Increased plasma plant sterol levels in heterozygotes with sitosterolemia and xanthomatosis

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Abstract Plasma sterol levels in a family of sitosterolemia and xanthomatosis were determined by a high performance liquid chromatography. Three affected siblings manifested marked xanthomatosis including subcutaneous soft tissues and generalized atherosclerosis. Two other siblings as well as children of the patients did not show such clinical symptoms and signs. Plasma levels of cholesterol, sitosterol, campesterol, and cholestanol in three affected subjects were 190 ± 18.5, 25.9 ± 11.6, 16.1 ± 7.8, 1.84 ± 0.92 mg/dl (mean ± SD), respectively. Four daughters of the affected subjects, who should be considered as obligatory heterozygotes, showed moderately increased levels of these sterols (195 ± 41.7, 1.33 ± 0.44, 1.56 ± 0.69, 0.80 ± 0.28 mg/dl), which were significantly higher than those of normal subjects. Treatment with cholestyramine had little effect on the increased plasma plant sterol levels, but markedly decreased plasma cholestanol concentrations in two affected siblings. This report presents the clinical features of the patients with sitosterolemia and xanthomatosis and also demonstrates that heterozygotes with this disorder have increased plasma levels of plant sterols as well as cholestanol, and suggests that this rare disease might be inherited as an autosomal co- dominant trait in certain cases. The data also indicate that cholestyramine administration was not effective in this family for treatment of sitosterolemia.—**Hidaka, H., T. Nakamura, T. Aoki, H. Kojima, Y. Nakajima, K. Kosugi, I. Hatanaka, M. Harada, M. Kobayashi, A. Tamura, T. Fujii, and Y. Shigeta.** Increased plasma plant sterol levels in heterozygotes with sitosterolemia and xanthomatosis. *J. Lipid Res.* 1990. **31:** 881–888.

Supplementary key words phytosterolemia • atherosclerosis • cholestanol • family study

Sitosterolemia and xanthomatosis is a rare inherited disorder characterized by increased plasma levels of plant sterols (1). The previous family studies showed that the disease is inherited as an autosomal recessive trait (2). In contrast to homozygotes, heterozygotes with the disease have been reported to show normal plasma levels of plant sterols and no clinical signs of xanthomatosis. However, some of the heterozygotes have been reported to have increased plasma cholesterol levels (3, 4).

We encountered a family with sitosterolemia one of whom had paraplegia caused by spinal compression of by multiple xanthomas (5). In this report, we further describe our findings of the clinical and biochemical characteristics in the family members of the patients by analyzing plasma plant sterols with a sensitive method for the determination of plasma sterols using high performance liquid chromatography (HPLC). In contrast to the previous studies, we demonstrate increased plasma levels of plant sterols in heterozygotes with sitosterolemia and xanthomatosis. Furthermore, we tested the efficacy of cholestyramine treatment for this disorder.

METHODS

Subjects

The clinical features of the patient (case 3 in Fig. 1) who presented with paraplegia have been reported elsewhere (5). Briefly, the patient had noticed multiple xanthomas since the age of 18 years and paraparesis since the age of 47. Myelography revealed the presence of an intradural mass which was surgically removed. Subsequently, plasma levels of sitosterol, campesterol, and cholestanol were found to be elevated, and the diagnosis of sitosterol-

Abbreviations: HPLC, high performance liquid chromatography; GLC-MS, gas–liquid chromatography–mass spectrometry; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins. The following systematic names are used for sterols and 5α-stanol referred to by trivial names: cholesterol, 5-cholesten-3β-ol; sitosterol, 24-ethyl-5-cholesten-3β-ol; campesterol, 24-methyl-5-cholesten-3β-ol; cholestanol, 5β-cholestan-3β-ol.

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emia was established. The family pedigree of the patient is shown in **Fig. 1**. Cases 2 and 4 also presented with marked tendon and subcutaneous xanthomas. Other family members who were examined did not show any clinical signs of xanthomas.

Determination of plasma lipids

Laboratory examination was performed by routine methods using automated analyzers. Plasma lipid levels were determined by enzymatic methods, where 3β-hydroxy sterols were measured as cholesterol because of the lack of substrate specificity of cholesterol oxidase (6). Phospholipid levels were also determined by an enzymatic (phospholipase D) method (7) with a commercial kit (Sanko Jun-yaku, Tokyo, Japan) and the automated analyzer. HDL-cholesterol concentrations were measured after precipitation by a dextran sulfate method (8).

Lipoprotein measurement

Plasma lipoprotein fractions were separated by an ultracentrifugation method in a Beckman tabletop ultracentrifuge (TL. 100) with a TL 100.3 rotor. Briefly, 2 ml of fasting plasma was overlayed with 1 ml of 0.195 M NaCl, 0.3 mM EDTA (pH 7.4, d 1.006) and centrifuged for 2 h at 100,000 rpm. One ml of the supernatant was taken as very low density lipoproteins (VLDL), and the density of the remaining infranatant was adjusted to 1.063 g/ml. The fraction was further ultracentrifuged for 4 h, and 1 ml of the supernatant was taken as low density lipoproteins (LDL), and the remainder was the high density lipoprotein fraction (HDL).

Determination of plasma sterols

Plasma sterol concentrations were determined at fasting by the method described by Kasama, Byun, and Seyama (9). Briefly, 0.1 ml of plasma with 10 μg of 5β-cholestane 3α-ol (as an internal standard for the calculation of recovery) was treated with 1 M ethanolic KOH and extracted twice with n-hexane. The sterols in the extracts were converted into their benzoate derivatives with benzoyl chloride reagent which was freshly prepared for each assay. The benzoate derivatives of the sterols were re-extracted with 1.2-dichloroethane, and dissolved again in 250 μl of acetonitrile–dichloroethane 2:1 after evaporation under a stream of nitrogen. Five μl of the solution was injected into the HPLC system. The separation of the sterols was performed on a reverse-phase column (SBC-ODS 150 × 2.5 mm, Shimadzu, Kyoto, Japan) maintained in an incubator at 50°C and monitored at 228 nm. The instrument was an LC-6A system (Shimadzu), equipped with a column oven and chromatogram data processor (Chromatopac C-R5A). The solvent used for the elution was acetonitrile–water–acetic acid 97:3:0.2 at a flow rate of 0.5 ml/min. A calibration was done after the derivatization of authentic sterols obtained from Sigma (St. Louis, MO) or Nacalai Tesque (Kyoto, Japan). The elution pattern of benzoate derivatives of the authentic sterols is shown in **Fig. 2**. Relative retention times were 0.903 for cholesterol, 1.000 for internal standard, 1.043 for campesterol, 1.104 for cholestanol, and 1.221 for sitosterol. Separation of campesterol and cholestanol from the internal standard was relatively small, but separation of sitosterol was enough to determine the sterol levels as shown by Salen et al. (10) by a reverse-phase C_{18} column. Standard curves of the sterols were linear up to 400 mg/dl for cholesterol, and up to 40 mg/dl for sitosterol, campesterol, and cholestanol. Extinction coefficients of these sterols at 228 nm seemed to be similar to those reported by Kasama et al. (9), inasmuch as peak areas of the same weights of the sterols separated by the HPLC method were inversely related to their relative molecular weight. As low as 0.16 mg/dl of campesterol, 0.08 mg/dl of cholestanol or sitosterol can be detected by this method (Fig. 2). The coefficients of variation in five different assays in case 7 were 1.8% for cholesterol, 4.2% for sitosterol, 2.2% for campesterol, and 7.3% for cholestanol. Cochromatography of the plasma sterols of case 7 with derivatives of authentic sterols in HPLC showed identical retention times for each sterol derivative, and recovery rates of the added sterols and cholestanol were more than 85%. Plasma sterols after trimethylsilyl derivatization were also analyzed qualitatively by GLC–MS (QP-1000S; Shimadzu), using a capillary column CBP-1 (25 m × 0.2 mm, Shimadzu) maintained at 250–310°C.

Morphological analysis

For morphological observation of erythrocytes, the cells were washed in isotonic phosphate-buffered saline, and

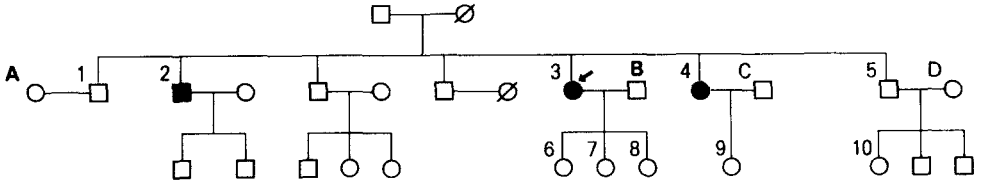

Fig. 1. Family pedigree of sitosterolemia and xanthomatosis; (■, ●) subjects with xanthomatosis.

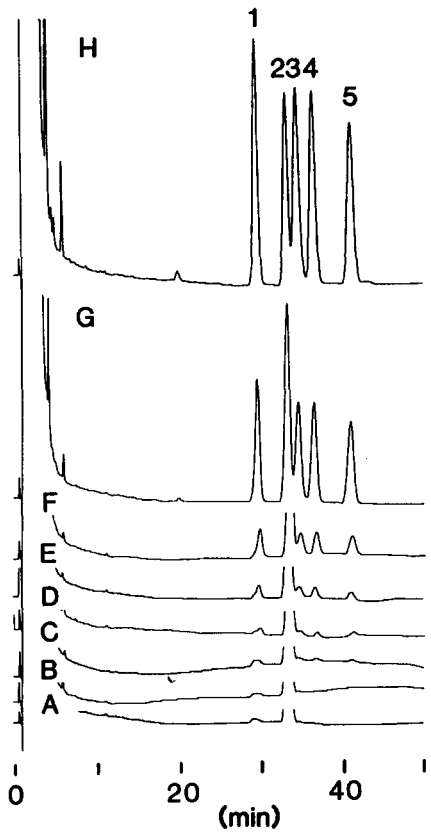

Fig. 2. Elution pattern of benzoate derivatives of authentic sterols by HPLC. Samples containing 0–10 μg of each sterol and cholestanol with 10 μg of internal standard were derivatized by the same procedure for plasma sterol determination. Peak 1, cholesterol; peak 2, internal standard; peak 3, campesterol; peak 4, cholestanol; peak 5, sitosterol. A, 0 μg; B, 0.08 μg; C, 0.16 μg; D, 0.31 μg; E, 0.63 μg; F, 1.25 μg; G, 5 μg; H, 10 μg.

fixed by glutaraldehyde solution. The specimens were observed under a scanning electron microscope (JSM-35, JEOL, Tokyo, Japan).

RESULTS

Clinical manifestations

Clinical manifestations of the three affected subjects are presented in **Table 1**. Cases 2, 3, and 4 had marked xanthomatosis including soft tissues. Achilles tendon thickness measured radiographically was more than 20 mm in the patients. Two siblings (cases 1 and 5) did not show any xanthomas including Achilles tendon (tendon thickness 7 mm in case 1, 6 mm in case 5). Case 3 and case 4 had mild anemia, while the second brother, case 2, was not anemic. Scanning electron microscopy of red blood cells revealed stomatocytic deformation (**Fig. 3**). Osmotic fragility of the red blood cells was also demonstrated in cases 3 and 4. Splenomegaly was found in cases 3 and 4 by abdominal echograms. Electrocardiograms showed no abnormality at resting in any examined family member, but there was marked ST depression after exercise in cases 3 and 4. Clinical signs of arteriosclerosis obliterans, i.e., arterial bruit in femoral arteries and decreased ankle blood pressure, were present in cases 3 and 4.

Lipid analysis

Determination of plasma levels of cholesterol, triglyceride, and HDL cholesterol by enzymatic methods showed mild hyperlipidemia in some family members (**Table 2**). Lipoprotein analysis of the patients and siblings by an ultracentrifugation method showed mild elevation of LDL cholesterol in some cases but not in others (data not shown).

Plasma concentrations of sitosterol, campesterol, and cholestanol determined by HPLC (**Fig. 4**) were markedly elevated in cases 2, 3, and 4 (**Table 3**). GLC-MS confirmed the presence of large amounts of plant sterols in the patient's plasma consisting of three major peaks identified as cholesterol, sitosterol, and campesterol by the

TABLE 1. Clinical manifestation of subjects with sitosterolemia and xanthomatosis

Case	Xanthomas			Hb	Splenomegaly	IHD	API	
	ATT		Others					
	Rt	Lt					Rt	Lt
	mm			*g/dl*				
2	21	19	+	15.8	NE	NE	NE	
3	34	35	+	8.7	+	+	1.02	0.70
4	22	24	+	11.2	+	+	1.01	1.00[a]
Normal values	5–8			11.3–17			1.0–1.2	

ATT, Achilles tendon thickness determined radiographically; Hb, hemoglobin; IHD, ischemic heart disease; API, ankle pressure index (ankle blood pressure/brachial blood pressure) determined by a Doppler method; NE, not examined.

[a]API after exercise were 1.23 (Rt) and 0.83 (Lt).

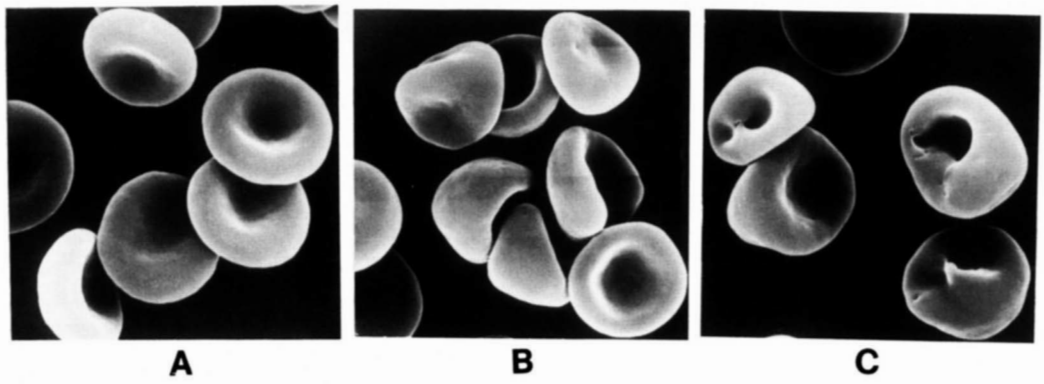

Fig. 3. Scanning electron micrograph of red blood cells of sitosterolemia (× 6000). A, control; B, case 3; C, case 4. Control red blood cells were obtained from one of the authors (H. H.).

mass spectrograms (data not shown). These compounds were similarly distributed as cholesterol in plasma lipoproteins in cases 3 and 4 (**Table 4**). Other family members related to the patients also showed significant elevation of these plasma sterol levels compared with normal values, but the spouses of cases 1, 3, 4, and 5 did not. The increased plasma plant sterol levels were confirmed in plasma samples taken at an interval of 10 months in cases 6, 7, and 8. Cholesterol levels were 180, 225, 182 mg/dl; sitosterol 1.46, 1.49, 1.33 mg/dl; campesterol 1.38, 1.96, 1.33 mg/dl; and cholestanol 0.67, 1.00, 0.48 mg/dl in cases 6, 7, and 8, respectively. Plant sterol contents were also elevated in red blood cell ghosts in cases 3 and 4 (plant sterol 18.7% of total sterol in case 3, 31.4% in case 4).

Administration of cholestyramine to cases 3 and 4 resulted in a 23–46% decrease of plasma cholesterol levels (**Table 5**). However, decrease of plant sterol concentrations was relatively small (0–32%) especially in case 3, while plasma cholestanol levels were markedly decreased to the normal ranges. The small decrease of plant sterol levels has been observed up to 7 months after the initiation of the treatment even though the dosage of cholestyramine had been increased to 12 g/day in case 3.

DISCUSSION

According to the recent review by Björkhem and Skrede (1), 12 families or 22 cases of sitosterolemia and xanthomatosis have been reported in the literature. In addition, one case has been reported from New Zealand (11). Clinical pictures of the reported patients are multiple xanthomas in all cases, and premature atherosclerosis, intravascular hemolysis, splenomegaly, and hypercholesterolemia in some cases (12). None of these papers reported neurological signs and symptoms in this rare disorder. One of our patients (case 3) showed paraplegia due to spinal compression by multiple xanthomas of spinal ligaments (5). This is the first patient who presented with neurological abnormalities in sitosterolemia and xanthomatosis.

Four daughters of the affected patients, who should carry the abnormal gene (obligatory heterozygotes), had elevated levels of plant sterols as well as cholestanol. Plas-

TABLE 2. Plasma lipid levels in family members

Case	Sex	Age	Total Cholesterol	TG	HDL-Cholesterol	Phospholipids
		yr		*mg/dl*		
1	M	59	263	72	54	254
2	M	55	189	318	56	262
3	F	48	267	142	43	286
4	F	45	246	52	81	277
5	M	42	287	152	40	255
6	F	20	167	40	NE	167
7	F	18	245	62	NE	259
8	F	15	198	74	47	204
9	F	17	165	64	53	175
10	F	12	180	30	57	182
Normal values			125–250	45–150	40–65	145–265

Lipid concentrations were determined by enzymatic methods; NE; not examined.

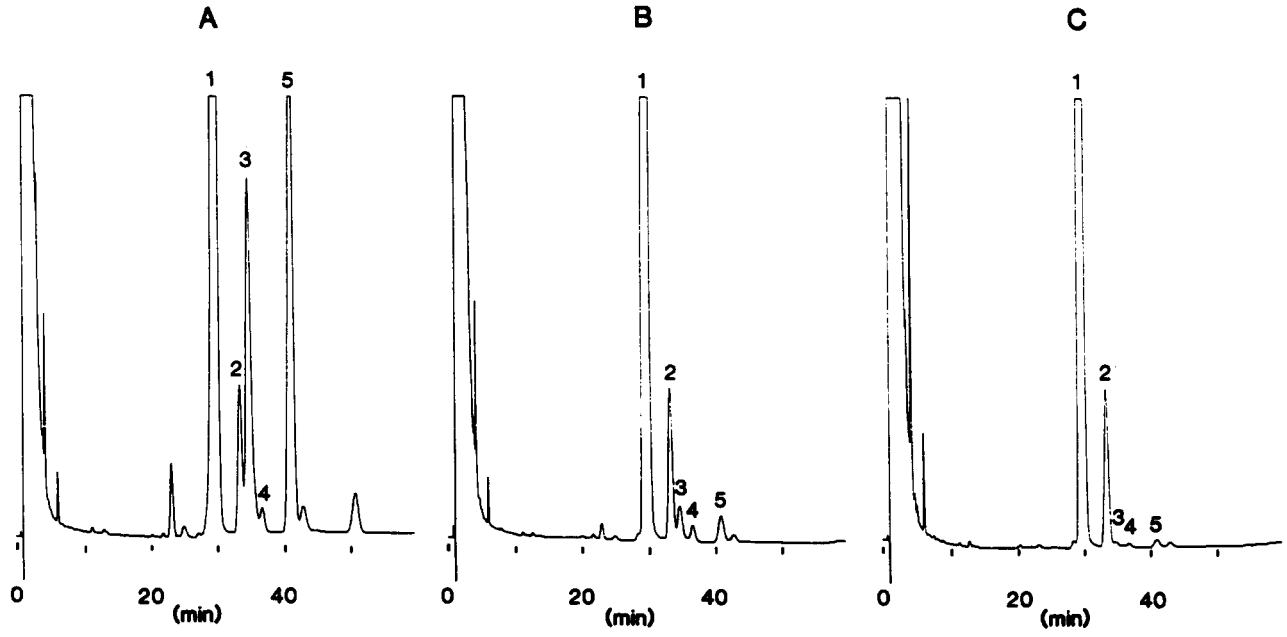

Fig. 4. Elution pattern of plasma sterols in subjects with sitosterolemia and xanthomatosis. A, subjects with xanthomatosis (case 4); B, obligatory heterozygote (case 8); C, control. Peak 1, cholesterol (relative retention time (RTT) 0.903); peak 2, internal standard (RTT 1.000); peak 3, campesterol (RRT 1.043); peak 4, cholestanol (RRT 1.104); peak 5, sitosterol (RRT 1.221).

ma levels of plant sterols (determined by gas-liquid chromatography) in the first degree relatives of the subjects with this disorder were reported to be within a normal range in eight out of nine families (1, 11). Only one case report from Hong Kong (13) has shown the elevation of plant sterols in children and mother of a clinically symptomatic patient. There are several possibilities to explain the discrepancy between our results and the previous re-

TABLE 3. Plasma sterol levels determined by HPLC

Case	Cholesterol	Sitosterol	Campesterol	Cholestanol
		mg/dl		
1	222	1.57	1.76	1.03
2	178	19.52	12.00	0.96
3	211	18.98	11.61	2.79
4	180	39.26	24.67	1.77
5	269	1.95	2.08	0.86
6	174	1.18	1.52	0.68
7	254	1.98	2.55	1.17
8	193	1.16	1.16	0.84
9	159	1.00	1.02	0.50
10	177	1.21	1.46	0.74
A	247	0.99	0.87	0.77
B	227	0.74	0.29	0.41
C	193	0.74	0.63	0.34
D	223	0.84	0.51	0.57
Homozygote (cases 2, 3, 4)	190 ± 18.5[a]	25.9 ± 11.6[b]	16.1 ± 7.8[b]	1.84 ± 0.92[b]
Obligatory Hetrozygote (cases 6, 7, 8, 9)	195 ± 41.7	1.33 ± 0.44[c,d]	1.56 ± 0.69[c,d]	0.80 ± 0.28[b]
Spouses (cases A, B, C, D)	222 ± 22.3	0.82 ± 0.12	0.58 ± 0.24	0.52 ± 0.19
Normal control (n = 10)	187 ± 24.5	0.71 ± 0.13	0.48 ± 0.23	0.39 ± 0.18
Range	140–240	0.51–0.99	0.20–0.87	0.10–0.79

[a]Mean ± SD; [b] $P < 0.05$; [c] $P < 0.01$ vs control; [d] $P < 0.05$ vs spouses by Wilcoxon rank sum test.

TABLE 4. Sterol concentrations in lipoprotein fractions in subjects with sitosterolemia and xanthomatosis

Lipoproteins	Cholesterol	Sitosterol	Campesterol	Cholestanol
		mg/dl		
Case 3				
Plasma	213 (100)	19.6 (100)	12.1 (100)	2.73 (100)
VLDL	20 (9.4)	1.29 (6.6)	0.97 (8.0)	0.22 (8.1)
LDL	113 (53)	10.60 (54)	6.42 (53)	1.45(53)
HDL	47 (22)	4.91 (25)	2.76 (23)	0.62 (23)
Case 4				
Plasma	160 (100)	36.9 (100)	23.6 (100)	1.69 (100)
VLDL	5 (3.1)	0.82 (2.2)	0.67 (2.8)	0.05 (3.0)
LDL	86 (54)	19.55 (53)	12.72 (54)	0.89 (53)
HDL	53 (33)	13.33 (36)	7.99 (34)	0.60 (36)

Number in parentheses is percent of plasma concentrations of each sterol, calculated as each sterol concentration in the lipoprotein fraction divided by plasma concentration of each sterol. Average recovery of each sterol in the lipoprotein fractions by ultracentrifugation was 87.7%.

ports. First, it is possible that the family members habitually consumed diets containing high levels of plant sterols. It has been reported that the average plant sterol content in Japanese diets is approximately 400 mg/day (14), which might be higher than those of typical diets in Western countries (up to 250 mg/day) (15). Therefore, large amounts of dietary plant sterol in oriental meals may cause the increased plasma plant sterols in heterozygotes with this disorder since another family of sitosterolemia in which the patient's children had increased plasma levels of plant sterols was Chinese (13). Secondly, the method of the plant sterol determination, done mainly by gas-liquid chromatography in previous studies, was not sensitive enough to detect the small amounts of plant sterols in the heterozygotes with sitosterolemia and xanthomatosis, while normal values are similar to our results. A careful determination of plasma plant sterol levels in other families with this disorder by a sensitive method is necessary to confirm this phenomenon. Thirdly, it is also possible that our patients may have had a different inherited genetic and biochemical abnormality compared with the patients reported previously, since most of inherited disease have various degrees of genetic heterogeneity even though the clinical symptoms and signs are similar (16).

This report also demonstrates that plasma plant sterols are similarly distributed in various lipoproteins compared with cholesterol. Bhattacharyya and Connor (17) reported that LDL in two sisters was the main carrier of plant sterols in plasma and that VLDL contained little plant sterols in plasma of the affected sisters. Since plant sterols are not synthesized in humans (18), the fact of the distribution of the plant sterols similar to cholesterol in

TABLE 5. Effect of cholestyramine on plasma sterol levels in two sitosterolemic patients

Date	Cholestyramine	Cholesterol	Sitosterol	Campesterol	Cholestanol
	g/day			*mg/dl*	
Case 3					
3/23/89		211	19.0	11.6	2.79
4/18/89		213	19.6	12.1	2.73
5/1/89	8	151	16.7	9.9	1.35
5/31/89	8	158	19.5	10.9	1.11
6/28/89	8	164	19.6	10.8	0.62
7/27/89	8	137	18.4	10.2	0.70
8/17/89	8	157	21.0	11.3	0.58
8/28/89	12	146	20.7	11.0	0.85
9/13/89	12	137	19.5	10.2	0.62
9/27/89	12	130	17.8	9.3	0.47
10/18/89	12	114	16.3	8.8	0.93
11/26/89	12	156	20.7	10.8	0.63
Case 4					
2/22/89		180	39.3	24.7	1.77
4/12/89		160	36.9	23.6	1.69
5/17/89	4-8	112	25.9	16.8	0.36
6/28/89	8	136	33.6	20.2	0.41
7/26/89	8	132	32.1	20.1	0.36
8/16/89	8	113	29.8	17.4	0.63

Cholestyramine treatment started on 4/18/89 in case 3, and on 4/12/89 in case 4. Dosage of cholestyramine was increased to 12 g/day on 8/17/89 in case 3.

lipoproteins suggests that sterol exchange between lipoproteins is not specific for different sterols.

Cholestyramine treatment reduced the elevated plant sterol levels in our patients as reported by Salen et al. (19). However, the decrease of plasma plant sterol levels is small compared with the decrease of plasma cholesterol concentrations even at a dosage of 12 g/day in case 3. Body weights of our patients may be smaller than those of previously reported patients (45 kg in case 3 and 37 kg in case 4), therefore the smaller dosage could not account for the small decrease of plant sterols by cholestyramine. The discrepancy between this study and the previous reports is difficult to explain. Since it has been reported that the concentrations of plant sterols are sensitive to dietary sterols (10), it is possible that the fluctuation of dietary sterol intake might have affected the treatment. Even though it is difficult to explain the consistency of the relative ineffectiveness of the cholestyramine treatment for more than 6 months on plasma plant sterol levels in our patients by the sterol intake alone, a dietary factor cannot be ruled out for the explanation of the discrepancy, since the Japanese diet may contain larger amounts of plant sterols as discussed above. The decrease of plasma cholestanol concentrations was more remarkable. The biochemical significance of this discrepancy between plant sterol and cholestanol is not clear, since mechanisms of increased 5α-saturated sterols in sitosterolemic subjects have not been elucidated. It is possible that the increased 7α-hydroxylase activity induced by cholestyramine treatment (20) may change cholestanol metabolism, since it has been reported that the enzyme activity is inhibited in liver microsomes of sitosterolemic patients (21).

Plasma cholestanol concentrations were increased in siblings without xanthoma and in children of the patients. The increased ranges were similar to those observed in another rare inherited xanthomatous disease: cerebrotendinous xanthomatosis (CTX) (1, 9). Kasama et al. (9) reported that the ratio of cholestanol to cholesterol levels in plasma is a better marker for diagnosis of CTX than cholestanol concentrations alone. The ratios were 9.48 ± 3.95, 4.00 ± 0.64, 2.31 ± 0.65, and 2.06 ± 0.81 (× 1000) in subjects with xanthoma, obligatory heterozygotes, spouses of the family members, and control subjects, respectively. Even though the ratios in the affected subjects as well as heterozygotes were lower than those reported in CTX patients, both the ratios and cholestanol concentrations were significantly elevated compared with control values. Since these subjects showed no clinical symptoms and signs including xanthomas, the clinical significance of the increased plasma cholestanol levels is not clear at present.

In summary, we have determined plasma plant sterol levels in a family with sitosterolemia and xanthomatosis by an HPLC method, and found that plasma levels of plasma plant sterols as well as cholestanol were elevated in heterozygotes of the disease, suggesting that this rare disorder may be inherited as an autosomal co-dominant trait in certain cases. The treatment with cholestyramine normalized plasma cholestanol levels in two clinically symptomatic patients, but had little effect on plasma plant sterol concentrations.

The authors wish to thank Miss Yuka Horie for her excellent technical assistance, and also Drs. T. Kasama and Y. Seyama for their valuable suggestions on the measurements of plasma sterols. This study was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

Manuscript received 12 September 1989 and in revised form 17 January 1990.

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