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Gangliosides as Markers of Cortisone-Sensitive and Cortisone-Resistant Rabbit Thymocytes: Characterization of Thymus-Specific Gangliosides and Preferential Changes of Particular Gangliosides in the Thymus of Cortisone-Treated Rabbits†

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ABSTRACT: Neutral glycosphingolipids and gangliosides in rabbit thymus, spleen, bone marrow, and erythrocyte ghosts were analyzed by conventional chemical and enzymatic procedures and negative ion fast atom bombardment mass spectrometry (FABMS). Thymus gangliosides showed a characteristic composition. Major gangliosides comprising 75% of the total thymus gangliosides were sialosyl lacto-*N*-*neo*-tetraosyl- and sialosyl lacto-*N*-*nor*-hexaosylceramides containing NeuGc and palmitic acid. These major thymus gangliosides were not detected in spleen, bone marrow, or erythrocytes, whereas GD1a, which was not present in the thymus even in a trace amount, was present in spleen and bone marrow. In addition, the major gangliosides in rabbit thymus were preferentially reduced when an animal was given an intraperitoneal injection of cortisone acetate, as found on analysis 48 h later. The decrease was accompanied by a concomitant increase in NeuAc-containing GM3 with longer chain fatty acids.

By application of the ganglioside-mapping technique (Iwamori & Nagai, 1978c) to studies on the tissue distribution of gangliosides, we found in rabbit that the ganglioside distribution in the thymus is uniquely different from those in other tissues (Iwamori & Nagai, 1981b,c) and that several gangliosides only occur in this tissue. Recently, considerable attention has been paid to the possible involvement of glycosphingolipids in various important immunological functions such as in the case of cell surface markers of immunocyte subpopulations (Kasai et al., 1980; Momoi et al., 1980; Nakano et al., 1980; Schwarting et al., 1980; Young et al., 1980; Taki et al., 1981; Rosenfelder et al., 1982; Nagai et al., 1984; Ugorski et al., 1984), differentiation antigens (Habu et al., 1980; Schwating et al., 1980; Akagawa et al., 1981; Kannagi, et al., 1983; Taki et al., 1983), lymphokine receptors (Riedl et al., 1982), mitogen-induced activation (Rosenfelder et al., 1978; Ryan & Shinitzky, 1979; Whisler & Yates, 1980; Sela, 1981), and immune recognition (Hakomori, 1981). In mouse, for example, globoside was identified as a serological marker of alloantigen-stimulated T lymphocyte percursors of both helper and cytolytic T cells (Muhlradt et al., 1984), and the

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cytolytic T cells could be distinguished from natural killer cells by their lack of serologically detectable asialo-GM1 (Kasai et al., 1980). Therefore, to elucidate the role of glycosphingolipids in the thymus, we compared the thymus glycosphingolipids with those in spleen, bone marrow, and erythrocytes by using the powerful FABMS[1] procedure for structural analysis. Moreover, in view of the possible relationship of the distribution of gangliosides in the thymus to the functional heterogeneity of thymocytes, comparative ganglioside analysis was performed on the thymus and spleen of rabbits with or without steroid administration.

Materials and Methods

Preparation of Erythrocyte Ghosts and Steroid Administration. Rabbits (New Zealand White, 3 months old) were purchased from Japanese Biological Materials Co., Tokyo. Blood was collected from the carotid artery with a catheter with an anticoagulant, heparin. After centrifugation at 300*g* for 10 min, the buffy coat cell layer on the surface of erythrocytes was carefully removed, and the erythrocytes were washed 5 times with phosphate-buffered saline. The erythrocytes were hemolyzed with hypotonic buffer (phosphate buffer, pH 7.4), and then the erythrocyte ghosts were washed 3 times with water until the supernatant solution after centrifugation was clear. The steroid was administered as follows. Cortisone acetate (Wako Chemicals, Tokyo) suspended in phosphate-buffered saline at a concentration of 50 mg/mL was injected intraperitoneally into five rabbits (125 mg/kg of body weight), and 48 h after the injection, glycolipids of the thymus and spleen were compared with those in five control rabbits.

Quantitative Analysis of Lipid Components. Thymus, spleen, bone marrow, and erythrocyte ghosts from the same rabbit were used for the analysis of lipid components, and quadruplicate analyses were performed to evaluate the results. Extraction of crude lipids, fractionation of the lipid extracts into neutral and acidic fractions, and isolation of neutral glycosphingolipids by acetylation and Florisil column chromatography were carried out according to the methods reported previously (Iwamori et al., 1982, 1984). Lipid-bound phosphorus and free cholesterol in the crude lipid fraction were determined by a colorimetric procedure (Bartlett, 1959) and by GLC with cholestane as an internal standard (Iwamori & Moser, 1975), respectively. Also, lipid-bound sialic acid in the acidic lipid fraction was determined by the resorcinol–HCl method (Miettinen & Takki-Luukainen, 1959). The individual neutral glycosphingolipids and gangliosides were quantitated densitometrically with a dual-wavelength TLC densitometer (CS-910, Shimadzu Co., Kyoto) after the spots were located with orcinol–H_2SO_4 and resorcinol–HCl reagents, respectively (Iwamori & Nagai, 1978b, 1979). The analytical wavelengths for neutral glycosphingolipids and gangliosides were set at 430 and 570 nm, respectively, and the control wavelength was set at 710 nm. CMH, CDH, CTH, globoside, IV$^3\alpha$-Gal-nLc$_4$Cer, GM3, GM2, GM1, GD1a, GD1b, and GT1b, 0.2–2 μg, were used as standards for quantification, and the mean values were determined for five separate determinations. The same procedure was used for comparison of the ganglioside compositions of the thymus and spleen or rabbits with or without steroid administration.

Table I: Chemical Compositions of Thymus, Spleen, Bone Marrow, and Erythrocyte Ghosts of Rabbit (NZW, 3 Months Old)[a]

	μmol/g of dry weight			
	thymus	spleen	bone marrow	erythrocyte ghosts
cholesterol	26.6	39.8	5.9	266.3
lipid-bound phosphorus	56.8	72.2	11.9	159.7
phosphatidylethanolamine	11.5	14.0	2.0	20.7
phosphatidylcholine	32.6	36.7	4.8	56.1
sphingomyelin	5.3	11.9	3.7	55.4
phosphatidylserine and -inositol	5.2	6.6	1.3	21.1
	nmol/g of dry weight			
	thymus	spleen	bone marrow	erythrocyte ghosts
lipid-bound sialic acid	900.0	1200.0	100.0	9.0
GM3	91.8	380.4	71.8	6.5
GD3	105.3	289.2	13.8	0.8
SLT	285.3			
GD1a		69.0	10.6	
SLH	307.8			
CMH	147	229	10	tr
CDH	38	52	5	146
CTH	31	110	14	2922
CTetH	31	7	2	tr
CPH	87	112	31	5050

[a] Globoside and nLc$_4$Cer were included in CTetH, and neutral glycosphingolipids having more than six carbohydrate residues were not measured because of the lack of suitable standard glycosphingolipids for quantitation. tr, trace amount.

Structural Analysis of Glycosphingolipids. The glycosphingolipids were purified by a combination of anion-exchange and silica gel column chromatographies (Iwamori & Nagai, 1978a,c, 1981a; Iwamori et al., 1982), and their structures were elucidated by comparison of their mobilities on TLC with those of standard glycosphingolipids, carbohydrate quantitation by GLC, linkage analysis of carbohydrate residues by permethylation studies as partially methylated aldohexitol acetates, exoglycosidase treatment, and a double immunodiffusion test on agar plates using rabbit anti-asialo-GM1 antiserum as reported previously (Iwamori & Nagai, 1978a, 1981a; Iwamori et al., 1982). In addition to the above conventional methods, negative ion FABMS was used for structural elucidation, which was quite useful for the analysis of molecular species, molecular weights, and carbohydrate sequences of glycosphingolipids (Arita et al., 1983a,b; Iwamori et al., 1984). Briefly, about 5 μg of glycosphingolipids was dissolved in 1 μL of 1,1,3,3-tetramethylurea/triethanolamine, 1:1 (v/v), and the solution was put in a stainless steel sample holder (1 × 5 mm) for the FAB ion source; then, about 1 μL of triethylenetetraamine was added. Analysis was performed by bombardment with a neutral xenon beam with a kinetic energy of 4–6 keV and detection of negative ions with a mass spectrometer (JMS DX-300,JEOL Ltd., Tokyo) equipped with a JMA-3500 computer system (JEOL Ltd.). (Perfluoroalkyl)phosphazine (Ultra mark, PCR Laboratories, Gainesville, FL) was used as a mass marker.

Results

Lipid Contents of Rabbit Thymus, Spleen, Bone Marrow, and Erythrocytes. The lowest contents of cholesterol and lipid-bound phosphorus per gram dry weight were found in

[1] Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; SLT, IV^{3}NeuGc-nLc$_4$Cer; SLH, VI^{3}NeuGc-nCl$_6$Cer; CTetH, ceramide tetrahexoside; CPH, ceramide pentahexoside; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; FABMS, fast atom bombardment mass spectrometry. The nomenclature of Svennerholm (1972) for gangliosides is used throughout.

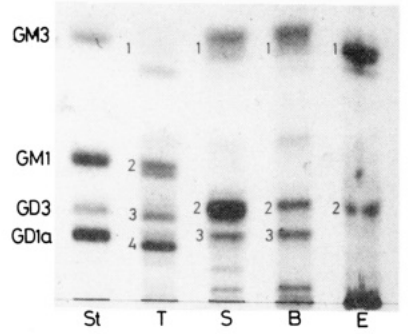

FIGURE 1: TLC of gangliosides from rabbit thymus (T), spleen, (S), bone marrow (B), and erythrocyte ghosts (E). The developing solvent was chloroform/methanol/0.5% $CaCl_2 \cdot 2H_2O$, 55:45:10 v/v/v, and the spots were located with resorcinol–HCl reagent. St, standard gangliosides.

bone marrow (Table I), but the ratios of lipid-bound sialic acid and cholesterol to lipid-bound phosphorus (=100) in thymus, spleen, and bone marrow were almost the same as each other (1:50:100). On the contrary, the cholesterol content of erythrocyte ghosts was higher than that of lipid-bound phosphorus, and the ganglioside content was extremely low, amounting to 9 nmol/g of dry weight. The molar ratio of lipid-bound sialic acid and cholesterol to lipid-bound phosphorus in rabbit erythrocyte ghosts was 0.01:166.7:100.0. As to phospholipids analyzed, the molar ratio of sphingomyelin to phosphatidylcholine was found to be the most variable among the tissues examined so far, it being the highest for erythrocyte ghosts and lowest for the thymus.

Gangliosides of Rabbit Thymus, Spleen, Bone Marrow, and Erythrocyte Ghosts. TLC of gangliosides obtained from thymus, spleen, bone marrow, and erythrocyte ghosts of a New Zealand White rabbit (3 months old) is shown in Figure 1. The thymus contained four major gangliosides, and the pattern essentially coincided with that for the NIBS strain of rabbit reported previously (Iwamori & Nagai, 1981b,c). The fourth band from the top (ganglioside 4 in Figure 1), whose mobility on TLC was close to that of GD1a, showed the highest concentration in the thymus, and its structure was determined to be VI^3NeuGc-nLc_6Cer from the following observations. (a) The ganglioside gave 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol, and 3,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,4,5-tri-*O*-acetylglucitol in a ratio of 1:3:2 (Table II). (b) The molar ratio of Glc, Gal, GlcNAc, and sialic acid was 1:3:2:1. (c) As shown in Figure 2, the carbohydrate sequence of the ganglioside as analyzed by negative ion FABMS was completely identical with that of VI^3NeuGc-nLc_6Cer (SLH). The molecular ion was obtained in the form of a negative ion with loss of a proton, and all fragment ions with the ceramide portion, which corresponded to sequential ions cleaved at the glycosidic linkages from the nonreducing to the reducing end of the carbohydrate chain, were easily recognized in the spectrum. The assignment of each ion is shown in Figure 2. Because each fragment ion was single, the ceramide portion

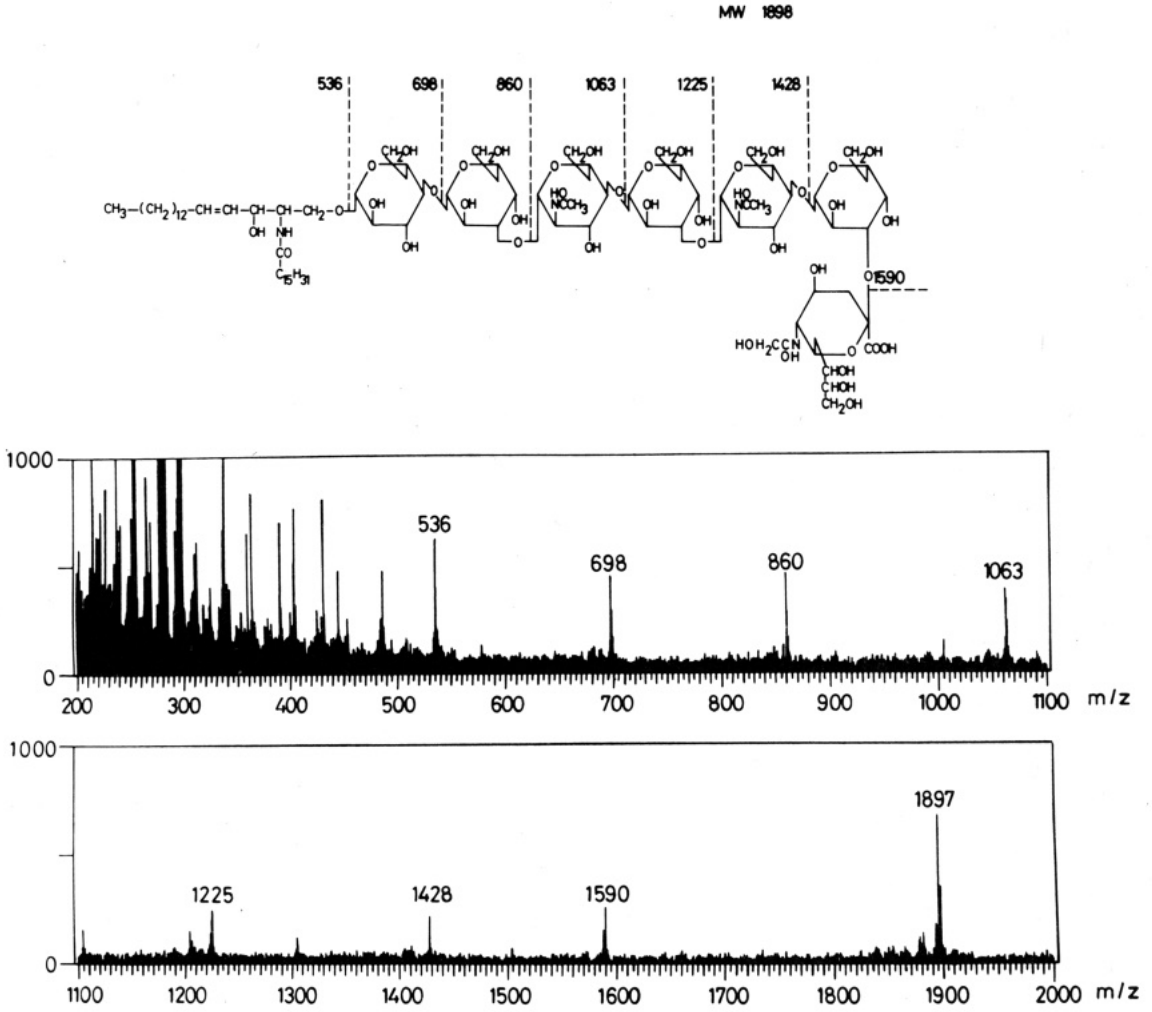

FIGURE 2: Negative ion FABMS spectrum of the fourth band from the top of rabbit thymus gangliosides (ganglioside 4 in Figure 1). About 3 μg of the ganglioside was used for the analysis.

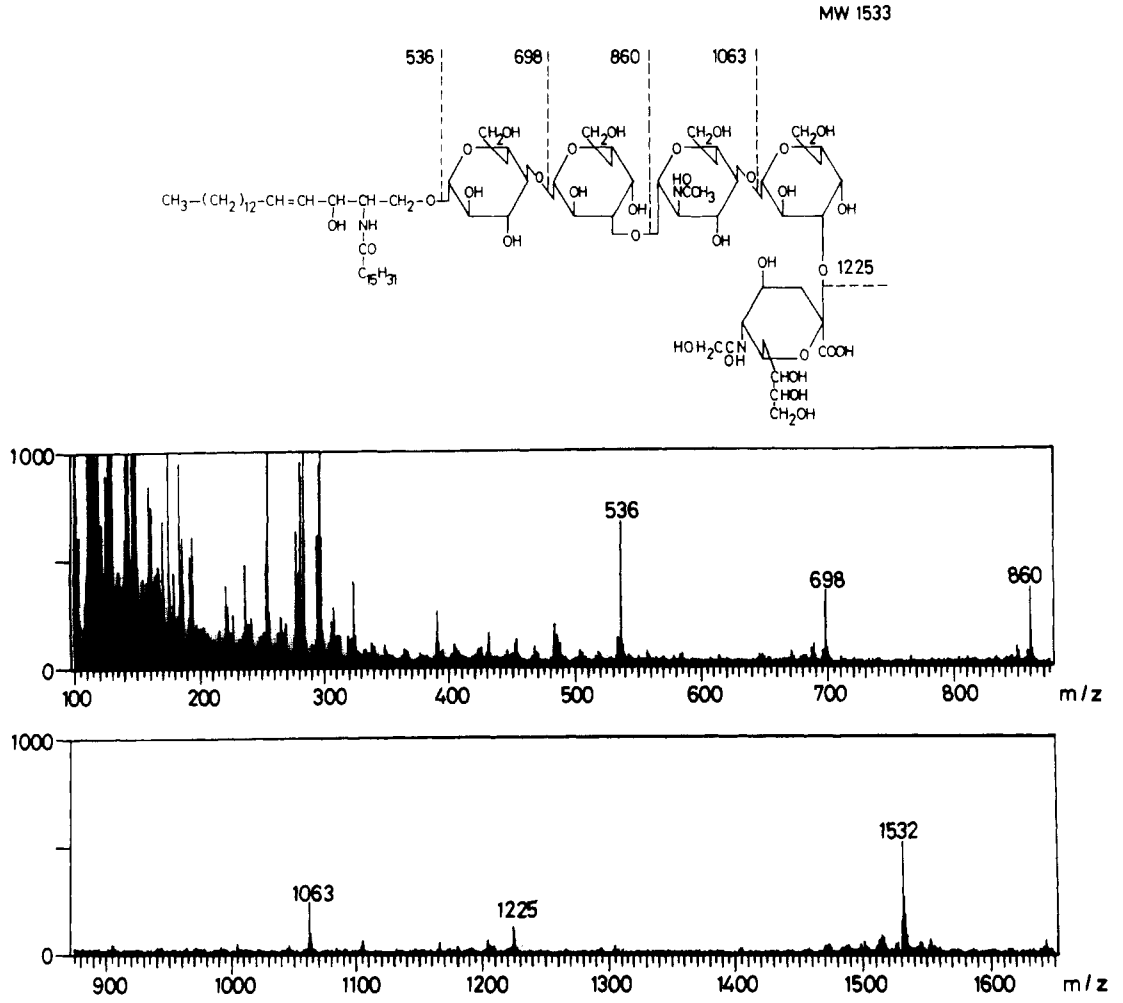

FIGURE 3: Negative ion FABMS spectrum of the second band from the top of rabbit thymus gangliosides (ganglioside 2 in Figure 1). About 3 μg of the ganglioside was used for the analysis.

of the ganglioside was clearly composed of a single molecular species, *N*-palmitoylsphingosine, which is in accord with the previous reports (Iwamori & Nagai, 181b,c). The fatty acid and long-chain base compositions of the gangliosides determined by GLC coincided with the structure. Also, from the spectrum, sialic acid at the nonreducing terminal was shown to be exclusively composed of NeuGc. (d) Negative ion FABMS of the ganglioside after treatment with neuraminidase showed a molecular ion at m/z 1590, and the same fragment ions as in Figure 2, that is, m/z 1428, 1225, 1063, 860, 698, and 536, were also observed, indicating that NeuGc is definitely located at the terminal of the carbohydrate chain. (e) Sequential cleavage with neuraminidase, β-galactosidase, and β-hexosaminidase, as performed in the previous studies (Iwamori & Nagai, 1981b, c), confirmed the structure shown in Figure 2. Thus, the ganglioside present in the thymus of New Zealand White rabbit in the highest concentration was concluded to be NeuGc(α2–3)Gal(β1–4)GlcNAc(β1–3)-Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1)–*N*-palmitoylsphingosine, which was identical with the ganglioside from the thymus of the NIBS rabbit. In a similar way, ganglioside 2, which was the second band from the top and whose mobility on TLC can be seen to be close to that of GM1 in Figure 1, was characterized as IV^{3}NeuGc-nLc$_4$Cer (SLT) from the following evidence. (a) The molar ratio of Glc, Gal, GlcNAc, and sialic acid determined by GLC was 1:2:1:1. (b) nLc$_4$Cer was produced on treatment with neuraminidase. (c) Permethylation analysis of the ganglioside showed 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitiol, and 3,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,4,5-tri-*O*-acetylglucitol in a ratio of 1:2:1 (Table II). (d) As shown in Figure 3, $(M - H)^-$ and the fragment ions corresponding to the sequential ions cleaved at the glycosidic linkages appeared at m/z 1532, 1225, 1063, 860, 698, and 536, so the spectrum indicated the complete sequence of the carbohydrate chain of the ganglioside. From the spectrum, NeuGc and palmitic acid were found to be the sole sialic acid and fatty acid of the ganglioside, respectively. On analysis of the fatty acid and long-chain base compositions by GLC, the structure of ceramide of SLT was found to be identical with that of SLH. Also, the top and third bands of thymus gangliosides (Gangliosides 1 and 3) in Figure 1, which showed R_f values similar to those of GM3 and GD3, respectively, were characterized as exactly GM3 and GD3 as follows. For GM3, (a) the molar ratio of Glc, Gal, and sialic acid was 1:1:1, (b) lactosylceramide was produced on treatment with neuraminidase, (c) permethylation analysis of the ganglioside showed 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol and 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol in a ratio of 1:1 (Table II), and (d) glucosylceramide was produced on treatment of the asialoganglioside with β-galactosidase. For GD3, (a) neuraminidase treatment at 20 °C gave GM3 and lactosylceramide, (b) 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol and 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol were obtained in permethylation studies (Table II), and (c) the carbohydrate sequence was found to be NeuGc–NeuGc–hexose–hexose–ceramide by negative ion FABMS. The contents of NeuGc–NeuGc–lactosylceramide and NeuGc–Ne-

Table II: Ratios of Partially Methylated Aldohexitol Acetates from the Purified Gangliosides Analyzed by GLC[a]

	ganglioside	2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl-glucitol	2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-galactitol	2,6-di-*O*-methyl-1,3,4,5-tetra-*O*-acetyl-galactitol	3,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,4,5-tri-*O*-acetylglucitol	4,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,3,5-tri-*O*-acetylgalactitol
thymus	1	1.00	1.05			
	2	1.00	2.03		0.99	
	3	1.00	1.01			
	4	1.00	3.10		1.97	
spleen	1	1.00	0.99			
	2	1.00	1.02			
	3	1.00	1.01	1.12		0.89
bone marrow	1	1.00	0.99			
	2	1.00	1.02			
	3	1.00	0.98	1.10		0.92
erythrocytes	1	1.00	1.02			
	2	1.00	1.01			

[a] Peak areas were compared with that of 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol (=1.00). Ganglioside numbers correspond to those in Figure 1.

uAc–lactosylceramide were estimated to be 92 and 8%, respectively, but NeuAc–NeuAc–lactosylceramide and NeuAc–NeuGc–lactosylceramide were not detected in the thymus. On the other hand, spleen and bone marrow showed distinctly different compositions from the thymus. Three major gangliosides (gangliosides 1–3 in Figure 1) showing identical mobilities with those of GM3, GD3, and GD1a, respectively, were recognized in spleen and bone marrow, as shown in Figure 1. By application of the same procedure used for the characterization of GM3 and GD3 from thymus, the top band and the second band from the top were clearly identified as GM3 and GD3. The contents of NeuAc–lactosylceramide in total GM3 from spleen and bone marrow were 63.3 and 77.1%, respectively. And the molar concentrations of di-NeuAc-lactosylceramide, NeuAc–NeuGc– or NeuGc–NeuAc–lactosylceramide, and di-NeuGc–lactosylceramide in GD3 from spleen were 73.9, 6.2, and 19.9%, respectively, whereas GD3 of bone marrow as exclusively in the form of di-NeuAc–lactosylceramide. The third bands from the top of gangliosides (ganglioside 3 in Figure 1) from spleen and bone marrow were clearly characterized as GD1a with NeuAc as the sialic acid. Evidence for this structure was as follows. (a) The hydrolysates of the ganglioside after treatment with 1 M formic acid at 80 °C for 1 h formed a fused precipitin line with asialo-GM1 on an Ouchterlony double immunodiffusion agar plate with anti-asialo-GM1 antiserum. (b) The ganglioside gave the following partially methylated aldohexitol acetates in equimolar amounts: 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol, 2,6-di-*O*-methyl-1,3,4,5-tetra-*O*-acetyl-galactitol, 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol, and 4,6-di-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)-1,3,5-tri-*O*-acetylgalactitiol (Table II). (c) the carbohydrate sequence of the ganglioside as analyzed by negative ion FABMS was found to be hexose(NeuAc)–*N*-acetylhexosamine–hexose-(NeuAc)–hexose–ceramide. A typical negative ion FABMS spectrum of GD1a was presented in the previous paper (Iwamori et al., 1984). In addition, two bands of gangliosides (gangliosides 1 and 2 in Figure 1) from erythrocyte ghosts, whose R_f values were similar to those of standard GM3 and GD3, were identified as GM3 and GD3 according to the methods described above.

Neutral Glycosphingolipids of Rabbit Thymus, Spleen, Bone Marrow, and Erythrocyte Ghosts. TLC of neutral glycosphingolipids from rabbit thymus, spleen, bone marrow, and erythrocyte ghosts is shown in Figure 4. As is well-known (Eto et al., 1968), IV$^3\alpha$Gal-nLc$_4$Cer (CPH) with blood group

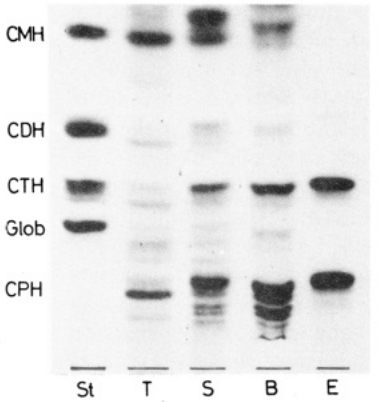

FIGURE 4: TLC of neutral glycosphingolipids from rabbit thymus (T), spleen (S), bone marrow (B), and erythrocyte ghosts (E). The developing solvent was chloroform/methanol/water, 70:30:4 v/v/v, and the spots were located with orcinol–H_2SO_4 reagent. St, standard neutral glycosphingolipids; Glob, globoside.

B determinant activity is present in rabbit erythrocytes in the highest concentration. Structures of CMH, CDH, CTH, CTetH, and CPH were confirmed by permethylation analysis and negative ion FABMS as described above. The same CPH was also detected in thymus, spleen, and bone marrow, and in addition, CMH, CDH, CTH, and CTetH were present in all tissues examined (Table I). The neutral glycosphingolipids with more than six carbohydrates were found to differ in their compositions and their relative concentrations among the tissues examined.

Glycosphingolipid Compositions of Rabbit Thymus, Spleen, Bone Marrow, and Erythrocyte Ghosts. The concentrations of glycosphingolipids of rabbit thymus, spleen, bone marrow, and erythrocyte ghosts are shown in Table I. The concentration of glycosphingolipids in erythrocyte ghosts was quite high as well as those of cholesterol and phospholipids when compared to those of thymus, spleen, and bone marrow, because isolated plasma membranes and not tissues were subjected to the analysis. However, a large percentage of glycosphingolipids in erythrocyte ghosts was due to CPH and CTH, and the concentration of gangliosides was found to be extremely low. GM3, which is the ganglioside present in erythrocytes in the highest concentration, comprised only 0.1% of CPH. On the other hand, the molar ratio of glycosphingolipids including gangliosides to liquid-bound phosphorus in erythrocytes was 3.7:100, which was rather similar to those of spleen (2.4:100), thymus (2.7:100), and bone marrow

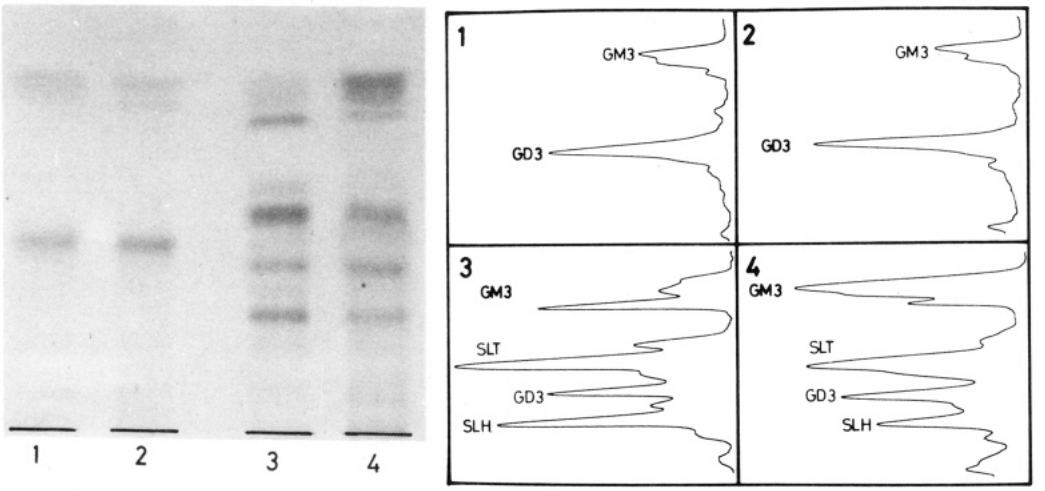

FIGURE 5: TLC of gangliosides from thymus and spleen of control and cortisone-administered rabbits. Spleen (1) and thymus (3) from the control rabbit. Spleen (2) and thymus (4) from a rabbit 48 h after intraperitoneal injection of cortisone acetate.

(2.6:100), indicating that the amount of glycosphingolipids as compared with matrix lipids such as cholesterol and phospholipids is relatively constant among the different tissues. In contrast with erythrocytes, in which CPH was present in the highest concentration, SLH was the highest in thymus and GM3 in spleen and bone marrow. SLH and SLT were characterized as thymus-specific gangliosides, whereas GD1a detected in spleen and bone marrow was not present in thymus or erythrocytes. However, such a distinct difference in ganglioside composition was not observed in the neutral glycosphingolipid composition. In order to demonstrate the tissue specificity of gangliosides more clearly on a molecular basis, when gangliosides were classified into three groups according to their asialocarbohydrate structures, lactose, ganglio-*N*-tetraose, and lacto-*N-neo*-tetraose, and lacto-*N-nor*-hexaose, thymus gangliosides showed the unique characteristic that the majority of the gangliosides can be classified into the area of lacto-*N-neo*-tetraose and lacto-*N-nor*-hexaose. SLH and SLT comprised 75% of the total gangliosides in thymus, but in spleen, bone marrow, and erythrocytes, more than 89% of gangliosides were lactose-containing molecules. On the other hand, GD1a, classified as a ganglio-series ganglioside, was present in spleen and bone marrow at a concentration of about 10%. Also, the concentration of NeuGc in thymus gangliosides was higher than those in the cases of spleen, bone marrow, and erythrocytes.

Changes in Ganglioside Composition of Rabbit Thymus with Cortisone Administration. Five controls and five steroid-administered rabbits were used for this experiment. At 48 h after intraperitoneal injection of cortisone acetate, thymus and spleen tissue weights were greatly reduced as shown in Table III. Especially, that of thymus was about one-third of the control. The concentrations of cholesterol and lipid-bound phosphorus per gram of dry tissue weight were increased in both tissues, whereas that of lipid-bound sialic acid in thymus was not changed after steroid administration. Figure 5 shows TLC of gangliosides from spleen and thymus of rabbits treated with or without cortisone acetate. Although the relative ratio of GM3 and GD3 in the spleen was not significantly altered by cortisone, gangliosides in the thymus were found to be changed as to the composition and the molecular species of GM3. In Figure 5, a decrease of SLT and SLH and an increase of GM3 relative to that of GD3 can be clearly observed, and of the three bands of GM3, the top two became major in the thymus of cortisone-administered rabbits, which was in contrast to the lowest band being abundant in the control thymus. As shown in Table III, the concentration of GD3 was not significantly changed by cortisone administration, whereas that of GM3 was doubled and those of SLT and SLH were halved compared to the control levels. The total amounts of SLH in the control and cortisone-administered thymuses were 163.6 and 26.8 μmol, respectively, indicating that SLH was reduced to one-sixth of the total amount in the control by cortisone administration. All rabbits used in this experiment showed identical results, indicating that SLT and SLH are selectively localized on the cortisone-sensitive thymocytes (the immature cells in the cortex). Since a change in the relative concentrations of the three bands of thymus GM3 was observed on cortisone administration (Figure 1), the fatty acid, long-chain base, and sialic acid compositions of GM3 were analyzed to clarify the molecular basis for the change. NeuGc-containing GM3 amounted to 56% of the total GM3 in the control thymus but was almost negligible in the cortisone-administered thymus. In addition, GM3 from the thymus of cortisone-administered rabbits contained more abundant longer chain fatty acids than that of control rabbits. On the other hand, sphingosine was the major long-chain base of GM3 from both thymuses. Thus, the alteration of GM3 molecular species on cortisone administration is ascribed to the compositional changes of the fatty acid and sialic acid moieties, and as a consequence, GM3 with NeuAc and longer chain fatty acid is assumed to be localized on the cortisone-resistant thymocytes (the medullary mature cells) and/or the thymus matrix.

Table III: Chemical Compositions of Thymus and Spleen of Control (n = 5) and Cortisone-Administered (n = 5) Rabbits (NZW, 1.5 Months Old)[a]

	thymus		spleen	
	control	steroid administered	control	steroid administered
weight (g)	1.67	0.48	0.78	0.47
water (%)	74.9	76.1	78.2	79.1
cholesterol[b]	16.6	26.2	38.3	40.5
lipid-bound phosphorus[b]	22.7	30.3	68.9	89.2
lipid-bound sialic acid[b]	0.4	0.4	2.0	2.3
GM3[c]	72.9	148.2	582.6	612.2
SLT[c]	99.3	49.7		
GD3[c]	64.7	58.4	682.1	862.1
SLH[c]	98.0	55.9		

[a] At 48 h after intraperitoneal injection of cortisone acetate, analysis was performed as described in the text. [b] Micromoles per gram of dry weight. [c] Nanomoles per gram of dry weight.

Discussion

There are several lines of evidence showing that a certain glycosphingolipid serves as a marker of lymphocytes. Work on characterization of different cell types has mainly been conducted with the use of carbohydrate-specific anti-glycosphingolipid antibodies or lectins. But the antigen detectable with the immunochemical procedure was not always directly related to glycosphingolipid per se. Accordingly, we felt that it was necessary to characterize the tissue or cellular specificity of glycosphingolipis on the basis of the chemical structures. Therefore, we undertook the precise analysis, using a new FABMS procedure, of all lipid classes in the lymphoid organs of rabbit to extend our previous findings (Iwamori & Nagai, 1978d, 1981b,c; Nagai & Iwamori, 1980a,b). The usefulness of FABMS for structural elucidation of glycosphingolipids with underivatized material was clearly demonstrated in this study. Since glycosphingolipids in mammalian tissues and cells can be classified into six types according to their principal carbohydrate sequences, FABMS information on glycosphingolipids, such as molecular weight, molecular species, and the carbohydrate sequence, is quite adequate for determining the glycosphingolipid class and the complete structure. On the basis of observations that repeated sequences of hexose and *N*-acetylhexosamine were present in the molecule and that NeuGc was located at the nonreducing terminal of the carbohydrate chain, we could conclude that one of the thymus-specific gangliosides belonged to the lacto series having a core carbohydrate of lacto-*N-nor*-hexaose. Furthermore, the unique structure of the ganglioside as to ceramide and sialic acid residues could be easily elucidated from the spectrum. Thus, the tissue specificity of the thymus as to ganglioside molecular species observed in the NIBS strain of rabbit (Iwamori & Nagai, 1981b,c) could be reconfirmed in the New Zealand White strain for rabbit. Rabbit thymus gangliosides showed the following unique characteristics: (a) Lacto-series gangliosides having lacto-*N-neo*-tetraose and lacto-*N-nor*-hexaose as the asialocarbohydrate, SLT and SLH, comprised 75% of the total gangliosides. (b) The NeuGc was the sole sialic acid of SLT and SLH in the thymus. (c) The proportion of palmitic acid in the glycosphingolipid fraction of the thymus was significantly higher than those in the other tissues, and SLT and SLH from the thymus were exclusively composed of palmitic acid. The structural uniqueness of SLT and SLH as to the exclusive NeuGc and palmitic acid composition is quite interesting with regard to their antigenic properties. NeuGc is known to be involved in the antigenic determinant of the Hanganutziu–Deicher antigen (Higashi et al., 1977; Merrick et al., 1978). Furthermore, the structural specificity as to the hydrophobic portion of glycosphingolipids has been recognized to affect and regulate the antigenicity of the carbohydrate portion. For example, Le^x and H antigens and IV^3NeuAc-nLc$_4$Cer with NeuAc(α2–6)Gal and NeuAc(α2–3)Gal showed different fatty acid compositions (Kannagi et al., 1982), respectively, and a tumor-associated glycosphingolipid detected with monoclonal antibody directed toward human pancreatic carcinomas had a unique structure, *N*-α-hydroxy fatty acyl phytosphingosine (Falk et al., 1983). Thus, the immunochemical properties of SLT and SLH in rabbit thymus are of considerable interest. We expected the existence of these specific gangliosides in bone marrow and spleen; however, they were not found in either tissue even in a trace amount. This finding strongly suggests the possibility that these unique molecules may participate in the surface antigens of lymphocytes, especially of the T cell lineage developing under the microenvironmental influence of the thymus. One of the methods for resolving each functionally heterogeneous thymocyte type separately is cortisone administration, with which the remarkable reduction of thymocytes was observed, and the differential functions of cortisone-sensitive and cortisone-resistant thymocytes were well characterized by combination with other surface markers (Smith, 1984; Reichert et al., 1984). The present investigation also showed the differential expression of glycosphingolipid molecules, especially of gangliosides in the cortisone-sensitive and cortisone-resistant thymocytes. At 48 h after intraperitoneal injection of cortisone acetate, SLT and SLH in the thymus were selectively reduced in concentration, which was in contrast to the concomitant increase in NeuAc-containing GM3 with longer chain fatty acids. Thus, SLT, SLH, and NeuGc-containing GM3 were thought to be localized predominantly in the cortisone-sensitive thymocytes (the immature cells in the cortex) and NeuAc-containing GM3 in the cortisone-resistant thymocytes (the medullary mature cells) and/or the thymus matrix. These gangliosides should be useful markers to characterize the thymocyte subpopulations. Further studies on the cellular function and the metabolism of glycosphingolipids in relation to their immunological properties should be conducted on the cellular level by correlating the marker glycosphingolipids with the well-known phenotypic properties of immunocyte subpopulations. A study along these lines is now in progress in our laboratory.

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Registry No. GD1a, 12707-58-3; GM3, 54827-14-4; IV^3NeuGc-nLc$_4$Cer, 72626-26-7; VI^3NeuGc-nLc$_6$Cer, 73379-94-9; GD3, 62010-37-1; NeuGc(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)-GlcNAc(β1–3)Gal(β1–4)Glc(β1–1)-*N*-palmitoylsphingosine, 99397-76-9; cortisone, 53-06-5; cholesterol, 57-88-5.

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Biosynthesis of Porphyrins and Corrins. 1. ^{1}H and ^{13}C NMR Spectra of (Hydroxymethyl)bilane and Uroporphyrinogens I and III†

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ABSTRACT: High-field NMR spectroscopic methods have been applied to study the reactions catalyzed by porphobilinogen (PBG) deaminase and uroporphyrinogen III (uro'gen III) cosynthase, which are the enzymes responsible for the formation of the porphyrin macrocycle. The action of these enzymes in the conversion of PBG, [2,11-^{13}C]PBG, and [3,5-^{13}C]PBG to uro'gens I and III has been followed by ^{1}H and ^{13}C NMR, and assignments are presented. The principal intermediate that accumulated was the correspondingly labeled (hydroxymethyl)bilane (HMB), the assignments for which are also presented.

The biosynthesis of uroporphyrinogen III (uro'gen III,[1] **11**; Scheme I) from porphobilinogen (PBG, **2**) has been the subject of extensive investigation for the past 35 years. Pioneering work by Bogorad and Granick (1953) and Booij and Rimington (1957) has shown that two enzymes are required to catalyze the condensation of 4 mol of PBG to give uro'gen III. These two enzymes are PBG deaminase (EC 4.3.1.8, deaminase) and uro'gen III cosynthase (EC 4.2.1.75, cosynthase). In the absence of cosynthase, deaminase catalyzes the conversion of PBG into uro'gen I (**7**) and the latter is not converted into uro'gen III by the action of cosynthase. Since the natural

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[1] Abbreviations: HMB, (hydroxymethyl)bilane; PBG, porphobilinogen; uro'gen I, uroporphyrinogen I; uro'gen III, uroporphyrinogen III; NMR, nuclear magnetic resonance; ALA, 5-aminolevulinic acid; FID, free induction decay.

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