FORSSMAN GLYCOLIPID HAPTEN VARIANTS OF DOG GASTRIC MUCOSA

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1. Introduction

Forssman hapten is a genetically defined heterophilic antigen that specifically inhibits the reaction between sheep erythrocytes and anti-sheep erythrocyte antibody derived from different species. This antigen, first described by Forssman [1] has been shown to be a glycosphingolipid [2–4]. The structure of this glycolipid, earlier thought to be a tetraglycosylceramide, was later established as a pentaglycosylceramide [5] and shown to be common for the hapten isolated from various sources [6–8]. Variation with regard to the internal carbohydrate chain of Forssman glycolipid has been also reported [9].

In this study, three water-soluble glycolipids containing *N*-acetylgalactosamine and exhibiting Forssman activity were isolated from dog gastric mucosa. One of the isolated glycolipids was found to be a pentaglycosylceramide with identical sugar composition to that of Forssman hapten. The other two were heptaglycosylceramide and octaglycosylceramide.

2. Experimental

2.1. *Materials*

Dog stomachs used for mucosa preparation were obtained from Pel-Freez Biological (Rogers, AR). Heterophile Forssman antiserum was from Difco Laboratories (Detroit, MI) and IDF cells for microimmunodiffusion from Cordis Laboratories (Miami, FL). DEAE–Sephadex A-25 was supplied by Pharmacia (Piscataway, NJ) and silicic acid (100–200 mesh) by Bio-Rad Laboratories (Richmond, CA). Silica-gel HR plates, 250 nm coating thickness were purchased from Analtech (Newark, DE). Human red cells A, B, O types, human blood-grouping serum anti-A, anti-B and anti-H (*Ulex europeus* extract) were from Biological Corporation of America (Port Reading, NJ).

2.2. *Isolation of water-soluble glycolipids*

Extraction of lipids from dog gastric mucosa scrapings was performed by the tetrahydrofuran procedure [10,11]. The aqueous phase was concentrated to a small volume, dialyzed and lyophilized. The crude glycolipid fraction contained in the lyophilizate was dissolved in propan-1-ol/isopropanol/NH_4OH (35:35:30 v/v/v) and chromatographed on silicic acid column [12]. Fractions, 5 ml, were collected and every fifth was monitored for glycolipids by thin-layer chromatography. The eluates showing the same chromatographic pattern were pooled to give three different fractions (I, II and III). Each fraction was dialyzed, lyophilized, dissolved in methanol/chloroform/water (60:30:8 v/v/v) and separately applied to a column of DEAE–Sephadex [13]. The neutral glycolipids were eluted from the column with the above mixture and the gangliosides and other acidic lipids, with 0.2 M sodium acetate in methanol/chloroform/water (60:30:8 v/v/v). The neutral glycolipid fractions were then subjected to mild alkaline methanolysis [14], dialysis and lyophilization. The final purification of the glycolipids contained in the lyophilizates was accomplished by preparative thin-layer chromatography in chloroform/methanol/water (60:35:8 v/v/v), chloroform/methanol/NH_4OH (40:80:25 v/v/v) and chloroform/methanol/acetic acid/water (55:45:5:5 v/v/v/v).

2.3. *Analytical methods*

Thin-layer chromatography was performed on silica-gel HR plates activated at 130°C for 1 h. Neutral glycolipids were visualized with orcinol, gangliosides with resorcinol and sulfatides with rhodizonate [15]. Iodine was used for preparative purposes.

Methyl esters of fatty acids and methyl glycosides were obtained by methanolysis of the glycolipids in 1.2 M methanolic HCl at 85°C for 20 h. Hexosamines were re-*N*-acetylated with acetic anhydride [12]. The alditol acetate derivatives of monosaccharides were obtained from the glycolipids according to the procedure of Yang and Hakomori [16].

Gas–liquid chromatography was performed with a Beckman GC-65 instrument equipped with glass columns 6 ft × 1/8 in packed with 3% SE-30 on chromosorb, W, AW, DMCS (80–100 mesh), and packed with 1% ECNSS-M on Gas Chrom-Q. The alditol acetates were analyzed on ECNSS-M columns programmed at 2°C/min from 150–210°C. For the analysis of trimethylsilyl derivatives of methyl glycosides on SE-30 columns, the temperature was programmed at 1°C/min. from 140–210°C. The long-chain bases were quantitized as described previously [11]. Removal of fucose residue from fucose-containing glycolipid was accomplished by hydrolysis in 0.1 M trichloroacetic acid at 100°C for 2 h.

2.4. *Immunological assays*

Hemagglutination and hemagglutination-inhibition assays were performed with the Takatsy microtitrator using 0.025 ml loops and 2% suspension of human red cells. The anti-A and anti-B serums were diluted to 4 units/0.025 ml, the anti-H was commercially available at potency of 2 units/0.025 ml. Forssman hapten activity was tested by a double diffusion micromethod as described by Wadsworth [17]. The wells were filled with 10 μl undiluted antiserum and 10 μl 1 mg/ml solutions of the glycolipids in saline. All immunological assays were performed in the presence of ten-fold excess of a mixture of lecithin–cholesterol (1:1) as auxiliary lipids [18].

3. Results

Each fraction from silicic acid column contained one major and several minor neutral glycolipids. The major components were purified to homogeneity on thin-layer plates to give three glycolipids (I, II and III). Glycolipid I was eluted from silicic acid column mainly in fraction I. Fraction II contained glycolipid II and fraction III contained glycolipid III. The relative migration rates (R_F) of the isolated glycolipids are given in table 1. The yields of the purified glycolipids (fig.1) per 100 g wet gastric mucosa scrapings were: glycolipid I, 3.9 mg.; glycolipid II, 2.4 mg.; glycolipid III, 1.1 mg.

The composition and molar ratio of carbohydrates present in the isolated glycolipids is given in table 2. Glucose, galactose and *N*-acetylgalactosamine in a molar ratio of 1:2:2 were found in glycolipid I. The same carbohydrates, but in a molar ratio of 1:3:3, were present in glycolipid II. The carbohydrate portion of glycolipid III revealed the presence of glucose fucose, galactose and *N*-acetylgalactosamine in a molar

Table 1
Relative migration rates (R_F) of the purified glycolipids

Solvent system	R_F Values of		
	Glycolipid I	Glycolipid II	Glycolipid III
Chloroform/methanol/water (65:35:8 v/v/v)	0.25	0.22	0.18
Chloroform/methanol/NH_4OH (40:80:25 v/v/v)	0.47	0.42	0.15
Chloroform/methanol/acetic acid/water (55:45:5:5 v/v/v/v)	0.40	0.41	0.22

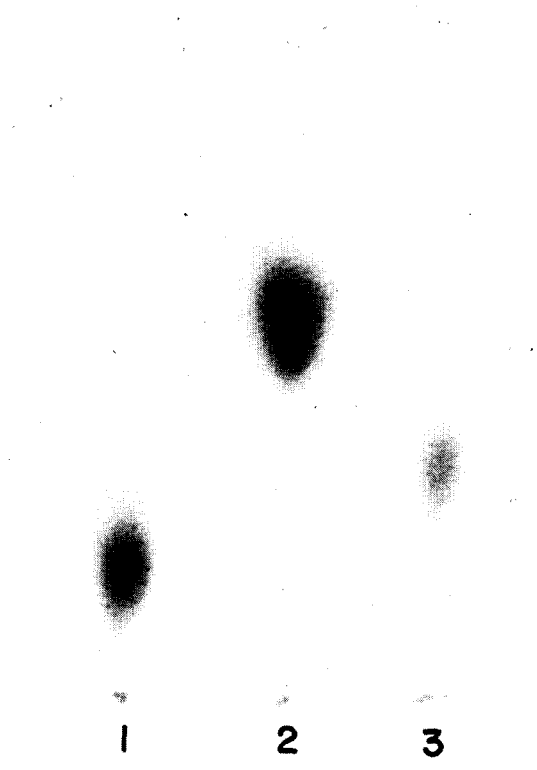

Fig.1. Thin-layer chromatography of the purified glycolipids with Forssman activity. (1) Glycolipid III; (2) glycolipid I; (3) glycolipid II. Conditions: silica-gel HR-250 nm developed in chloroform/methanol/water (60:35:8 v/v/v). Visualization: orcinol reagent.

ratio of 1:1:3:3. The above data indicate that the glycolipid I is a pentaglycosylceramide, glycolipid II, heptaglycosylceramide and glycolipid III, octaglycosylceramide. Partial acid hydrolysis of glycolipid III (defucosylation) resulted in a glycolipid which corresponded in its chromatographic behavior and sugar compositions to that of glycolipid II.

In the double diffusion micromethod, all three glycolipids gave a single precipitation line with anti-Forssman antiserum. In the presence of auxiliary lipids, all three glycolipids were equally reactive and, according to the precipitation pattern, showed identity. In hemagglutination-inhibition assays all three glycolipids completely inhibited agglutination of human group-A red cells by human anti-A serum at concentration of

Table 2
The composition and molar ratios of carbohydrates in the isolated glycolipids

Glycolipid	Molar ratios			
	Fuc	Gal	Glc	GalNAc
Glycolipid I	–	1.98	1.0	1.95
Glycolipid II	–	2.91	1.0	2.97
Glycolipid III	0.98	2.95	1.0	2.91

2.5–5.0 μg. Furthermore, glycolipid III also showed strong activity in H anti-H system. This activity was completely abolished by the removal of fucose (partial hydrolysis).

4. Discussion

Dog gastric and intestinal mucosa has been recognized for some time as an exceptionally rich source of pentaglycosylceramide [19,20]. This glycolipid was shown to have the antigenic activity and structure identical with that of horse spleen Forssman glycolipid hapten, i.e., GalNAcα(1→3)GalNAcβ(1→3)Galα(1→4)Galβ(1→4)Glc(1→1)ceramide [5–8]. Recent studies on glycolipids of hamster fibroblasts [9] indicate that this antigen exists in the cultured cells in two polymorphic variations with regard to its internal carbohydrate chain. Both variants, however, share the common terminal structure composed of three sugar residues, GalNAcα(1→3)GalNAcβ(1→3)Gal. Our data indicates that Forssman glycolipid hapten of dog gastric mucosa exists at least in three different structural forms, with the pentaglycosylceramide being predominant. It is apparent that glycolipids I, II and III must share the common terminal structure which determines the immunological specificity of Forssman antigen. Furthermore, all three glycolipids exhibited blood-group A activity which is in accordance with the results obtained by other investigators [21] and is thought to be due to the presence of terminal α-*N*-acetylgalactosaminyl residue in both the Forssman antigen and blood group-A determinant. The H-activity of glycolipid III could be explained only if the carbohydrate portion of this compound

consisted of a branched saccharide chain with the α-*N*-acetylgalactosaminyl(1→3)β-*N*-acetylgalactosaminyl and α-L-fucosyl-(1→2)β-galactosyl residues being terminal. That the H-activity resides in the latter disaccharide portion of the glycolipid III is supported further by the results of partial acid hydrolysis. Defucosylation of glycolipid III resulted in the loss of its H-activity, but had no effect on its reactivity with Forssman antiserum nor on its ability to inhibit hemagglutination in A anti-A system. Accordingly, the glycolipid II which differs from glycolipid III only in one sugar residue, i.e., fucose, probably consists of two non-reducing termini, α-*N*-acetylgalactosaminyl-(1→3)β-*N*-acetylgalactosamine and galactose. Our recent structural studies [12] support this assumption.

The presence of multiple forms of glycolipids carrying blood-group determinants in gastrointestinal tract of mammals is well established [22–24]. Also, existence of the glycolipids of a branched structure carrying more than one antigenic determinant has been reported [13,24]. It has been suggested earlier by Rapport and Graf [21] that the Forssman antigen may not be a single compound. The data presented in this report indicate that Forssman glycolipid hapten exists as a number of variants.

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