridine-hexamethyldisilazane-trimethylchlorosilane (10:13:8, v/v), at 80°C for 15 min. Approximately 1 µl was injected per assay, and each sample was assayed in triplicate. For analysis of avian cell lines and tissue, 3 mg of dry tissues was similarly hydrolyzed in 1 ml of 0.05 N HCl-CH₃OH and the hydrolysates together with 500 or 50 ng of each internal standard were trimethylsilylated for NeuAc or NeuGc determination, respectively.

A Shimadzu GC-MS QP 1000 apparatus equipped with a glass column (2.5 mm × 2 or 1 m) packed with 2% OV-1-coated Chromosorb W was used under the following conditions: column temperature, 250°C for NeuAc analysis or 260°C for NeuGc analysis; helium carrier gas flow rate, 34 ml/min; injection temperature, 300°C; separator temperature, 250°C; ion source temperature, 250°C; ionization voltage, 70 eV.

RESULTS

GC-MS Determination of NeuAc and NeuGc. In the mass spectra of the TMS derivatives of methyl ester methyl glycosides of NeuAc and NeuGc, two of the most intense ion peaks were *m/e* 298 and 420 and *m/e* 386 and 508, respectively, as shown by Kamerling *et al.* (27) and Siddiqui *et al.* (28). The peaks at *m/e* 298 and 420 in the *N*-acetyl group were shifted 88 m.u. to *m/e* 386 and 508 in the *N*-glycolyl group (34). By trideuteriomethyl substitution in each sialic acid, the peaks at *m/e* 298 and 386 and *m/e* 420 and 508 were shifted 3 and 6 m. u. to *m/e* 301 and 389 and *m/e* 426 and 514, respectively (Fig. 1). The deuterium-labeled compound was stable under the

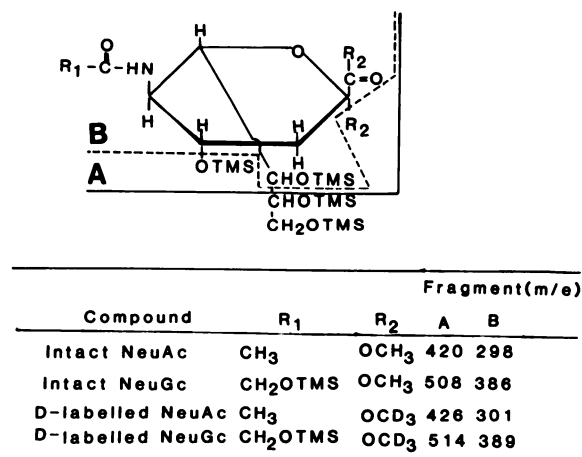


Fig. 1. Chemical structures and molecular masses of the fragments from TMS derivatives of the methyl ester methyl glycosides of both sialic acids and those of the trideuteriomethyl ester trideuteriomethyl glycosides of both sialic acids. Scheme from Kamerling *et al.* (15).

neutral condition and could be used as an internal standard to quantify NeuAc and NeuGc by GC-MS with MF analysis. A pair of the fragment ions at *m/e* 420 and 426 was chosen for NeuAc determination and pairs at *m/e* 386 and 389 and/or *m/e* 508 and 514 were chosen for NeuGc determination. The peak area ratios of each pair of ions were calibrated against different molar ratios of each sialic acid to the internal standard added for analysis (Fig. 2). The detectable limits of the calibration curves in pairs of ions at *m/e* 386 and 389 and *m/e* 508 and 514 for NeuGc detection, which demanded the most strict sensitivity, were 40 and 200 pg, respectively, when we injected 5 ng of internal standard of NeuGc for each analysis.

NeuAc and NeuGc Contents of GSLs from Human Cancerous Tissues and Normal Tissues. Crude GSL fractions, which were prepared from various cancerous tissues from 8 patients and normal lung, gastric, liver, and spleen tissues, were analyzed for sialic acid by GC-MS with MF. NeuGc which has been known as an antigenic epitope of a tumor-associated antigen (15) was detected in 5 of 8 samples from cancer patients when we used a pair of ions at *m/e* 386 and 389 and in 4 of 8 when we used a pair of ions at *m/e* 508 and 514. In the former pair of ions, a false peak at the position of NeuAc, which had a faster retention time, slightly overlapped the true NeuGc peak as shown in Fig. 3. Therefore, we thought that the former pair of ions was more sensitive but the latter pair was more accurate. The appearance of NeuGc was recognized in gastric cancer (1 of 2), liver cancer (1 of 1), malignant lymphoma (2 of 2), and teratoma (1 of 1) as shown in Fig. 3 and Table 1. The contents of NeuGc in these cancerous tissue GSLs were approximately 0.02–0.5% of the total sialic acid in molar ratio.

NeuAc and NeuGc Contents of Avian Lymphoma Cell Lines and Normal Tissue. NeuGc is not synthesized in chickens or humans but NeuGc-containing ganglioside antigens were detected in chicken Marek's disease lymphoma and the lymphoma-derived cell lines (29). In GC-MS with MF analysis, 5 different cell lines derived from Marek's disease lymphoma synthesized NeuGc in glycoconjugate molecules. The NeuGc contents were computed to be 0.03–0.11% of the total sialic acid contents when we used a pair of ions at *m/e* 386 and 389

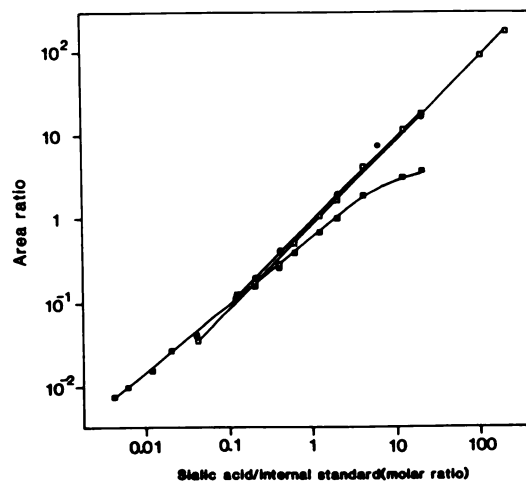


Fig. 2. Calibration curves for determination of NeuAc and NeuGc by GC-MS with MF. Various amounts of NeuAc or NeuGc were mixed with 750 or 75 ng, respectively, of each deuterium-labeled compound as an internal standard. The mixtures were converted to TMS derivatives and one-fifteenth of the total amounts was analyzed in triplicate by GC-MS with MF using a pair of ions at *m/e* 420 and 426 (○) for NeuAc or at *m/e* 386 and 389 (■) and *m/e* 508 and 514 (□) for NeuGc.

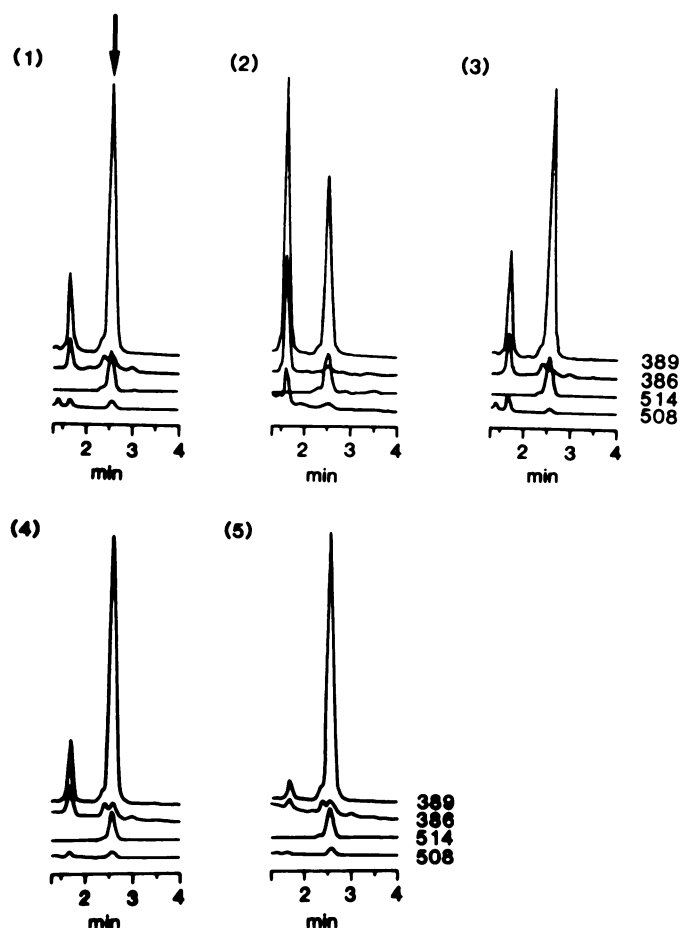


Fig. 3. GC-MS with MF analyses of NeuGc in positive cases of various human cancers. NeuGc contents of GSLs extracted from gastric cancer A (1), liver cancer (2), malignant lymphoma A (3), malignant lymphoma B (4), or teratoma (5) were quantified by GC-MS analyses using a 2% OV-1 column (2.5 mm x 2 m). Arrow, retention time of NeuGc; that of NeuAc was 1.6 min. Results are shown in Table 1.

(Table 2). Cell line 1104X5, which was established from avian lymphoid leukemia lymphoma, and normal chicken skeletal muscle did not have detectable amounts of NeuGc.

DISCUSSION

Quantitative determination of NeuAc using GC-MS with MF has been reported by several research groups. The deuterium-

labeled derivatives, methoxyneuraminic acid (30) and *N*-trideuterioacetylneuraminic acid (35), were used as internal standards to obtain an accurate retention time and similar levels of background interference by monitoring of ions, which had analogous structures. Mononen and Kärkkäinen (32) used the same trideuteriomethyl ester trideuteriomethyl glycoside of sialic acid that we used as an internal standard and they monitored a pair of ions at either *m/e* 298 and 301 or *m/e* 420 and 426. They prepared a sample by heating glycoproteins in 0.5 N methanolic HCl at 85°C for 18 h followed by *N*-acetylation. Their method is not applicable for separate detection of NeuAc and NeuGc because both sialic acids produce the same derivatives in this procedure. Therefore, we used a different hydrolyzing condition by heating in 0.05 N methanolic HCl at 80°C for 1 h (33). This condition releases most of the bound sialic acid from gangliosides with only small amounts of de-*N*-acylation (2).

In the present paper, both pairs of ions at *m/e* 386 and 389 and *m/e* 508 and 514 were monitored for NeuGc determination. The ion intensity of *m/e* 386 was always higher than that of *m/e* 508 in NeuGc fragments (28), and therefore, monitoring of the former ion was more sensitive than that of the latter one, although the background interference was always higher in the former ion (Fig. 3). Monitoring of both pairs of ions was therefore necessary so as not to misjudge a false peak. In human cancerous tissues, we used purified GSL fractions for sialic acid analysis to minimize background interference for more sensitive detection. When lyophilized samples of chicken cell lines and skeletal muscle tissue were directly applied for hydrolysis, the background level became higher. The detectable limit of NeuGc was 40 pg when we used GSLs and a pair of ions at *m/e* 386 and 389 for monitoring, but it decreased to 100 pg for analyzing lyophilized powder without any purification procedure. The latter sensitivity was enough to detect 0.01% NeuGc of total sialic acid. Direct use of freeze-dried tissues can save the preparation time and it is very convenient for clinical diagnosis.

Recently, Furukawa *et al.* (34) reported that no melanoma tissues or melanoma cell lines expressed NeuGc in amounts within the detectable limit by gas chromatography. Usually, GC-MS analysis has a sensitivity 10-fold or more than that of GC analysis. In the present case, NeuGc in cancerous tissues was only 0.03–0.14% of total sialic acid. It is difficult to make a definite peak for comparison with the NeuAc peak on the same gas chromatogram. In chickens, NeuGc-containing gangliosides were first found as MD tumor-associated surface

Table 1 Sialic acid contents of human cancerous tissues and normal tissues in GSLs

Tissue	NeuAc content (nmol/mg GSL) by <i>m/e</i> 420 and 426	NeuGc content (pmol/mg GSL) in total sialic acid	
		By <i>m/e</i> 386 and 389	By <i>m/e</i> 508 and 514
Cancer tissue			
Lung	57.8 ± 4.5 ^a	<0.089 ^{ac}	<0.89
Gastric	30.7 ± 0.4	43.6 ± 0.8 (0.14)	26.9 ± 0.7 (0.088)
Gastric	52.0 ± 0.8	<0.0036	<0.036
Liver	51.6 ± 2.6	12.4 ± 5.6 (0.024)	<0.036
Colon	20.8 ± 1.8	<0.0086	<0.086
Malignant lymphoma	41.6 ± 0.1	24.1 ± 1.4 (0.058)	6.6 ± 1.3 (0.016)
Malignant lymphoma	25.1 ± 0.3	48.1 ± 3.2 (0.19)	41.9 ± 1.0 (0.17)
Teratoma	9.7 ± 0.2	44.6 ± 8.0 (0.46)	34.3 ± 6.5 (0.35)
Normal tissue			
Lung	2.1 ± 0.1	<0.089	<0.89
Gastric	9.6 ± 0.1	<0.019	<0.19
Liver	25.9 ± 0.5	<0.0017	<0.017
Spleen	2.7 ± 0.7	<0.069	<0.69

^a Mean ± SD of triplicate determinations.

^b NeuGc was not detected and it was assumed to contain <0.089 molecular % from the detection limit.

^c Numbers in parentheses, %.

Table 2 Sialic acid contents of avian lymphoma cell lines in glycoconjugates

Cell line or tissue	NeuAc content* (nmol/mg dry tissue)	NeuGc content* (pmol/mg dry tissue)	NeuGc molecular % ^c in total sialic acid
MSB1	1.48 ± 0.09 ^c	0.71 ± 0.10	0.05
MSB1 E ^d	2.71 ± 0.10	2.68 ± 0.02	0.10
HP1	1.00 ± 0.09	0.62 ± 0.02	0.06
HP2	2.69 ± 0.15	3.08 ± 0.08	0.11
MOGA2	3.74 ± 0.11	3.05 ± 1.30	0.08
RP1	2.38 ± 0.04	0.62 ± 0.02	0.03
1104X5	2.10 ± 0.04		<0.01
Chicken	0.56 ± 0.05		<0.007
Skeletal muscle			

* NeuAc was determined by a pair of ions at *m/e* 420 and 426.

^b NeuGc was determined by a pair of ions at *m/e* 386 and 389.

^c Means ± SD of triplicate determinations.

^d Original MSB1 was used within 3 months after it had been recovered from a liquid N₂ tank.

antigens.

HD antigenic expression was demonstrated in MD lymphoma and the lymphoma-derived cell lines but not in lymphoid leukosis lymphoma and lymphoid leukosis lymphoma-derived cell lines by immunological methods (29). In an MD cell line, MSB1, the content of HD antigen-active gangliosides (HD3, HD5, and HD7) was 0.55% of the total gangliosides by the thin layer chromatography-immunostaining technique (12), but that of other cell lines has not been examined so far. In this paper, all 5 different MD cell lines examined were confirmed to contain NeuGc with a range of 0.03–0.11% of total sialic acids. This paper deals with a mixture of glycoproteins and gangliosides from MSB1, and therefore, the lower NeuGc content (0.1%) might be a result of dilution with more abundant amounts of NeuAc content of the glycoprotein fraction which did not show HD antigenicity.

Synthesis of NeuGc which was recognized in many animals, even in the primate monkey (35), was blocked during the evolution process to human beings. The block is broken in about 50% of various cancers (15). The synthesized NeuGc in cancerous tissues is not so much but immunogenic. If NeuGc is detected in surgically removed cancerous tissue by GC-MS, an immunotherapy with NeuGc-containing gangliosides (HD3 and/or HD5) prepared from animal tissues or erythrocytes may possibly reduce the mortality from tumor recurrence after the surgery. Increasing of ganglioside content is a typical characteristic of cancers (36). Serum lipid-associated sialic acid levels also increase in cancer patients (37, 38). If serum gangliosides are derived from tumor cells by shedding (39), quantitative determination of NeuAc and NeuGc in serum samples by the GC-MS analysis may be applicable to diagnosis of cancer.

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Toru Kawai, Akira Kato, Hideyoshi Higashi, et al.

Cancer Res 1991;51:1242-1246.

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