Occurrence of a new hematoside in the kidney of guinea pig

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We have isolated a new hematoside from guinea pig kidney. Like the usual hematoside (II^3NeuAc-LacCer), isolated from human erythrocytes, this new hematoside contained glucose, galactose, and *N*-acetylneuraminic acid in an equimolar proportion. By thin-layer chromatography (TLC), however, it migrated faster than the usual hematoside. After mild alkaline hydrolysis the TLC mobility of this ganglioside became identical to that of the usual hematoside. The sialic acid in this ganglioside was susceptible to Clostridial neuraminidase. Based on TLC mobility and the results of periodate oxidation, the sialic acid of the new hematoside was identified as 9-*O*-acetyl-*N*-acetylneuraminic acid. Therefore, the structure of this new hematoside is 9-*O*-Ac-NeuAcα2→3Galβ1→4GLcβ1→1'Cer.

Ganglioside *O-Acetylhematoside* *Sialic acid* *Guinea pig* *Glycosphingolipid* *Hematoside*

1. INTRODUCTION

The possible involvement of gangliosides as tissue antigens, receptors, and in cell transformation has been reported [1–3]. Gangliosides, however, especially those found in extraneural tissues, have not been well characterized. Hematoside is one of the major gangliosides found mainly in extraneural tissues. Until now, 3 kinds of hematosides containing different types of sialic acids have been isolated [4]. They are the hematosides containing *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid or 4-*O*-acetyl-*N*-glycolylneuraminic acid. Here, we describe the isolation and characterization of a new hematoside which is 9-*O*-Ac-NeuAcα2→3Galβ1→4GLcβ1→1'Cer.

2. MATERIALS AND METHODS

2.1. *Isolation of glycolipids*

One hundred guinea pig kidneys (purchased from Pel-Freeze Biologicals, Rogers, ARK) were chopped and homogenized in 5 vol. water using a Polytron grinder (Brinkman Instruments, Westbury NY) and subsequently lyophilized. The dried tissue was treated with 500 ml of acetone to obtain 42 g dried powder. This powder was then homogenized with 10 vol. C–M (2:1) and the mixture left overnight at room temperature. After filtering through a Buchner funnel, the residue was re-extracted twice with 500 ml of C–M (1:1). The 2 extracts were combined and dried under vacuum. The total lipid extract was applied to a DEAE–Sephadex A-25 column (acetate form, 2.0 × 28 cm) as in [5]. The neutral lipids were eluted with 1 l of C–M–W (30:60:8, by vol.) followed with 0.5 l methanol. The monosialosylgangliosides were then eluted with 0.5 l of methanol containing 0.01 M sodium acetate. The eluate was evaporated to dryness using a flash evaporator, dissolved in a small volume of C–M–W (30:60:8, by vol.) and dialyzed exhaustively against water for 24 h. The non-dialyzable materials were lyophilized to obtain 400 mg crude gangliosides. This material was dissolved in chloroform and applied to an Iatrobeads 6RS-8060 (Iatron, Tokyo) column (1 × 90 cm) which had been equilibrated with chloroform. The column

Abbreviations: C–M–W, chloroform–methanol–water; TLC, thin-layer chromatography; GLC, gas–liquid chromatography

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was first washed with 200 ml chloroform, and the gangliosides were eluted with a linear gradient using 450 ml C–M–W (83:16:0.1, by vol.) in the mixing chamber and 650 ml C–M–W (45:55:5, by vol.) in the reservoir [6]. Fractions of 8 ml each were collected. Fractions containing the new hematoside were pooled and further purified by repeating the Iatrobeads column chromatography. The yields of the new hematoside and the usual hematoside from 100 kidneys were 1.1 mg and 10.0 mg, respectively.

2.2. *Preparation of sialic acid from the new hematoside*

The sialic acid of the new hematoside (500 μg) was set free by Clostridial neuraminidase as in [7]. After incubation with the enzyme, the mixture was dialyzed against 2 ml water for 2 days in a cold room with 2 changes of water. The combined dialysates that contained free sialic acid were lyophilized, and further purified by ion-exchange column chromatography [8].

2.3. *Method of analysis*

The purity of the new hematoside was examined by high performance TLC using the following solvent systems (by vol.): solvent I, C–M–W (60:32:7); solvent II, C–M–2.5 N ammonia (60:32:7); and solvent III, C–M–W (65:25:4). Resorcinol reagent [9] was used to detect gangliosides. The sugar composition of the new hematoside was analyzed by GLC as trimethylsilyl ethers of *O*-methylglycosides [10]. GLC analyses were performed by using Hewlett-Packard Model 5830 A gas chromatography unit equipped with a 3% SE 30 column. Fatty acids were analyzed as their methyl esters by GLC using a 10% DEGS column at 190°C. The TLC of sialic acids was performed both on cellulose and silica gel plates (high-performance TLC plates). The cellulose plates were prewashed with 0.1 N HCl before use [8]. The solvent system of *n*-butanol–*n*-propranol–0.1 N HCl (1:2:1, by vol.) was used for cellulose plates, and *n*-propanol–water (7:3, v/v), *n*-propanol–2.5 N ammonia (7:3, v/v) and C–M–W (5:5:1, by vol.) for the high-performance TLC plates.

3. RESULTS AND DISCUSSION

Fig.1 shows the elution profile of the first Iatrobeads column chromatography. The new hematoside was eluted between phospholipids and the usual hematoxide *N*-acetylneuraminyllactosylceramide). The fractions containing the new hematoside were pooled and purified again with Iatrobeads column chromatography. This procedure effectively removed phospholipids from the new hematoside. Using solvents I and II, the new hematoside migrated as a single band on TLC with a mobility faster than the usual hematoside, but slightly slower than 4-*O*-acetyl-*N*-glycolyllactosylceramide obtained from horse erythrocytes [11] (fig.2). The TLC mobility of this ganglioside was found to be identical to that of *N*-acetylneuraminylgalactosylceramide (GM4). As in the case of 4-*O*-acetyl-*N*-glycolylneuraminyllactosylceramide, this hematoside was easily converted into *N*-acetylneuraminyllactosylceramide by exposure to ammonia vapor as shown in fig.2.

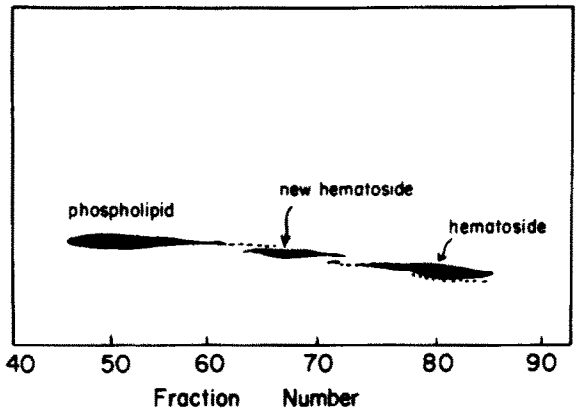

Fig.1. Thin-layer chromatography showing the elution profile of monosialosylgangliosides from Iatrobeads column. A 50 μl aliquot of each fraction was analyzed by TLC with solvent I.

The carbohydrate composition of the new ganglioside was found to contain an equimolar ratio of galactose, glucose and *N*-acetylneuraminic acid. This composition is identical to that of the usual hematoside. The new hematoside from guinea pig kidneys is different from 4-*O*-acetyl-*N*-glycolylneuraminyllactosylceramide isolated from horse erythrocytes in their susceptibility to Clostridial neuraminidase. The hematoside from horse erythrocytes was resistant to neuraminidase [11], while the new hematoside was easily hydrolyzed by the same enzyme to produce lactosylceramide. The hematoside has two possible alkaline-labile sialic

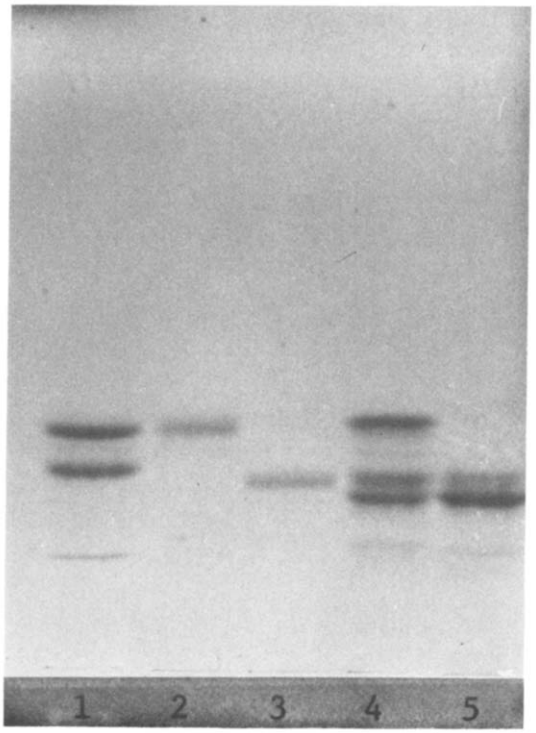

Fig.2. Thin-layer chromatography showing the mobility of the new hematoside isolated from guinea pig kidney: (1) standard gangliosides, from top, *N*-acetylneuraminylgalactosylceramide and *N*-acetylneuraminyllactosylceramide; (2) the new hematoside; (3) the new hematoside after exposure to ammonia vapor; (4) hematosides from horse erythrocytes, from top, 4-*O*-acetyl-*N*-glycolylneuraminyllactosylceramide and two bands of *N*-glycolylneuraminyllactosylceramide; (5) horse hematosides after exposure to ammonia vapor; solvent II was used.

acids: the sialic acid in lactone form and the sialic acid containing *O*-acyl groups. Since the lactone form of sialic acid is known to be resistant to neuraminidase [12], the alkaline-labile sialic acid in the new hematoside should be one of the *O*-acylated acids. The sialic acid released by neuraminidase was isolated and characterized by TLC as shown in fig.3. The sialic acid from the new hematoside moved at the same position as 9-*O*-acetyl-*N*-acetylneuraminic acid. The sialic acid released from the new hematoside was converted into *N*-acetylneuraminic acid by the treatment with ammonia. The same conclusion was also obtained from the experiment using a cellulose thin-layer plate with solvent I.

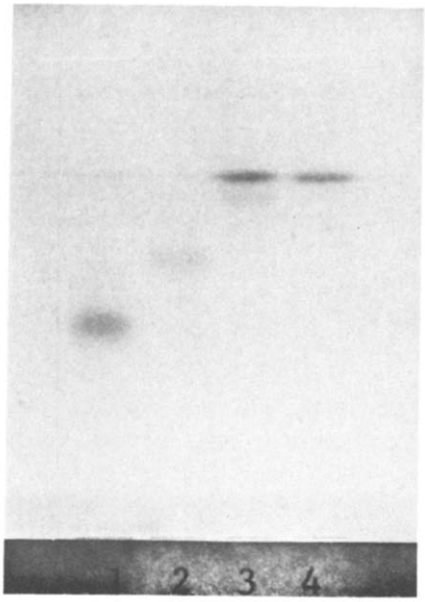

Fig.3. Thin-layer chromatogram of sialic acid released from the new hematoside by neuraminidase: (1) *N*-glycolylneuraminic acid; (2) *N*-acetylneuraminic acid; (3) 9-*O*-acetyl-*N*-acetylneuraminic acid; (4) the sialic acid from the new hematoside; solvent system, *n*-propanol–15 N ammonium hydroxide–water (7:0.5:2.5, by vol.).

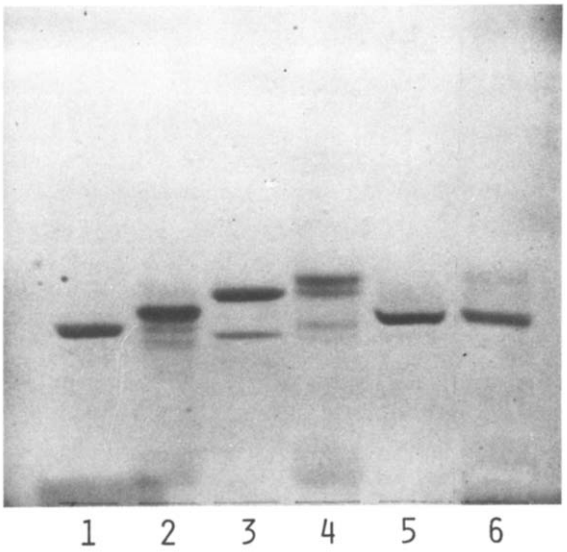

Fig.4. Thin-layer chromatogram of the new hematoside after mild periodate oxidation: (1) *N*-acetylneuraminyllactosylceramide; (2) 1 after periodate oxidation; (3) hematosides from horse erythrocytes; (4) 3 after periodate oxidation; (5) the new hematoside; (6) 5 after periodate oxidation; solvent I was used.

Table 1

Fatty acid composition of hematosides from guinea pig kidney[a]

Fatty acid	Hematoside	New hematoside
C16:0	2.1	7.1
C16:1	tr.	0.4
C18:0	15.8	28.3
C18:1	5.3	10.3
C20:0	4.1	1.7
C21:0	4.9	1.1
C22:0	25.8	17.5
C22:1	4.1	tr.
C22:2	8.3	6.6
C24:0	14.4	10.7
C24:1	8.7	10.5

[a] Each fatty acid is expressed as percentage of total fatty acids in each hematoside

Fig.4 shows the effect of periodate oxidation on the new hematoside according to the condition in [13]. Like 9-*O*-acetylneuraminic acid [13], the new hematoside was resistant to periodate oxidation. Under the same condition, more than 70% of *N*-acetylneuraminyllactosylceramide was oxidized. From these results, the position of the *O*-acetyl group in the sialic acid of the new hematoside is concluded to be at C-9 of the sialic acid.

The anomeric configuration and sugar sequence were determined by using exoglycosidases. The lactosylceramide which was produced after the treatment with Clostridial neuraminidase, was converted into glucosylceramide by jack bean β-galactosidase. From these results, the structure of the new hematoside was assigned as 9-*O*-acetyl-*N*-acetylneuraminyllactosylceramide.

The fatty acid composition of the new hematoside is presented in table 1. Qualitatively, the new hematoside contained the same fatty acid composition as that found in the usual hematoside. However, the new hematoside contained twice as much of the shorter chain fatty acids such as C16:0 and C18:1 than the usual hematoside.

The existence of different molecular species of sialic acids has been reported [8]. The attachment of one or more acyl groups to the sialic acid in gangliosides may affect the biological properties of these gangliosides which have been shown to play many important biological roles. The exact biological roles of *O*-acetylated gangliosides, however, remain to be elucidated.

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