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Research Article

The urine of patients with proteinuria of various etiologies was examined to determine if proteinuria alone was associated with significant glycosphingolipiduria. In all cases of proteinuria examined, the level of glycosphingolipids in the urine was found to be markedly elevated. There was no evidence of a glycosphingolipid storage disorder in any case. It was concluded that significant glycosphingolipiduria may occur in proteinuria as well as in the glycosphingolipid storage disorders.

Increased Glycosphingolipid Excretion Associated with Proteinuria

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Abstract The urine of patients with proteinuria of various etiologies was examined to determine if proteinuria alone was associated with significant glycosphingolipiduria. In all cases of proteinuria examined, the level of glycosphingolipids in the urine was found to be markedly elevated. There was no evidence of a glycosphingolipid storage disorder in any case. It was concluded that significant glycosphingolipiduria may occur in proteinuria as well as in the glycosphingolipid storage disorders.

INTRODUCTION

With the identification of deficient enzymes (1) and improved methods of glycosphingolipid analysis (2), knowledge of the hereditary glycosphingolipidoses has advanced profoundly in recent years. Improved diagnostic methods for these diseases have been developed. These techniques have included assay of enzyme deficiency in leukocytes, cultured fibroblasts, plasma, and amniotic fluid (3). One of the major characteristics of the glycosphingolipidoses is the accumulation of glycosphingolipids in various tissues. Analysis of accumulated glycosphingolipids has proved very effective in diagnosis (4). In addition, it has been proposed that analysis of glycosphingolipids in the urinary sediment can be a useful adjunct in the diagnosis of a wide variety of glycosphingolipidoses including: Krabbe's leukodystrophy, Gaucher's, Fabry's, Sandhoff's diseases, and metachromatic leukodystrophy (5). Analysis of urinary sediment for trihexosylceramide and digalactosylceramide has been used to screen for hemizygotes and heterozygotes with Fabry's disease (5–12). In screening for patients with lipidoses it was found that both urinary sediment and supernate (sediment-free urine) of patients with proteinuria of various etiologies contained elevated levels of glycosphingolipids. These lipids included globoside, (GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ → Cer); Trihexosylceramide, (Galα1 → 4Galβ1 → 4Glcβ → Cer); and lactosylceramide, (Galβ1 → 4Glcβ → Cer).

METHODS

Voided urine samples were collected at 4°C at 12 h intervals and were centrifuged at 6,000 *g* for 30 min in a Sorval RC-2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) using a GS-3 large capacity rotor. The sediment was collected. The pH of the supernate was then adjusted to 7.0. The supernate was concentrated fivefold with an Amicon DC-2 hollow fiber dialyzer/concentrator (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) with an H1OP1O cartridge. The concentrated supernate was then lyophilized. Glycosphingolipids were isolated from both the lyophilized urinary sediment and supernate according to the method described by Desnick et al. (7). Glycosphingolipids were further purified by DEAE-cellulose chromatography according to Ledeen et al. (13). Separation and quantitation of glycosphingolipids by thin-layer chromatography were performed according to the procedures described (14, 15). Each glycosphingolipid was isolated in pure form by preparative thin-layer chromatography. Specific glycosidases were used to sequentially hydrolyze the saccharide units in the glycolipids (16). Various glycosidase activities in leukocytes were assayed according to the procedure of Wenger et al. (17). Urine from a patient with Fabry's disease was kindly provided by Dr. Robert Hurst, University of Alabama Medical Center, Birmingham, Alabama. Cases studied are presented in Table I.

RESULTS

Fig. 1 shows the thin-layer chromatography of glycosphingolipids derived from the urinary sediment of a normal male subject (lane 1), a patient with classical Fabry's disease (lane 2), and case 1 (lane 3). The major glycosphingolipid excreted in the urine of a patient

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TABLE I
Pertinent Clinical Data of Cases Studied

Case	Age	Sex	Urinary protein	Clinical diagnosis	Kidney biopsy
			g/24 h		
1	43	M	0.6–4.8	Fabry's disease	Glomerular hyalinization
2	14	F	0.6–18.0	Nephrotic syndrome	Chronic membranoproliferative glomerulonephritis with benign nephrosclerosis
3	34	M	2.0–5.5	Nephrotic syndrome	Lipoid nephrosis with focal glomerulosclerosis
4	46	M	6.0–7.7	Diabetes mellitus	No kidney biopsy, suspected diabetic nephropathy
5	12	M	3.2–4.8	Nephrotic syndrome	No kidney biopsy, suspected chronic glomerulonephritis
6	13	M	1.4–11.9	Nephrotic syndrome	Chronic proliferative glomerulonephritis
7	18	M	8.8–37.4	Nephrotic syndrome	Lipoid nephrosis

with Fabry's disease is trihexosylceramide. Case 1 excreted significant amounts of trihexosylceramide as well as globoside. Normal control urinary sediment contained very little detectable glycosphingolipids. Globoside and trihexosylceramide were separately isolated in pure form by preparative thin-layer chromatography. Incubation of the globoside with jack bean β-*N*-acetylhexosaminidase (18) converted this glycolipid into trihexosylceramide; the trihexosylceramide in turn was converted into lactosylceramide and glucosylceramide by the actions of fig α-galactosidase (19) and jack bean β-galactosidase (20), respectively. Trihexosylceramide isolated from the urinary sediment of case 1 was also converted into lactosylceramide and glucosylceramide, sequentially, by the action of fig α-galactosidase and jack bean β-galactosidase. The structures for these two glycolipids were therefore concluded to be GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ Cer and Galα1 → 4Galβ1 → 4Glcβ Cer, respectively. If the patient was afflicted with Fabry's disease, one should see a decrease of α-galactosidase activity. However, the activity of this as well as other leukocyte lysosomal glycosidases (β-galactosidase, α-mannosidase, and α-L-fucosidase) were found to be within the normal range. Because globoside contains β-linked *N*-acetylgalactosamine at the nonreducing end of the saccharide chain, the possibility that this patient may be deficient in β-*N*-acetylhexosaminidase was considered. With either *p*-nitrophenyl-β-*N*-acetylglucosaminide or *p*-nitrophenyl-β-*N*-acetylgalactosaminide as substrates, no aberration in β-*N*-acetylhexosaminidase was detected. At autopsy, this patient was found to have primary amyloidosis with no evidence of glycosphingolipid accumulation in any major organ. Because this patient had extensive glomerular pathology by light microscopy (Table I) and a persistent proteinuria, we investigated the urine from several patients with proteinuria.

Fig. 2 shows the thin-layer chromatogram of glycosphingolipids isolated from the urinary sediment of cases 1–3. It can be noted that the urinary sediment from these patients demonstrated high levels of glycosphingolipids including globoside, trihexosylceramide, and lactosylceramide. We further examined the excretion patterns of glycosphingolipids in urinary supernate and urinary sediment of cases 4–7. As shown in Fig. 3, the 6,000 *g* supernate contained more glycosphingolipid from the 6,000 *g* sediment.

To determine if the glycosphingolipids found in the 6,000 *g* supernate are associated with membrane fragments, the 6,000 *g* supernate of case 4 was centrifuged at 100,000 *g* for 2 h. Glycosphingolipids were isolated

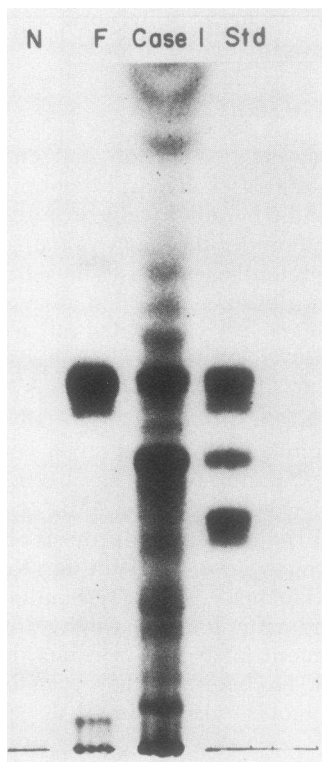

FIGURE 1 Thin-layer chromatography of glycosphingolipids in the urinary sediment of normal (N), classical Fabry's disease (F), and case 1. Standards (Std) are from top to bottom: trihexosylceramide, globoside, and Forssman hapten.

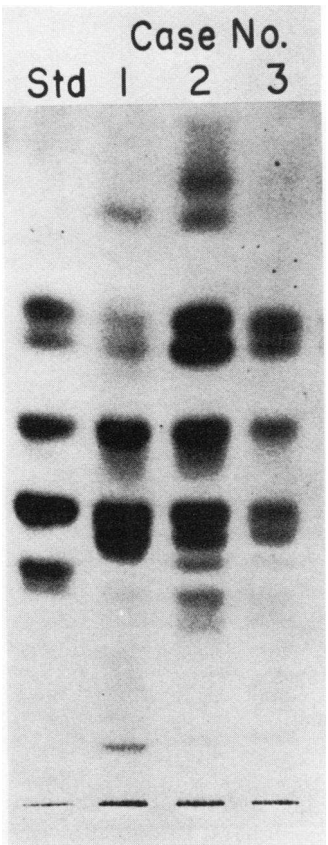

FIGURE 2 Thin-layer chromatography of glycosphingolipids in the urinary sediment of patients (cases 1, 2, and 3) with proteinuria. Standards (Std) are from top to bottom: lactosylceramide, trihexosylceramide globoside, and Forssman hapten.

from the resulting 100,000 *g* supernate and sediment. As shown in Fig. 4, the 100,000 *g* supernate still contains ≅50% of the glycosphingolipids found in the 6,000 *g* supernate. The nature of the association of the "soluble"glycosphingolipids remains to be determined.

Table I and II summarize some pertinent clinical data of the cases studied. Table III shows the range of the various glycosphingolipids excreted in 24 h of the patients examined. For comparison, we have included the reported ranges of glycosphingolipids in the urinary sediment of normal controls and patients with Fabry's disease.

DISCUSSION

It has been proposed that analysis of the glycosphingolipids in the urinary sediment can be used in the diagnosis of various glycosphingolipids (5). In recent years, analysis of the urinary sediment has been extensively used as a screening technique in the diagnosis of patients with various lipidoses (5–12). In an attempt to confirm the clinical diagnosis of Fabry's disease in case 1, analysis of glycosphingolipids in urinary sediment and assay of specific glycosidases in the leukocytes were performed. Although glycosidase activities were within the normal range, the thin-layer chromatograms showed increased excretion of urinary glycosphingolipids; however, as shown in Fig. 1, the glycosphingolipid pattern does not correspond to that reported in Fabry's disease. After the patient's death, we found that he did not have a glycosphingolipid storage dis-

TABLE II
Urinalyses of Samples Analyzed for Glycosphingolipids

Case	Erythrocytes per high power field	Leukocytes per high power field	Casts per low power field
1	0–1	0–1	1–2 coarse granular casts
2	not available	not available	—
3	1–3	10–15	3–5 granular casts
4	1–3	2–3	2–4 coarse granular casts
5	0–1	1–2	1–4 coarse granular casts
6	0–1	10–20	1–2 hyaline casts 1–2 granular casts
7	1–2	3–4	5–10 fine granular casts

TABLE III
Glycosphingolipid Excretion in Normal and Pathological Urinary Sediment

	Lactosyl ceramide	Trihexosyl ceramide	Globoside	Reference
	nmol/24 h			
Normal	10–24	14–23	12–16	12
Normal	18–68	17–28	15–28	7
Fabry's disease:				
Hemizygote	19–400	240–4,910	31–640	7
Heterozygote	—	130–980	—	5
Variants of Fabry's disease	54–450	227–749	12–58	12
Nephrotic syndrome	25	96	97	12
Case				
1	100–200	400–900	750–1,200	—
2	300	150	250	—
3	100	50	75	—
4	30	150	150	—
5	10	150	350	—
6	350	300	350	—
7	100	75	100	—

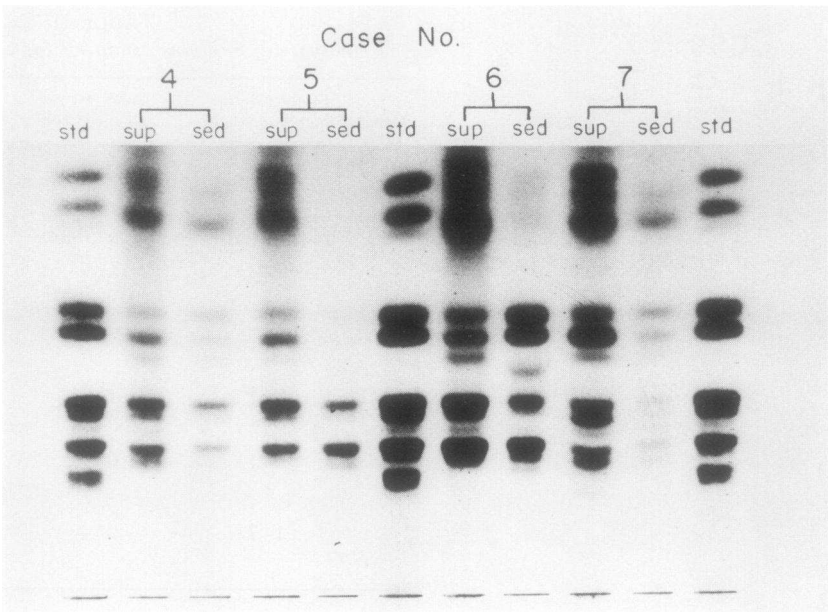

FIGURE 3 Thin-layer chromatography of glycosphingolipids in the 6,000 *g* urinary sediment (sed) and 6,000 *g* supernate (sup) of patients (cases 4–7) with proteinuria. Standards (Std) are from top to bottom: galactosylceramide, lactosylceramide, trihexosylceramide, globoside, and Forssman hapten. Sup, is 6,000 *g* supernate and sed, is 6,000 *g* sediment.

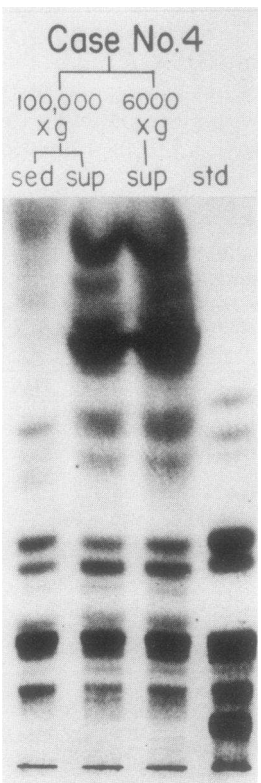

FIGURE 4 Thin-layer chromatography of glycosphingolipids in the 100,000 *g* urinary sediment (sed), supernate (sup), and 6,000 *g* supernate (sup) of case 4. Standards are from top to bottom: galactosylceramide, lactosylceramide, trihexosylceramide, globoside, and Forssman hapten.

order despite marked elevation of glycosphingolipids in the urinary sediment. Examination of other patients with proteinuria demonstrated that these patients also had elevated glycosphingolipid levels in the urine as shown in Figs. 2 and 3. Kidney biopsy (performed in all but two cases) revealed no evidence of a storage disorder.

Table III shows that the 24 h excretion of various glycosphingolipids in these patients with proteinuria overlaps the lower range of excretion of some Fabry hemizygotes and most Fabry heterozygotes. Patients with lipidoses excrete a large amount of a specific glycosphingolipid. Patients with proteinuria tend to excrete high levels of several glycosphingolipds such as globoside, trihexosylceramide, lactosylceramide, and to a lesser extent monohexosylceramide. Also, there is considerable variation from case to case in the ratio of each glycosphingolipid. Whether this pattern variation is related to the etiology in each case remains to be determined.

The source of glycosphingolipids in the above cases has not been ascertained. Two major contributing sources are possible: (*a*) from cellular elements of the urinary tract; and (*b*) from plasma via abnormal glomerular filtration. The membranes of erythrocytes and leukocytes could contribute significant amounts of globoside and lactosylceramide, respectively (21–23). Inasmuch as the kidney contains globoside and trihexosylceramide (24, 25), exfoliated renal tubular epithelium could contribute to the glycosphingolipiduria. Except for

cases 1 and 4 (Figs. 1–4), the glycosphingolipid pattern does not resemble that of normal kidney (24, 25, 26). Also, kidney is the only visceral organ which contains appreciable amounts of galactosylceramide in addition to glucosylceramide (24). The ratio of glucosylceramide to galactosylceramide is reported to be 1:0.6 (24). By using borate-impregnated thin-layer chromatography plates (27), it was found in cases 4 and 6 that the ratio of glucosylceramide to galactosylceramide was ≅1:0.7. The results suggest that the monohexosylceramide is renal in origin; however, the source of the other three major glycosphingolipids is not known.

The results presented above demonstrate that abnormal neutral glycosphingolipid excretion occurs in all cases of proteinuria examined as well as in the lipid storage disorders. Furthermore, the 24 h excretion in some cases of proteinuria is equal to that seen in lipidoses.

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