Relationship between Susceptibility to Hemolytic-Uremic Syndrome and Levels of Globotriaosylceramide in Human Sera

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Received 19 May 2000/Returned for modification 24 August 2000/Accepted 9 November 2000

The relationship between differential susceptibility to hemolytic-uremic syndrome (HUS) and levels of globotriaosylceramide (Gb3) in serum was studied in patients infected with verotoxin-producing *Escherichia coli* (VTEC). The serum Gb3 levels in patients with HUS were lower than these in diarrheal patients without subsequent HUS or in patients without clinical symptoms, indicating that individuals with a lower content of serum Gb3 show a higher incidence of HUS following VTEC infection.

Verotoxin (VT)-producing *Escherichia coli* (VTEC) produces either VT1 or VT2 or both (8). Epidemiological data show a strong correlation between these VTs and the development of hemolytic-uremic syndrome (HUS) (7, 14). However, it is well recognized that not all patients who have VTEC-associated enterocolitis develop HUS (2), and the nature of the underlying host susceptibility is not understood. It has been reported that a nonimmunoglobulin fraction of human serum (lipoproteins) shows VT-neutralizing activity (1), and in human serum, neutral glycosphingolipids (GSLs), including globotriaosylceramide (Gb3), which is recognized as the functional receptor for VTs (3, 10–12), are closely associated with serum lipoproteins (4). These findings raise the possibility that the heterogeneity of Gb3 content in the serum might be related to susceptibility to VT, leading to HUS. Therefore, we compared levels of the neutral GSLs glucosylceramide (GlcCer), lactosylceramide (LacCer), Gb3, and globotetraosylceramide (Gb4) in sera of patients with HUS, with appropriate controls.

Serum samples. Serum samples were obtained from Okayama National Hospital (Okayama, Japan), Okayama Rosai Hospital (Okayama, Japan), and Ibara City Hospital (Ibara, Japan). Blood was collected from 12 HUS patients in the acute phase (group A), 11 patients who had VTEC-associated diarrhea without development of HUS (group B), and 12 VTEC-infected patients who had no obvious gastrointestinal symptoms although they ate the same type of food as the other patients and showed relatively high serum antibody titers against the organism (group C). Blood samples were allowed to clot at 4°C, and following centrifugation (1,600 × *g*, 5 min), the sera were collected and stored at –20°C. Serum samples obtained were used for the extraction of neutral GSLs within 1 week. Six serum samples were also obtained from HUS patients in group A at the convalescent phase (group D) as described above. Clinical data for the patients are summarized in Table 1.

Extraction, purification, and quantitative analysis of neutral GSLs from serum samples. Each serum sample (1.5 to 3.0 ml) was placed in a flask, and ethanol was added to a concentration of 70%. The mixture was stirred vigorously at room temperature for 1 h and filtered through filter paper. The insoluble portion was reextracted sequentially with 10 volumes each of 70% ethanol, chloroform-methanol (2:1 [vol/vol], 1:1 [vol/vol], and 1:2 [vol/vol], and chloroform-methanol-water (30:60:8 [vol/vol/vol]) at 50°C for 1 h and filtered. The filtrates (ethanol, chloroform-methanol, and chloroform-methanol-water extracts) were combined and dried by rotary evaporation. The dried lipid extracts were washed with acetone followed by diethyl ether to remove neutral lipids and glycerophospholipids. Crude sphingolipids, which were insoluble in acetone followed by diethyl ether, were dissolved in a minimal amount of chloroform-methanol (1:1, vol/vol) and then incubated at room temperature for 3 h to cleave the ester-containing lipids after adjusting the pH of the solution to 12 with 1 N sodium methylate. The solution was neutralized with 1 N acetic acid in methanol and dialyzed against distilled water. The dialysate was concentrated by evaporation to dryness, and the residue was dissolved in a minimal amount of chloroform-methanol-water (30:60:8, vol/vol/vol) and applied to a DEAE-Sphadex A-25 column (acetate form) by the method of Ledeen et al. (9) to separate neutral GSLs and gangliosides. The neutral GSLs were eluted with chloroform-methanol-water (30:60:8, vol/vol/vol) and evaporated to dryness in vacuo.

The amount of lipid-bound hexose in the neutral GSL fraction was determined by the orcinol method (6). A neutral GSL solution containing 50 µl of serum extract was applied on high-performance thin-layer, chromatography (HPTLC) plate and developed with chloroform-methanol-water (65:35:8, vol/vol/vol). Neutral GSLs were visualized by spraying the plate with 0.2% orcinol in 2 N H₂SO₄, followed by heating in an oven at 120°C for 5 to 10 min. The spots were scanned for quantitation with a dual-wavelength TLC densitometer (CS-900; Shimadzu, Kyoto, Japan) at a wavelength of 520 nm. The amounts of individual neutral GSLs were calculated from the amount of lipid-bound hexose in the neutral GSL fraction on

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TABLE 1. Summary of clinical data

Group and patient no.	Age (yr)	Sex ^a	Clinical symptoms ^b	Antibody response to lipopolysaccharide antigen ^c
A				
1	69	F	HUS	0.45
2	75	F	HUS	0.29
3	6	F	HUS	0.60
4	6	F	HUS	2.30
5	7	M	HUS	0.30
6	7	F	HUS	1.20
7	12	F	HUS	NT ^d
8	6	M	HUS	0.60
9	6	F	HUS	1.20
10	6	F	HUS	1.40
11	6	F	HUS	2.70
12	7	M	HUS	NT
B				
13	20	F	WD	1.12
14	22	F	WD	0.24
15	20	F	WD	NT
16	20	F	WD	0.23
17	18	F	WD	0.83
18	20	F	BD	0.25
19	7	F	BD	NT
20	53	M	BD	NT
21	55	M	BD	NT
22	63	F	BD	NT
23	21	F	BD	0.75
C				
24	19	F	Normal	0.09
25	19	F	Normal	0.81
26	20	F	Normal	0.09
27	19	F	Normal	0.08
28	21	F	Normal	0.08
29	21	F	Normal	0.48
30	20	F	Normal	0.63
31	20	F	Normal	0.41
32	20	F	Normal	0.47
33	20	F	Normal	0.07
34	4	F	Normal	NT
35	8	M	Normal	NT

^a F, female; M, male.^b WD, watery diarrhea; BD, bloody diarrhea.^c Antibody activity was detected by enzyme-linked immunosorbent assay as absorbance at 492 nm using a 1/200 dilution of serum.^d NT, not tested.

the basis of the peak area ratios obtained from densitometric scanning of the HPTLC plate.

Student's *t* test was performed for statistical evaluation. Results are expressed as the arithmetic mean with the standard error of the mean.

Figure 1 shows an HPTLC profile of the neutral GSLs from sera of patients 1, 13, and 24 from groups A, B, and C, respectively. The neutral GSLs in the sera from patients 13 (group B) and 24 (group C) were composed of GlcCer, LacCer, Gb3, and Gb4. In patient 1 of group A, GlcCer, LacCer, and Gb3 were shown to be the major constituents of the neutral GSLs in the serum. Visually, however, Gb3 of patient 1 was a minor component compared with that in patients 13 and 24.

In order to clarify the relationship between susceptibility to HUS and the Gb3 content in the serum, the neutral GSL components in the sera from the patients in groups A, B, and C were quantitatively analyzed. The amounts of neutral

FIG. 1. TLC of neutral GSLs in sera from patients infected with *E. coli* O-157:H7. Lane 1, standard neutral GSLs GlcCer, LacCer, Gb3, and Gb4; lane 2, neutral GSLs from serum of patient 1 (group A); lane 3, neutral GSLs from serum of patient 13 (group B); lane 4, neutral GSLs from serum of patient 24 (group C). The bands marked with arrows were stained brown with orcinol spray.

GSL components in the sera from each group are shown in Table 2.

The concentrations of GlcCer, LacCer, Gb3, and Gb4 in group A were distinct from those in the other groups. The content of GlcCer in group A was suggestively lower than that found in group B ($P < 0.071$) and significantly lower than that in group C ($P < 0.002$). The LacCer content was lower in group A than in group B ($P < 0.0086$) or group C ($P < 0.0003$). Similarly, the amount of Gb3 in group A was also less than that in group C ($P < 0.0003$) and suggestively low relative to that in group B ($P < 0.068$). Gb3 is synthesized from LacCer. Thus, the low level of Gb3 in group A can reflect a lower LacCer content. Moreover, the content of Gb4 in group A was lower

TABLE 2. Contents of neutral GSLs in sera from patients infected with *E. coli* O-157:H7

Group ^a	No. of patients	Mean lipid-bound hexose $\pm$ SE (μ g/ml)				
		Total	GlcCer	LacCer	Gb3	Gb4
A	12	75.8 $\pm$ 7.2	10.4 $\pm$ 1.1	26.2 $\pm$ 2.9	23.0 $\pm$ 3.1 ^b	16.1 $\pm$ 1.6
B	11	124.4 $\pm$ 12.2	22.6 $\pm$ 7.3	43.5 $\pm$ 5.4	31.1 $\pm$ 2.8	31.3 $\pm$ 5.1
C	12	157.1 $\pm$ 15.2	21.5 $\pm$ 3.4	53.7 $\pm$ 5.8	46.0 $\pm$ 4.4	43.1 $\pm$ 5.7
D	6	105.8 $\pm$ 21.9	10.1 $\pm$ 2.9	26.4 $\pm$ 6.2	18.0 $\pm$ 4.8	51.4 $\pm$ 16.1

^a Group A, HUS patients in the acute phase; group B, patients who had VTEC-associated diarrhea without development of HUS; group C, VTEC-infected patients who had no obvious gastrointestinal symptoms; group D, HUS patients from group A in the convalescent phase.

^b $P < 0.0003$ between group A and group C; $P < 0.068$ between group A and group B.

than that found in group B ($P < 0.0076$) or group C ($P < 0.0002$). This is consistent with the decreased level of Gb3, which is the precursor of Gb4. The total amount of lipid-bound hexose was significantly lower in group A than in either group B ($P < 0.0022$) or group C ($P < 0.0001$). Neutral GSL components in the serum samples from group D were also analyzed and compared with those in group A. The amounts of GlcCer and LacCer in group D were similar to those in group A. The level of Gb3 in group D was slightly lower than that in group A, whereas the Gb4 content was higher in group D than in group A ($P < 0.007$). Gb4 is synthesized from Gb3 by the enzyme β -*N*-acetylgalactosaminyl transferase (13). Thus, the differences in serum Gb4 levels between group A and group D might be explained by differences in expression of this enzyme between the HUS patients in the acute phase and those in the convalescent phase. Higher *N*-acetylgalactosaminyl transferase activities in group D may relate to the increased Gb4 content. The total amount of lipid-bound hexose was slightly higher in group D than in group A.

In this study, the compositions of neutral GSLs in groups A, B, and C were quantitatively different. The most important point is that the level of Gb3 in the sera was significantly lower in group A than in group C ($P < 0.0003$) and was suggestively low relative to that in group B ($P < 0.068$). This suggests that there may be an association between the heterogeneity of Gb3 contents in the sera and outcome of VT-associated HUS. During VTEC infection, Gb3 in the serum should bind to circulating VTs and may reduce the amount of VTs binding to the target cells. Therefore, patients with lower serum Gb3 levels would show a higher incidence of HUS following VTEC infection. However, it is also possible that these differences in neutral GSLs in serum could reflect the change of neutral GSL composition in the serum associated with HUS. If this was so, the decreased neutral GSLs in group A would be expected to increase on recovery from the illness. The experimental results indicated that although the Gb4 level was higher in group D than in group A, the contents of GlcCer, LacCer, and Gb3 did not vary significantly between group A and group D, suggesting

that the lower neutral GSL contents are not a consequence of VTEC infection. Thus, quantitative differences in the neutral GSLs of groups A, B, and C may be due to innate differences in the metabolism of GSLs (4, 5).

It is suggested that individuals with a lower content of serum Gb3 may have a higher incidence of HUS following VTEC infection.

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