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Molecular species analysis of glycosphingolipids from small intestine of Japanese quail, *Coturnix coturnix Japonica* by HPLC/FAB/MS

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Neutral glycosphingolipids were isolated from quail small intestine and their structures were analysed. They contained:
Galβ1-4GlcCer(LacCer), Galα1-4GalCer(Ga_2Cer), Galα1-4Galβ1-4GlcCer(Gb_3Cer), GlcNAcβ1-3Galβ1-4GlcCer-(Lc_3Cer), GalNAcβ1-4Galβ1-4GlcCer(Gg_3Cer), GalNAcβ1-4[GalNAcβ1-3]Galβ1-4GlcCer($LcGg_4$Cer), and GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4GlcCer (Forssman glycolipid) as well as glucosylceramide, galactosylceramide (Nishimura K *et al.* 1984) *Biochim Biophys Acta* **796**:269–76) and the Le^X glycolipid, III^3 Fucα-nLc_4Cer (Nishimura K *et al.* (1989) *J. Biochem (Tokyo)* **101**:1315–18). The molecular species compositions of these glycosphingolipids were examined using fast atom bombardment-mass spectrometry linked with reversed-phase high-performance liquid chromatography. By such analysis, we could classify the quail glycosphingolipids into at least three classes: glycolipids rich in species having four hydroxyl groups in the ceramides (GalCer, Gg_3Cer, $LcGg_4$Cer and Le^X), those rich in the ceramides of *N*-acyl trihydroxysphinganine with normal fatty acids (Lc_3Cer), and glycolipids rich in the ceramides of *N*-acyl sphingenine with normal fatty acids (LacCer, Gb_3Cer and Forssman glycolipid). Immunohistochemical observation implies that the differences in the hydrophobic moieties specified the localization of glycosphingolipids in the tissue.

Keywords: quail; small intestine; glycosphingolipids; HPLC/FAB/MS; immunohistochemistry

Introduction

Diversities in glycosphingolipid composition in various tissues have been extensively studied. Most of these studies focused on their carbohydrate moieties, with regard to such biological phenomena as cell differentiation, cell to cell recognition, specific receptors for toxins and other factors, as well as their tumour specific antigenicities [1–4]. However, the analysis of their molecular species with respect to their hydrophobic moieties has been limited, although the significance of ceramide moieties of sphingolipids has recently received attention with regard to their possible role in the signal transduction mechanism [5].

Here, we studied glycosphingolipids of the small intestine of the Japanese quail, *Coturnix coturnix Japonica*, which is a useful animal in terms of developmental biology [6, 7]. The small intestine contains a large amount of glycosphingolipids, the ceramide moieties of which are rich in 4-D-hydroxysphinganine and hydroxy fatty acids [8, 9]. In previous reports, we have described major neutral glycolipids of the quail small intestine: GL1, a mixture of glucosylceramide and galactosylceramide [6] and GL6, III^3Fucα-nLc_4Cer [7]. In this paper, we describe the purification and characterization of other neutral glycolipids by HPLC/FAB/MS analysis, and discuss their molecular species and tissue-distribution as revealed by immunohistochemistry.

Material and methods

Animals

For preparation of glycolipids, frozen intestinal tissues of adult quails, *Coturnix coturnix Japonica*, were obtained from a local breeder. Immediately after thawing, jejunum and ileum between the bile duct and the ileo-caeco-colic junction were excised. The inner wall of the gut was washed with a gentle stream of cold saline, and stored at −20 °C until the preparation of acetone powder.

For the immunohistochemical study, adult quails of a domestic strain maintained for 46 years in one of our laboratories were used.

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Isolation and purification of glycolipids

The tissue was homogenized in 10 volumes of acetone. Lipids were extracted from the acetone powder of the tissue with chloroform:methanol (2:1 by vol.) and chloroform:methanol (1:2 by vol.), successively. The combined extracts were evaporated to dryness, redissolved with chloroform:methanol:water (30:60:8 by vol.) and applied to a column of DEAE-Sephadex A-25 (Pharmacia) equilibrated in the same solvent. The break-through fraction was evaporated to dryness and was acetylated in order to separate the neutral glycolipids from phospholipids [10]. The total neutral glycolipids thus obtained by deacetylation were then subjected to column chromatography [11, 12]. Approximately 500 mg of the neutral glycolipid fraction obtained from 1500 g of adult quail intestine was dissolved in 10 ml of chloroform:methanol (2:1 by vol.) and applied to a column (22 × 950 mm) of Iatrobeads 6RS-8060 (Iatron Laboratory, Tokyo) pre-equilibrated with 2-propanol:*n*-hexane:water (55:45:5 by vol.). The volume was eluted with a linear gradient of 2-propanol:*n*-hexane:water from 55:45:5 to 55:30:15 (by vol.) over a period of 400 min, followed by additional elution with a constant solvent composition of 55:30:15 (by vol.) for 100 min, at a flow rate of 5 ml min^{-1}. Fractions were collected and a small aliquot of each fraction was analysed on TLC. The fractions were roughly grouped and subjected to further purification by HPLC using Iatrobeads 6RSP-8010 (10 × 200 mm). Linear gradient elution was performed using 2-propanol:*n*-hexane:water mixtures at a flow rate of 2.0 ml min^{-1} with the following systems; A, (55:45:0 by vol.) to 55:40:5 (by vol.) for 60 min; B, 55:42:3 to 55:40:5 for 60 min; C, 55:40:5 to 55:42:8 for 120 min.

Thin-layer chromatography (TLC)

TLC was performed on Silica Gel 60 HPTLC plates (Merck). Glycolipids were visualized with a 1-naphthol/H_2SO_4 reagent. Preparative-TLC [12] was performed after acetylation when native glycolipids could not be separated by HPLC. A separated glycolipid band detected by iodine vapour was scraped and extracted with chloroform:methanol (2:1 by vol.). After deacetylation with 0.5% of sodium methoxide in chloroform:methanol (1:1, by vol.) for 30 min, the reaction mixture was desalted through Sep Pak C_{18} [13], and the glycolipid was purified by HPLC.

HPLC/FAB mass spectrometry

FAB mass spectra were obtained using a JMS-AX505H mass spectrometer/DA5500 data system (JEOL), HPLC was performed on a reversed-phase column of SFPAK-ODS (1.7 mm i.d. × 150 mm, JEOL) with methanol containing 0.7% glycerol as the elution solvent at a flow rate of 0.1 ml min^{-1}. The eluate was introduced through a splitter to a frit-FAB LC/MS interface. The split ratio was about 20:1. The primary beam for bombardment was 5 kV Xe^0.

Analysis of carbohydrates and fatty acids

Dried glycolipids were methanolysed with 0.5 ml of 3% HCl in anhydrous methanol at 80 °C for 16 h. The reaction mixture was washed three times with *n*-hexane to extract the methyl esters of fatty acids, and the lower phase was *N*-acetylated by the addition of 0.1 ml of pyridine and 0.1 ml of acetic anhydride. After 30 min, the mixture was dried and trimethylsilylated with pyridine:hexamethyldisilazane:trimethylchlorosilane (5:2:1 by vol.) and analysed by gas-liquid chromatography (GC). The trimethylsilylated methyl glycosides were applied to a glass column (3 mm i.d. × 4 m) packed with 3% SE-30. The temperature programme was 140–230 °C at 3 °C min^{-1}. The fatty acid methyl esters were analysed using the same column at 215 °C.

Methylation analysis

Permethylation of glycolipids was carried out by the method of Ciucanu and Kerek [14]. Identification of partially methylated alditol acetates was carried out by gas chromatography-mass spectrometry (GC/MS) as described by Levery and Hakomori [15]. GC/MS was performed using a Hewlett-Packard 5890A gas chromatograph interfaced to a JMS-AX505H (JEOL) mass spectrometer. Separation was on a DB-5 (0.32 mm i.d. × 30 m, J. & W. Scientific) bonded phase fused silica capillary column using splitless injection. The temperature programme was 140–250 °C at 4 °C min^{-1}. Electron impact mass spectra were acquired from 40 to 800 mass units at 0.5 s/scan.

Exoglycosidase cleavage of glycolipids

Glycolipid (about 50 μg) was dispersed in 200 μl of sodium citrate buffer containing 0.1% sodium taurodeoxycholate. Enzyme solution was added and incubated at 37 °C for 16 h. The incubation was stopped by the addition of 4 vol. of chloroform:methanol (2:1 by vol.) After removal of the upper layer, the lower layer was washed twice with methanol:water (1:1, by vol.). An aliquot of the product was taken for TLC analysis, and the rest was treated with the next enzyme in an analogous way. Enzymes and pH values of the buffer used were as follows: 0.125 units of α-galactosidase from green coffee bean (Sigma), pH 4.0; 1 unit of β-galactosidase from jack bean (Sigma), pH 4.0; 0.1 unit of α-*N*-acetylgalactosaminidase from squid liver (Wako), pH 4.0; 0.25 units of β-*N*-acetylhexosaminidase from jack bean (Sigma), pH 5.0.

Antibodies

Monoclonal antibodies J-1 (anti-Lc_3) [16], 1A4 (anti-Gb_3) [17] and FH-2 (anti-Le^X) [18] were gifts from Dr R. Kannagi (Aichi Cancer Center); rabbit anti-Forssman antiserum was a gift from Dr M. Hirabayashi (The Institute of Physical and Chemical Research); rabbit anti-human asialo GM2 (anti-Gg_3) antiserum was purchased from Dia-Iatron (Tokyo).

Immunostaining of glycolipids on TLC

TLC immunostaining was carried out as described by Magnani *et al.* [19] with slight modification. Following separation of the glycolipids on HPTLC silica gel 60 aluminium sheet (Merck), the sheet was dried and soaked for 90 s in 0.1% polyisobutylmethacrylate in *n*-hexane. The sheet was then covered with 1% bovine serum albumin (BSA) in 0.05 M Tris-HCl buffered saline pH 7.4 (TBS) for 30 min for blocking nonspecific adsorption. After removal of the blocking solution, the sheet was incubated with primary antibody appropriately diluted with BSA-TBS for 1 h. The sheet was then washed with TBS for 15 min changing the solution several times, and incubated with peroxidase conjugated secondary antibody diluted 1:500 in BSA-TBS for 1 h. After washing with TBS, the immunoreactive glycolipids were visualized by incubation with 0.3% 4-chloro-1-naphthol in methanol:TBS (1:5) containing 0.03% H_2O_2 for 5–10 min. All incubations were at room temperature.

Immunohistochemistry

The small intestine was fixed for 1 h in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. The tissue was cryoprotected by immersing for 1 h each in 10% and 20% sucrose, overnight in 30% sucrose in PBS at 4 °C, and then frozen with OCT compound in *n*-hexane at −85 °C. Cryosections of 7 μm were cut and collected on to poly-L-lysine coated slides and stored at −85 °C until use. Sections were successively treated with 3% H_2O_2 in water for 10 min and 1% BSA-TBS for 30 min to block non-specific reaction. They were then incubated with primary antibody, and finally with peroxidase-conjugated secondary antibody. All incubations were at room temperature and sections were washed with PBS between incubations. 3-Amino-9-ethylcarbazole was used as the substrate.

Results

Purification of neutral glycolipids from quail small intestine

Glycolipids were isolated by HPLC with various gradient systems of 2-propanol:*n*-hexane:water as described in the Methods section. Both GL1 and GL2 were purified with gradient system A, with which GL1 was eluted from 13 to 30 min, and GL2 from 30 to 50 min. GL3 was purified with gradient system B and separated into two subfractions designated as GL3a (retention time, 20–28 min) and GL3b (retention time, 30–38 min). Gradient system C applied for the purification of GL4–6 did not result in good separation. The mixtures of GL4 and 5 and those of GL5 and 6 were acetylated and subjected to preparative TLC using a solvent system of dichloroethane:acetone:water (60:40:0.1 by vol.). The acetylated glycolipids separated on the TLC were extracted from the gel, deacetylated, and finally purified by HPLC with gradient system C. GL5 was thus finally separated into two subfractions and designated GL5a and GL5b. Figure 1 shows chromatogram of isolated GL1 to GL6 on HPTLC plates with two solvent systems. It was noted that acetylated GL5a moved faster than acetylated GL5b on TLC. On the other hand, the latter moved faster than the former after deacetylation on TLC using a solvent system of chloroform:methanol:2.5 M ammonia (60:35:8 by vol.).

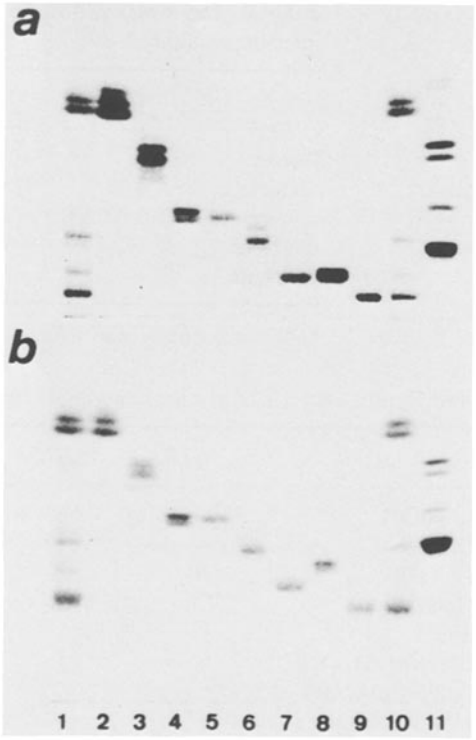

Figure 1. Thin-layer chromatograms of neutral glycosphingolipids isolated from quail small intestine. Lane 1 and 10, total neutral glycosphingolipids of quail small intestine; lane 2, GL1; lane 3, GL2; lane 4, GL3a; lane 5, GL3b; lane 6, GL4; lane 7, GL5a; lane 8, GL5b; lane 9, GL6; lane 11, total neutral glycosphingolipids of human red blood cells. Running solvents were *a*, chloroform:methanol:water (65:25:4 by vol.) and *b*, chloroform:methanol:2.5 M ammonia (60:35:8 by vol.). Glycolipids were visualized with Molish reagent.

Analysis of carbohydrate structure

The results of GC analysis of glycolipids as their trimethylsilylated methyl glycosides after methanolysis and the partially methylated alditol acetates are summarized in Tables 1 and 2, respectively. Figure 2 shows the results of enzyme treatment of GL3a and b, GL4 and GL5a and b, indicating anomer sequences in their sugar chains.

GL2 was a mixture of major lactosylceramide with a minor component of galabiosylceramide. The fraction was treated with exoglycosidases, β-galactosidase and α-galactosidase (Sigma, contaminated with β-galactosidase), and the monoglycosylceramides thus obtained were isolated by HPLC and analysed for their carbohydrate component

Table 1. The molar ratio of carbohydrates of glycosphingolipids isolated from small intestine of quail.

	GL2	*GL3a*	*GL3b*	*GL4*	*GL5a*	*GL5b*
Fucose	–	–	–	–	–	–
Galactose	1.39	1.62	1.31	1.26	1.28	2.19
Glucose	1.00[a]	1.00	1.00	1.00	1.00	1.00
GalNAc	–	–	–	0.96	1.07	1.86
GlcNAc	–	–	1.02	–	1.10	–

[a] Glucose is expressed as 1.00.

Table 2. Methylation analysis of glycosphingolipids from small intestine of quail.

	GL2	*GL3a*	*GL3b*	*GL4*	*GL5a*	*5a*[a]	*GL5b*	*5b*[b]
2,3,4,6-Me_4GalOH	+	+						+
2,3,6-Me_3GalOH	+	+		+		+	+	+
2,3,6-Me_3GlcOH	+	+	+	+	+	+	+	+
2,4,6-Me_3GalOH			+				+	
2,6-Me_2GalOH					+			
3,4,6-Me_3GlcN(Me)AcOH			+		+			
3,4,6-Me_3GalN(Me)AcOH				+	+	+	+	
4,6-Me_2GalN(Me)AcOH							+	

[a] Triglycosylceramide liberated from GL5a by β-*N*-acetylhexosaminidase.
[b] Triglycosylceramide liberated from GL5b by α-*N*-acetylgalactosaminidase and β-*N*-acetylhexasaminidase.

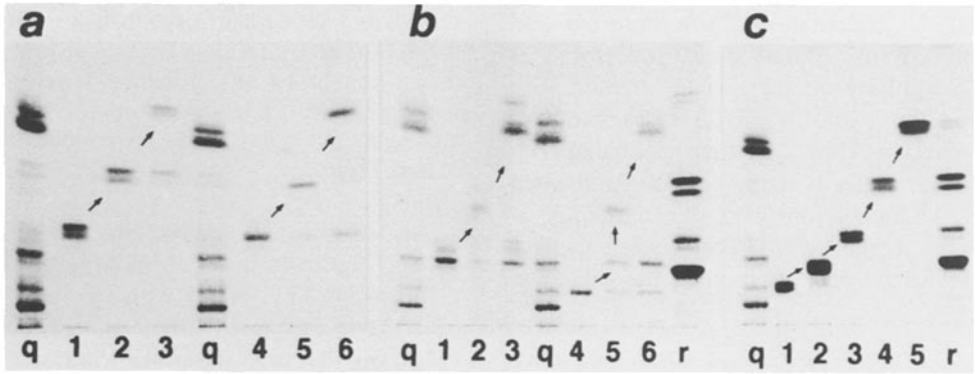

Figure 2. Sequential exoglycosidase treatment of GL3a and GL3b(a), GL4 and GL5a(b), and GL5b(c). *a*, GL3a (lane 1) was sequentially treated with α-galactosidase (lane 2) and then with β-galactosidase (lane 3); GL3b (lane 4) was treated with β-*N*-acetylhexosaminidase (lane 5) and β-galactosidase (lane 6). *b*, GL4 (lane 1) was treated with β-*N*-acetylhexosaminidase (lane 2) and β-galactosidase (lane 3); GL5a (lane 4) was treated with β-*N*-acetylhexosaminidase (lane 5) and β-galactosidase (lane 6). *c*, GL5b (lane 1) was treated with α-*N*-acetylgalactosaminidase (lane 2), β-*N*-acetylhexosaminidase (lane 3), α-galactosidase (lane 4) and β-galactosidase (lane 5), *q*, total neutral glycosphingolipids of quail intestine; *r*, total neutral glycosphingolipids of human red blood cells. Running solvent: chloroform:methanol:water (65:25:4 by vol.). Detection: Molish reagent.

by GC. Glucose was detected from the monoglycosylceramide after β-galactosidase treatment, indicating the presence of lactosylceramide in GL2. Against this, galactose and glucose were detected at a ratio of about 3:2 (Gal:Glc) from the monoglycosylceramide after α-galactosidase treatment, indicating the presence of galabiosylceramide together with lactosylceramide in the GL2 fraction.

The presence of 2,6-di-*O*-Me-Gal(-3[-4]Gal1-) in GL5a suggested the presence of a branched sugar chain. The fraction was hydrolysed by jack bean β-*N*-acetylhexosaminidase at pH 5.0 to yield two glycolipids, one having the same TLC mobility as GL4 and the other as a degradation product of GL4 cleaved by the same enzyme. Since the optimal pH of jack bean β-*N*-acetylhexosaminidase for β-GalNAc residues and β-GlcNAc residues is pH 3.5–4.0 and pH 5.0–6.0, respectively [20], GL5a was hydrolysed with this enzyme by the method of Kannagi *et al.* [21], at pH 6.0 for 6 h to obtain a ceramide trihexoside releasing

Table 3. Fatty acids in the glycosphingolipids from small intestine of quail.

Fatty acids	*GL2*	*GL3a*	*GL3b*	*GL4*	*GL5a*	*GL5b*
Normal acids						%
16:0	21.9	13.7	23.2	11.9	1.8	15.4
18:0	8.0	10.5	3.5	5.7	+[a]	16.7
18:1	+	+	+	–	–	1.0
20:0	5.2	6.1	5.3	+	+	7.2
21:0	+	+	1.9	1.6	+	+
22:0	16.5	20.1	28.5	6.3	+	20.4
23:0	5.3	14.8	9.6	1.8	–	13.0
24:0	13.8	20.0	14.9	2.3	–	16.7
24:1	13.2	10.4	9.1	–	–	5.3
25:0	1.5	+	+	–	–	+
Hydroxy acids						
16:0	2.1	–	–	19.3	26.3	–
18:0	+	–	–	6.0	1.1	–
20:0	+	–	–	4.4	7.2	–
21:0	–	–	–	1.9	2.3	–
22:0	4.2	–	–	26.2	34.7	–
23:0	1.8	–	–	5.9	9.9	–
24:0	2.4	–	–	3.2	4.3	–
24:1	–	–	–	1.9	9.1	–

[a] +: below 1%.

an *N*-acetylglucosamine residue. The hydrolysis product having the same TLC mobility as GL4 showed the presence of 2,3,6-tri-*O*-Me-Gal(-4Gal1-), 2,3,6-tri-*O*-Me-Glc(-4Glc1-), and 3,4,6-tri-*O*-Me-GalNAcMe (terminal *N*-acetylgalactosamine) by methylation analysis.

Using these data, the structures of GL2 to GL5b were identified as follows: GL2, Galβ1-4Glc1-Cer(LacCer) and Galα1-4Gal1-Cer (Ga_2Cer); GL3a, Galα1-4Galβ1-4Glc1-Cer(Gb_3Cer); GL3b, GlcNAcβ1-3Galβ1-4Glc1-Cer(Lc_3Cer); GL4, GalNAcβ1-4Galβ1-4Glc1-Cer(Gg_3Cer); GL5a, GalNAcβ1-3[GlcNAcβ1-4]Galβ1-4Glc1-Cer($LcGg_4Cer$); GL5b, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc1-Cer (Forssman). The identification of GL6 as III^{3}Fucα-nLc_4Cer, Le^x, has been published previously [7].

Figure 3 shows the results of TLC-immunostaining of GL5a and GL5b. GL5a and GL5b were recognized by anti-$LcGg_4$ mAb YI328-18 [22] and rabbit anti-Forssman antiserum raised against Forssman glycolipid, respectively. Isolated GL6 was recognized by anti-X hapten mAb FH2 [7]. However, at least three more bands with lower R_F values than GL6 reacted to mAb FH-2 (data not shown), suggesting the existence of several extended Le^x structures [18].

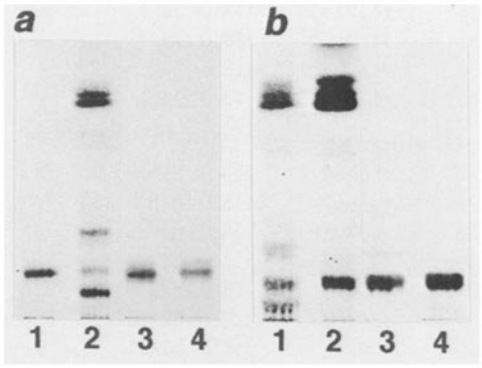

Figure 3. Immunostaining of GL5a (*a*) and GL5b (*b*), *a*, lane 1 and 3, isolated GL5a; lane 2 and 4, total neutral glycosphingolipids of quail intestine. *b*, lane 1 and 3, total neutral glycosphingolipids of quail intestine; lane 2 and 4, total neutral glycosphingolipids of chicken intestine. Running solvent: chloroform:methanol:water (65:25:4 by vol.). Detection: lane 1 and 2, staining with Molish reagent; lane 3 and 4, immunostaining with mAb YI328-18 (*a*) and with anti-Forssman antiserum (*b*).

Analysis of the fatty acids of the glycolipids

Table 3 shows the fatty acid composition of each glycolipid. About 90% of GL2 consisted of non-hydroxy fatty acids (C16–C24). GL3a, GL3b and GL5b contained exclusively non-hydroxy fatty acids. On the other hand, the majority of fatty acids in GL4 (about 70%) and GL5a (about 95%) were α-hydroxylated.

Molecular species of the glycolipids

Fast atom bombardment mass spectroscopy and other analytical methods of mass spectroscopy have recently been employed for the confirmation of glycolipid structure, because they give ample information on the sequences of

Table 4. Pseudo-molecular ions detected in the quail glycolipids.

				GL1	*GL2*	*GL3a*	*GL3b*	*GL4*	*GL5a*	*GL5b*	*GL6*
a	*b*	*c*	*d*								
Dihydroxyl group											
1	536	d34:1	d18:1–16:0	*698*	860	1022	1063	*1063*		*1428*	*1371*
2	538	d34:0	d18:0–16:0							*1430*	
3	564	d36:1	d18:1–18:0		888	1050	*1091*			1456	
4	592	d38:1	d18:1–20:0		916	1078	*1119*			1484	
5	620	d40:1	d18:1–22:0	*782*	944	1106	*1147*			1512	
6	634	d41:1	d18:1–23:0		958	1120				1526	
7	646	d42:2	d18:1–24:1		970	1132	*1173*			*1538*	
8	648	d42:1	d18:1–24:0		972	1134	1175			1540	
Trihydroxyl group											
9	552	dh34:1	d18:1–h16:0	*714*	876			*1079*	1282		1387
10	554	dh34:0	d18:0–h16:0					*1081*	1284		*1389*
11	580	dh36:1	d18:1–h18:0	*742*							
12	608	dh38:1	d18:1–h20:0	*770*	*932*						
13	636	dh40:1	d18:1–h22:0	*798*	960						
14	650	dh41:1	d18:1–h23:0	*812*	*974*						
15	664	dh42:1	d18:1–h24:0	*826*	988						
16	554	t34:0	t18:0–16:0	*716*	878		1081	*1081*	*1284*		*1389*
17	582	t36:0	t18:0–18:0		*906*		*1109*				
18	610	t38:0	t18:0–20:0	*772*	934	1096	1137				
19	624	t39:0	t18:0–21:0				1151				
20	638	t40:0	t18:0–22:0	*800*	962	1124	1165			*1530*	
21	652	t41:0	t18:0–23:0		*976*	*1138*	1179			*1544*	
22	664	t42:1	t18:0–24:1		*988*	1150	1191				
23	666	t42:0	t18:0–24:0		990	1152	1193			1558	
Tetrahydroxyl group											
24	570	th34:0	t18:0–h16:0	*732*	894			1097	1300		1405
25	598	th36:0	t18:0–h18:0	*760*				*1125*			
26	626	th38:0	t18:0–h20:0	788				1153	1356		1461
27	640	th39:0	t18:0–h21:0	802				1167	1370		1475
28	654	th40:0	t18:0–h22:0	816	978			1181	1384		1489
29	668	th41:0	t18:0–h23:0	830	992			1195	1398		1503
30	680	th42:1	t18:0–h24:1					*1207*	*1410*		*1515*
31	682	th42:0	t18:0–h24:0	884	1006			1209	1412		1517

a Peak no. in Fig. 4.
b *m/z* of ceramide ions or fragment ions due to the elimination of sugar moiety from $[M-H]^-$
c Number of aliphatic carbon: number of unsaturated bond. d, dihydroxysphingosine; t, trihydroxysphingosine; h, hydroxy fatty acid.
d Example of combination of LCB-fatty acid.
e *m/z* of pseudo-molecular ions detected. *Italic number*: detected at an appropriate retention time but its mass spectrum was not sufficiently clear.

carbohydrate moieties with fragment ions, as well as on molecular weights determined with pseudo-molecular ions. In the present study, GL1 to GL6 were analysed by HPLC/FAB/MS. The results are summarized in Table 4 and Fig. 4, and the procedures employed for the data analysis for some typical examples are shown in Figs 5 and 6.

In these studies, about 50 μg of glycolipids were applied to a semi-micro column, eluent was then introduced on line through a separator into the frit of the mass spectrometer for bombardment with a xenon beam. The reversed phase HPLC is able to separate the glycolipid sample into its molecular species, according to the number of carbon atoms as well as the number of hydroxyl groups and double bonds in their hydrophobic chains. The separation was monitored not only by the absorbance at 210 nm but also by the selected ion monitoring with *m/z* values corresponding to their pseudo-molecular ions. The mass spectrum of each peak gave further information for their identification.

It is noted that spectra obtained by this method revealed the structure of the ceramide moiety as well as that of carbohydrate moiety by the fragment ions which suggest

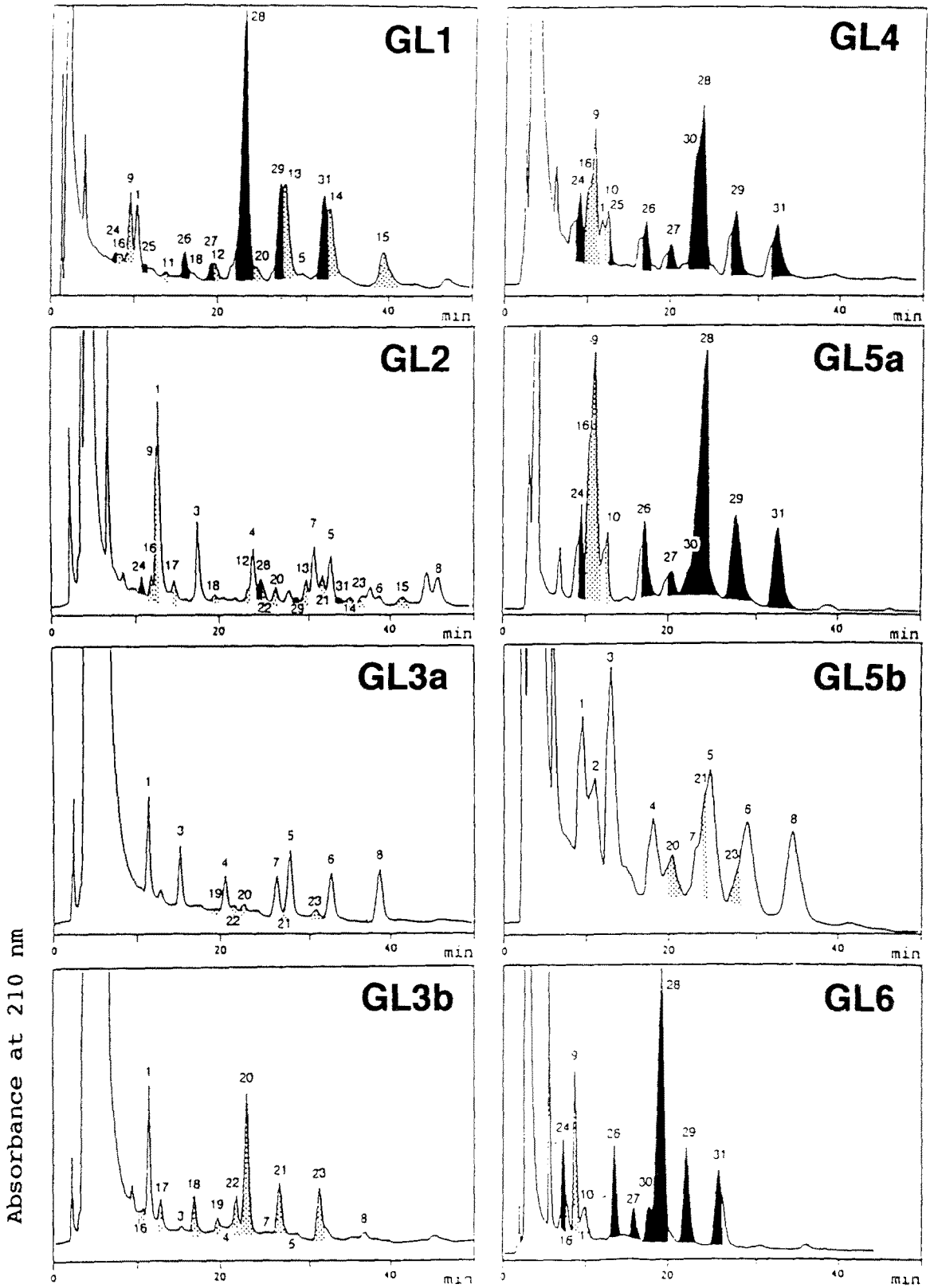

Figure 4. Reversed-phase HPLC of glycosphingolipids of quail small intestine. Detection: absorbance at 210 nm. Numbered peaks are identified in Table 4. Peaks are classified according to the number of hydroxyl groups in the ceramide moiety: unshaded peaks, molecular species with two hydroxyl groups; stippled peaks, three hydroxyl groups; black peaks, four hydroxyl groups.

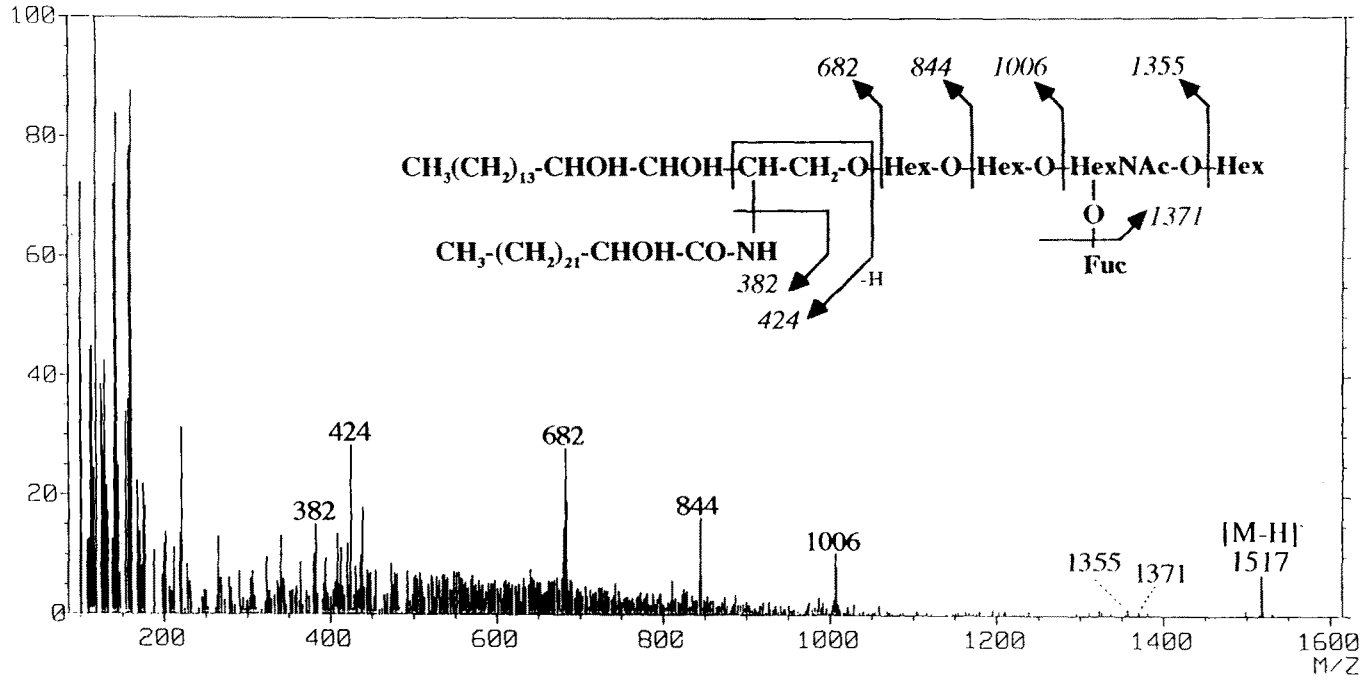

Figure 5. Negative ion FAB/MS spectrum of a molecular species of GL6 (Mol. Wt. 1518, peak no. 31).

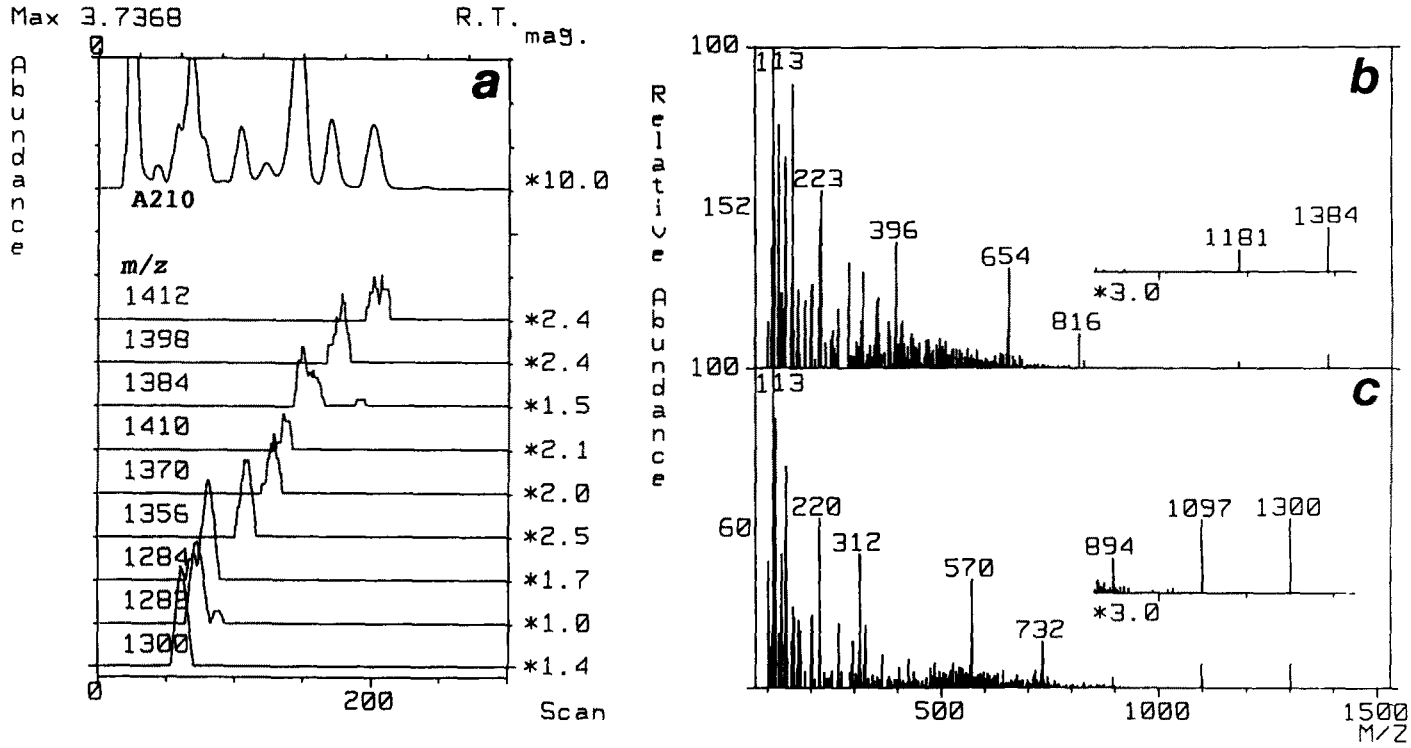

Figure 6. FAB/MS analysis of GL5a. *a*, mass-chromatograms of pseudo-molecular ions. *b* and *c*, negative ion FAB/MS spectra for the peaks of *m/z* 1384 and 1300, respectively.

the carbon number of the fatty acid, and, consequently, the long chain base of their parent molecule. In the case of a molecular species of GL6 shown in Fig. 5, the ceramide ion can be observed at *m/z* 682, suggesting the existence of 42 carbon and 4 hydroxyl groups in the ceramide moiety. Fragment ions at *m/z* 382 and 424 can be attributed to w-ion and y-ion as designated by Hemling *et al.* [23] (the latter was designated at T-ion according to Domon and Costello [24]), respectively, suggesting the existence of h24:0 fatty acid in the parent molecule. Thus the ceramide moiety of this molecular species was concluded to be a combination of t18:0–h24:0.

Figure 6a shows mass-chromatograms of GL5a. The peaks selected as the pseudo-molecular ions ($[M-H]^-$) of the molecular species of glycolipids having two HexNAc and two Hex residues at *m/z* 1300, 1282, 1284, 1356, 1370, 1410, 1384, 1398, and 1412 were observed in mass chromatograms at their appropriate retention times. The mass spectrum of each peak was obtained to confirm the structure. As typical examples, Figs 6b and 6c show mass spectra of two molecular species corresponding to the peaks detected by selected ions at *m/z* 1300 and 1384, respectively (Fig. 6a). Pseudo-molecular ions at *m/z* 1300 and 1384, and fragment ions at *m/z* 1097 and 1181, respectively, due to the elimination of an *N*-acetylhexosamine from the pseudo-molecular ions ($[M\text{-}1\text{-}203]^-$), *m/z* 732 and 816 due to the elimination of GalNAc-(GlcNAc-)Gal($[M\text{-}1\text{-}2x203\text{-}162]^-$), and *m/z* 570 and 654 due to the elimination of GalNAc-(GlcNAc-)Gal-Glc ($[M\text{-}1\text{-}2x203\text{-}2x162]^-$) were observed. The weak signal at *m/z* 894 in Fig. 6b is a fragment ion due to the elimination of two *N*-acetylhexosamines from the pseudo-molecular ion ($[M\text{-}1\text{-}2x203]^-$). The intense signals at *m/z* 312 (Fig. 6c) and 396 (Fig. 6b) can be attributed to y-ions (T-ions), suggesting that the fatty acids of their parent molecules are h16:0 and h22:0, respectively. These results indicate that the ceramide moieties of these molecular species consist of t18:0–h16:0 and t18:0–h22:0, respectively. In the same way, the mass spectrum of each peak was obtained and nine molecular species, t18:0–h16:0 (Mol wt, 1301), d18:1–h16:0 (1283), t18:0–16:0 (1285), d18:0–h16:0 (1285), t18:0–h20:0 (1357), t18:0–h21:0 (1371), t18:0–h22:0 (1385), t18:0–h23:0 (1399) and t18:0–h24:0 (1413) were identified. In the case of certain molecular species as shown by the ion at *m/z* 1410, the presence of t18:0–h24:1 species was suggested by its appropriate retention time, but the mass spectrum for this peak was not clear enough to confirm the structure. Two peaks could be observed by selected ion monitoring at *m/z* 1284 (Fig. 6a), corresponding to the molecular species of both d18:0–h16:0 and t18:0–16:0. The w- and y-ions were detected at *m/z* 312 and 270, respectively, in the spectrum for the main peak, indicating that their parent molecule was d18:0–h16:0. On the other hand, the minor peak could not be identified by these fragment ions. Although *m/z* 296 corresponded to the latter w-ion from 16:0 fatty acid, it could not be distinguished from the S-ion (designated by Domon and Costello [24]) of h16:0 derived from co-existing molecular species. The molecular species with t18:0–h24:1 and t18:0–25:0 are identical in their molecular weight, and indistinguishable from each other even by their w- and y-ions, but the existence of t18:0–h24:1 was suggested by the results of the analysis of fatty acid composition.

Other glycolipids were also subjected to HPLC/FAB/MS in the same way. The distribution of their molecular species was surveyed by selected ion monitoring, and their fragment patterns were analysed. Molecular species in each glygolipid detected are summarized in Table 4. The presence of certain species as shown in italics were detected by pseudo-molecular ions at appropriate retention times though their mass-spectra were not sufficient for their identification due to the small quantity or poor separation from other species.

Immunohistochemistry

Cryosections of quail intestine were immunostained with various anti-glycolipid antibodies in order to study the histological distribution of glycolipids (Fig. 7). Anti-Lc_3 mAb J1, anti-Gg_3 antiserum, anti-$LcGg_4$ mAb YI328-18 and anti-Le^x mAb FH-2 stained only epithelial cells. Forssman antigen was observed in the lamina propria mucosae and smooth muscle layer, but never in epithelial cells. Anti-Gb_3 mAb 1A4 stained almost all the area of the section.

In order to extract the glycolipids from the sections, they were treated with chloroform:methanol (2:1 by vol.) for 10 min and then immunostained. Immunoreactivities to YI328-18, anti-Forssman and anti-Gg_3 antisera were thoroughly removed by the treatment. Immunoreactivity to J-1 and FH-2 in epithelial cells was also lost. However, J-1 and FH-2 stained both goblet cells and the mucus on epithelial cells even after chloroform:methanol treatment. Immunoreactivity to 1A4 decreased but remained in all areas.

Discussion

Mass spectrometric analysis has become one of the routine analytical methods for the confirmation of glycolipid structure, because it affords valuable information on the sequence of carbohydrate moiety from the fragment ions, as well as on the molecular weight from the pseudo-molecular ion. In this study, HPLC/FAB/MS was used to analyse the chemical structure of glycolipids isolated from quail intestine. The structure of each molecular species of glycolipids having various ceramide moieties with an identical carbohydrate sequence was identified. The spectra obtained by HPLC/FAB/MS were obviously more discernible on fragment ions derived from the ceramide moieties, than those derived from direct application of mass spectrometry to a glycolipid, which was still heterogeneous in ceramide groups. It should also be emphasized that, by the method employed in the present study, information on the molecular species of glycolipids could be obtained without troublesome steps such as hydrolysis procedures for the component analysis.

Glycolipids of quail intestine can be sorted according to their ceramide moieties into at least three classes. Gg_3Cer, $LcGg_4Cer$ and Le^x glycolipid constitute a class rich in highly hydroxylated ceramide species which has four hydroxyl groups in its long-chain bases and/or fatty acids. Gb_3Cer and Forssman glycolipids make the second class with mostly di-hydroxylated long-chain bases in their ceramide moieties. They have similar fatty acid components

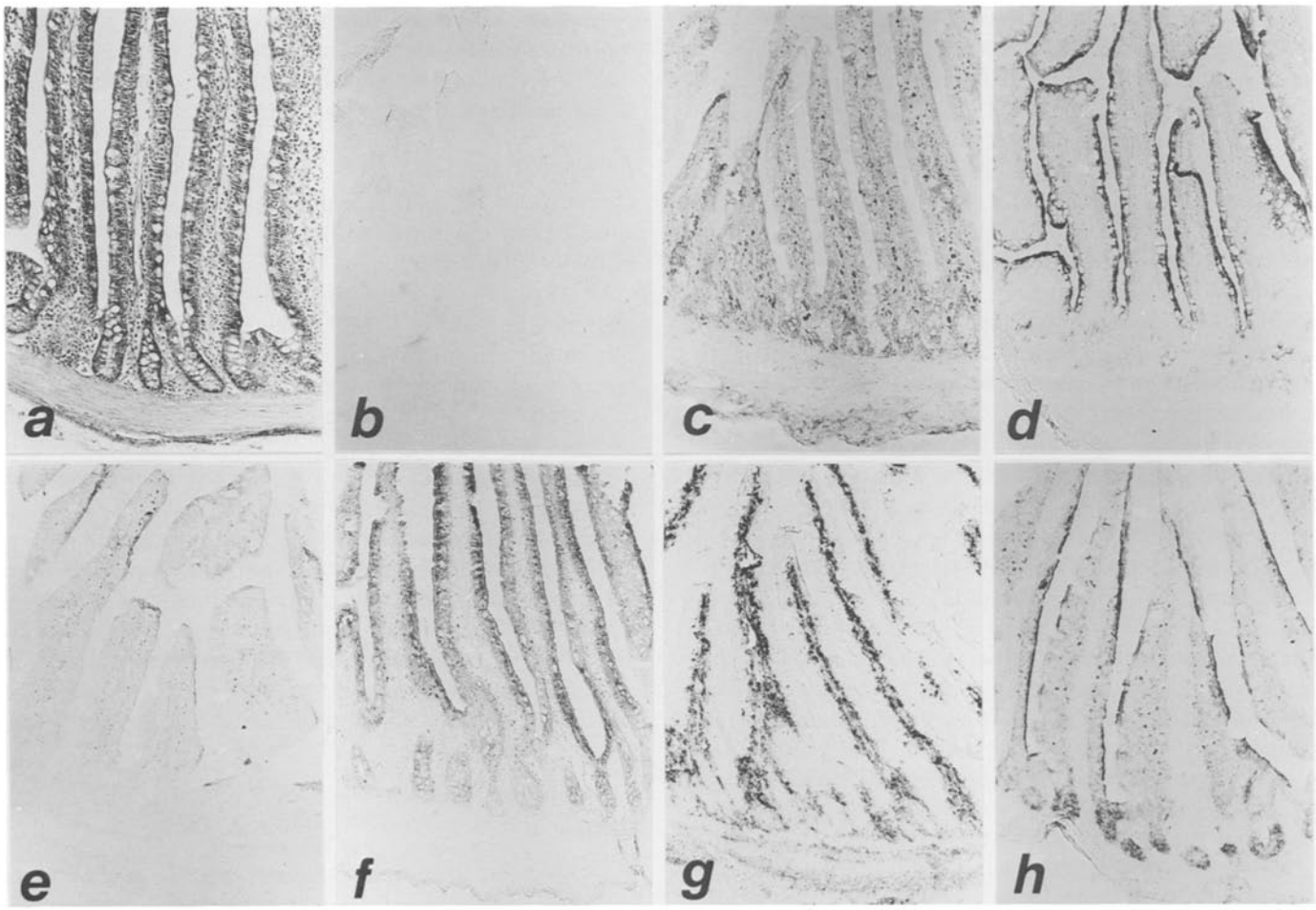

Figure 7. Cryosections of quail small intestine stained with haematoxylin-eosin (*a*) and those immunostained with various antibodies (*b–h*), *b*, negative control without primary antibody; *c*, anti-Gb_3Cer mAb 1A4; *d*, anti-Lc_3Cer mAb J1; *e*, anti-Gg_3Cer antiserum; *f*, anti-$LcGg_4Cer$ mAb YI328-18; *g*, anti-Forssman antiserum; *h*, anti-X hapten mAb FH2.

containing few hydroxy acids. It is also noted that Lc_3Cer has a similar fatty acid composition to Gb_3Cer and Forssman glycolipids. But it constitutes a different class because the ceramide moieties are rich in trihydroxyl long-chain bases. Mono- and dihexosylceramide were difficult to assign to such classes according to their ceramide moieties. Their complexity may possibly be explained by the fact that they are metabolic intermediates in the synthesis and/or catabolism of the three classes mentioned above. But other observations should also be taken into consideration. Monoglycosylceramide is a mixture of galactosyl- and glucosylceramide [6]. However the use of a semi-micro scale column in this experiment prevented sufficient separation of these, although this had been accomplished in previous experiments. It is thus suggested that the present analytical data on GL1 fraction predominant in the species with four hydroxyl groups in the ceramide moieties possibly reflects those of galactosylceramides, which comprise about 80% of this fraction. The apparent complexity of GL2 may also be attributed to the fact that this fraction is a mixture of lactosyl- and galabiosylceramide. However, the apparent type of molecular species may possibly be attributed to those of the major lactosylceramide since the latter quantity is too minor to make a measurable contribution to the analytical results.

The presence of $LcGg_4Cer$ is notable because this unique glycolipid of hybrid type was found in a cultured murine leukaemia cell line, suggesting that lacto-ganglio structures could be markers of maligant myeloid cells [21]. We previously described the isolation of $III^3Fuc\alpha\text{-}nLc_4Cer$ (GL6), which is also an embryonic antigen of human and mouse (SSEA-1), from quail intestine [7]. Although the domestic fowl belongs to the same family as the quail, such glycolipids have not been found in their tissues (our unpublished observations).

The complexities of molecular species of intestinal glycolipids and their different distribution in tissues have been previously discussed by several authors [25–28]. In those observations, long chain bases and fatty acids of epithelial cells were more hydroxylated than those of

non-epithelial tissue. Our immunohistochemical observations were mostly consistent with such results. Le^x, Gg_3Cer and $LcGg_4Cer$, glycolipids rich in the species having four hydroxyl groups in the ceramide moieties, were located only in epithelial cells. Lc_3Cer was also observed only in the epithelial region. On the other hand, Forssman glycolipid which is rich in normal fatty acids and sphingenine, was observed in non-epithelial cells including lamina propria mucosae and smooth muscle layer. The distribution of Gb_3Cer, a simple globo-series, could not be specified by immunoreactivity to mAb 1A4. The difficulty may be attributed to the presence of homologous glycoproteins, since the staining by the antibody was not changed after treatment with chloroform-methanol for the removal of glycolipids. However, by analogy with Forssman hapten. Gb_3Cer is also considered to be non-epithelial, for it has approximately identical ceramide moieties rich in dihydroxyl ceramides.

The complexities of the types of molecular species discriminating one group of glycolipids from another might be explained with regard to different metabolic pathways for glycolipid series; gala-, globo-, ganglio-, lacto-, neolacto-, and lactoganglio-types of glycolipids. However, glycolipids of different series such as ganglio- lactoganglio- and neolacto-series, all of which are distributed only in epithelial tissue, make one and the same class as mentioned above. It has been shown by the present study and by other authors [26, 27] that the differences in the hydrophobic moieties may possibly be related to their sorting route for microvillus membrane. It should be mentioned in this regard that tissues having microvillus structure such as urethral epithelial cell [29], kidney [30], and thymocyte [31] are also rich in glycolipids with highly hydroxylated ceramide moieties. Hydrogen bonding between ceramide moieties of membrane lipids has been suggested as a factor to stabilize the bilayer structure [32]. Thus hydroxyl groups abundant in the ceramide moieties of these membrane glycolipids may contribute to the stability of the proper structure of microvillus membrane. Significances in the heterogeneities in the ceramide moieties of intestinal glycosphingolipids with respect to various functional roles are to be elucidated by future experiments.

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