Dose-Dependent Enzyme Suppression in Spleen Induced by GM1 (Monosialoganglioside 1) Administration to Mice

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Our previous studies suggested that the administration of exogenous gangliosides to the body modulates enzymatic networks in the brain. In the present study, we tested whether that is the case with another organ, spleen. By testing the dose response relationship, we found that there is a optimum dose for the effect of enzymatic modulation of GM1 (monosialoganglioside 1) administration. Although the optimum level varied depending on each of the examined hydrolytic enzymes, it usually fell in the range around 50 μg/kg body weight. The findings led us to conclude that the enzyme-modulating actions of gangliosides come not merely from the bizarre actions *in vivo* of high molecular exogenous substances.

Keywords ganglioside; murine spleen; GM1 administration; protease; hydrolytic enzyme; enzymatic modulation

Introduction

Gangliosides are located in the outer membrane surface of neurons[1,2] and seem to be involved in a variety of cell surface events including cell-cell recognition.[3] Exogenous gangliosides have been shown to enhance neurite formation of cultured neuronal cells *in vitro*[4] as well as *in vivo* after injury to both the peripheral and central nervous system.[3,5-7] Biochemical, morphological, and functional recorvery has been found to be enhanced by exogenously administered gangliosides after central nervous system lesions.[8,9]

Prompted by these reports, we previously tested the effects of administration of exogenous gangliosides, finding that it caused sugar moiety-specific enzymatic changes in brain.[10] Furthermore, we found that the administration of glycosidase inhibitors modulates intracerebral glycosidase activities in the brain.[11]

In the present study, we attempted confirm the enzyme-modulating actions of gangliosides in another organ, spleen. The results clearly demonstrated the existence of the dose–response relationship.

Materials and Methods

Experimental Animals Nine-week-old male mice (SPF-Balb/cAnNCrj, body weight 24—29 g) were obtained from Charles River Japan Inc., Atsugi, Kanagawa, Japan. Twenty four hours after the final injection the mice were anesthetized with ethyl ether. After the blood was drawn by cardiocenthesis, various organs including spleen were taken out. The spleen homogenates were prepared in phosphate-buffered saline (PBS) using a tissue homogenizer, Ultraturrax, at the maximum speed for 1 min. The homogenate was centrifuged (3000 *g* for 20 min) and the supernatant fluid withdrawn for measurement of enzyme activity.

Gangliosides Monosialoganglioside 1 (GM1, bovine brain) and asialo-GM1 were purchased from Bachem Feinchemikalien AG, Budendorf, Switzerland. These samples were of high purity based on the thin-layer chromatographic analysis.

TABLE I. List of the Proteases Measured and Their Substrates

Enzyme	Abbreviation	Substrate	Reference for assay method
Aspartate aminopeptidase (EC 3.4.11.7)	AP-A	Glu·NA	12
Arginine aminopeptidase (EC 3.4.11.6)	AP-B	Arg·NA	12
Proline iminopeptidase (EC 3.4.11.5)	Pro-IP	Pro·NA	13
Dipeptidyl peptidase I (EC 3.4.14.1)	DPP-I	Gly–Arg·NA	14
Dipeptidyl peptidase II (EC 3.4.14.2)	DPP-II	Lys–Ala·NA	15
Dipeptidyl peptidase III (EC 3.4.14.4)	DPP-III	Arg–Arg·NA	16
Dipeptidyl peptidase IV (EC 3.4.14.5)	DPP-IV	Gly–Pro·NA	17
Prolyl endopeptidase (EC 3.4.21.26)	PEP	Z–Gly–Pro·NA	13
Cathepsin B (EC 3.4.22.1)	Cathepsin B	Z–Arg–Arg·NA	18
Tissue kallikrein (EC 3.4.21.35)	Kallikrein	Pro–Phe–Arg·MCA	19
Trypsin (EC 3.4.21.4)	Trypsin	Boc–Gln–Ala–Arg·MCA	20
Neuraminidase (EC 3.2.1.18)	SD	4MU–NANA	21
α-D-Glucosidase (EC 3.2.1.20)	Glc-ase	NP–Glc	12
β-D-Galactosidase (EC 3.2.1.23)	Gal-ase	NP–Gal	12
α-D-Mannosidase (EC 3.2.1.24)	Man-ase	NP–Man	12
α-L-Fucosidase (EC 3.2.1.51)	Fuc-ase	NP–Fuc	22
β-*N*-Acetyl-D-glucosaminidase (EC 3.2.1.30)	GlcNAc-ase	NP–GlcNAc	12

Abbreviations used: Glu·NA, L-glutamic acid β-naphthylamide hydrochloride; Arg·NA, L-arginine β-naphthylamide hydrochloride; Pro·NA, L-proline β-naphthylamide hydrochloride; Gly–Arg·NA, glycyl-L-arginine β-naphthylamide; Lys–Ala·NA, L-lysyl-L-alanine β-naphthylamide; Arg–Arg·NA, L-arginyl-L-arginine β-naphthylamide; Gly–Pro·NA, glycyl-L-proline β-naphthylamide; Z–Gly–Pro·NA, benzyloxycarbonylglycyl-L-proline β-naphthylamide; Z–Arg–Arg·NA, benzyloxycarbonylarginyl-L-arginine β-naphthylamide; Pro–Phe–Arg·MCA, L-prolyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide; Boc–Gln–Ala–Arg·MCA, *tert*-butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide; 4MU–NANA, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; NP–Glc, *p*-nitrophenyl-α-D-glucopyranoside; NP–Gal, *p*-nitrophenyl-β-D-galactopyranoside; NP–Man, *p*-nitrophenyl-α-D-mannopyranoside; NP–Fuc, *p*-nitrophenyl-α-L-fucopyranoside, NP–GlcNAc, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

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GM1 in amounts of 1, 10, 50, 200 and 1000 μg/kg (mouse) and asialo-GM1 in an amount of 200 μg/kg (mouse), all of which were dissolved in 0.2 ml of PBS, were given daily intraperitoneally for 20 d. Control animals were given 0.2 ml PBS for 20 d. Five mice were killed at a time.

Substrates for Enzyme Assay The sources of substrates were as follows (see Table I for abbreviations): Glu·NA, Arg·NA, Pro·NA, Gly-Arg·NA, Lys-Ala·NA, Arg-Arg·NA, and Gly-Pro·NA from Bachem Feinchemikalien AG, Budendorf, Switzerland; Pro-Phe-Arg·MCA and Boc-Gln-Ala-Arg·MCA from Peptide Institute, Inc., Osaka, Japan; 4MU-NANA from Nakarai Chemicals, Ltd., Kyoto, Japan; NP-Glc, NP-Gal, NP-Man, NP-Fuc, and NP-GlcNAc from Sigma Chemical Company, St. Louis, U.S.A. Z-Gly-Pro·NA and Z-Arg-Arg·NA were synthesized in our laboratory.

Determination of Enzyme Activities The supernatant fluids of homogenates were dispensed into microwell plates (nunclon, F96) for aminopeptidases and glycosidases, and into test tubes (1.5 × 10 cm) for endopeptidases, to which the respective substrates were added, followed by incubation for 1 h at 37 °C. For the aminopeptidase (AP) assay, the absorbance at 525 nm was determined by microplate reader model 3550 (Bio-Rad).[12-18] For the endopeptidase assay, the fluorescence with excitation at 380 nm and emission at 460 nm was determined by a Hitachi MDF-4 fluorometer.[19,20] For the sialidase assay, sialidase activity was determined by measuring the fluorescence intensity of released 4MU with a JASCO 820-FP fluorescence spectrophotometer (Ex 370 nm, Em 445 nm) using 4MU as a standard.[21] For the glycosidase assay, the absorbance at 400 nm was determined by a microplate reader.[12,22] The references for assay methods and substrates used are listed in Table I. All the enzyme assays in the supernatant fluids of homogenates were linear with time and enzyme concentration. All the enzyme assays were done in triplicate, and their standard deviations were within 10% of the average values.[12] For the assays, the units of enzyme activities were expressed as nmols of reaction products generated during one minute of incubation per mg protein (nmol/min/mg protein). Protein was determined by the method of Lowry *et al.*[23]

Factor analysis was chosen as the method of multivariate analysis.[24]

Results

Table II shows the effects of GM1 administration in the dose range of 1 to 1000 μg/kg body weight. At the dose of 1 μg/kg, the activities of AP-A, DPP-I, cathepsin B, trypsin, and Glc-ase in spleen significantly decreased when compared to controls. At the dose of 10 μg/kg, the activities of AP-A, AP-B, DPP-I, cathepsin B, and Glc-ase significantly decreased. At the dose of 50 μg/kg, the activities of AP-B, DPP-I, DPP-III, DPP-IV, cathepsin B, and Gal-ase significantly decreased. At the dose of 200 μg/kg, the activities of AP-B, DPP-I, cathepsin B, Glc-ase and Gal-ase significantly decreased. At the dose of 1000 μg/kg, the activities of AP-B, cathepsin B, and Glc-ase, significantly decreased. The table also included the data obtained from the animals treated with the asialo-GM1 (200 μg/kg). The activities of AP-A and DPP-I significantly decreased.

The dose response relationship for each enzyme is summarized in Figs. 1 and 2. As shown in Fig. 1, the degree of inhibition was maximal at the dose of 50 μg/kg for AP-B and SD, whereas at 10 μg/kg for PEP and at 200 μg/kg for Gal-ase. As shown in Fig. 2, the degree of inhibition was maximal at the dose of 50 μg/kg for DPP-I

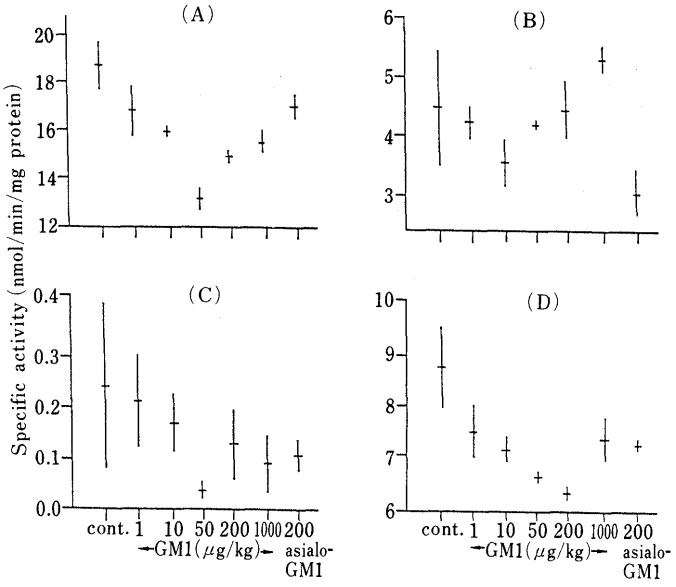

Fig. 1. The Activity Changes of AP-B (A), PEP (B), SD (C), and Gal-ase (D) Caused by the Administration of Various Doses of GM1 and Asialo-GM1

TABLE II. Enzymatic Changes in Spleen Caused by Ganglioside Administration

Enzyme	Specific activity ± S.E. (nmol/min/mg protein) $n=5$						
	Control	Ganglioside GM-1 (μg/kg)					Asialo-GM1
		1	10	50	200	1000	200
AP-A	0.42 ±0.03	0.31 ±0.03[a]	0.34 ±0.01[a]	0.33 ±0.03	0.35 ±0.02	0.41 ±0.03	0.32 ±0.02[a]
AP-B	18.9 ±0.94	16.94 ±0.99	16.1 ±0.27[a]	13.16 ±0.46[b]	15.02 ±0.32[b]	15.56 ±0.48[a]	17.12 ±0.44
Pro-IP	1.02 ±0.08	0.83 ±0.06	0.85 ±0.01	0.81 ±0.01	0.92 ±0.06	0.96 ±0.05	0.81 ±0.02
DPP-I	22.17 ±1.70	16.70 ±1.14[a]	15.63 ±0.36[b]	10.34 ±1.07[c]	13.52 ±1.11[b]	17.42 ±1.05	17.59 ±0.34[a]
DPP-II	9.54 ±1.23	7.95 ±0.47	7.57 ±0.74	7.83 ±0.40	8.15 ±0.80	10.91 ±1.35	7.22 ±0.29
DPP-III	4.90 ±0.37	4.30 ±0.15	4.23 ±0.15	3.66 ±0.19[a]	4.19 ±0.18	4.99 ±0.54	4.34 ±0.19
DPP-IV	2.41 ±0.18	2.02 ±0.12	2.03 ±0.08	1.91 ±0.08[a]	1.84 ±0.15	2.14 ±0.14	2.05 ±0.08
PEP	4.52 ±0.87	4.28 ±0.27	3.61 ±0.39	4.24 ±0.06	4.49 ±0.43	5.34 ±0.23	3.11 ±0.35
Cathepsin B	53.9 ±2.71	31.09 ±2.43[c]	38.14 ±2.21[b]	34.16 ±2.11[c]	32.55 ±1.44[c]	34.62 ±2.19[b]	45.51 ±2.25
Kallikrein	0.055±0.005	0.045±0.002	0.050±0.003	0.043±0.001	0.045±0.001	0.056±0.01	0.052±0.001
Trypsin	0.18 ±0.002	0.15 ±0.01[b]	0.17 ±0.01	0.15 ±0.01	0.16 ±0.01	0.17 ±0.02	0.20 ±0.01
SD	0.23 ±0.14	0.201±0.081	0.159±0.053	0.033±0.017	0.12 ±0.06	0.082±0.052	0.101±0.031
Glc-ase	0.87 ±0.06	0.69 ±0.03[a]	0.68 ±0.04[a]	0.71 ±0.01	0.68 ±0.04[a]	0.69 ±0.01[a]	0.76 ±0.01
Gal-ase	8.67 ±0.69	7.46 ±0.48	7.14 ±0.25	6.64 ±0.11[a]	6.31 ±0.11[a]	7.35 ±0.41	7.22 ±0.13
Man-ase	1.34 ±0.17	1.11 ±0.08	1.23 ±0.06	0.95 ±0.05	1.23 ±0.13	1.19 ±0.11	1.24 ±0.06
Fuc-ase	0.32 ±0.05	0.23 ±0.02	0.24 ±0.01	0.23 ±0.01	0.22 ±0.01	0.22 ±0.02	0.24 ±0.01
GlcNAc-ase	67.82 ±3.55	60.04 ±2.12	67.66 ±1.17	63.84 ±1.73	61.82 ±2.75	63.99 ±1.38	67.84 ±3.41

a) $p<0.05$, b) $p<0.01$, c) $p<0.001$ (according to Student's t-test), S.E. = standard error of mean.

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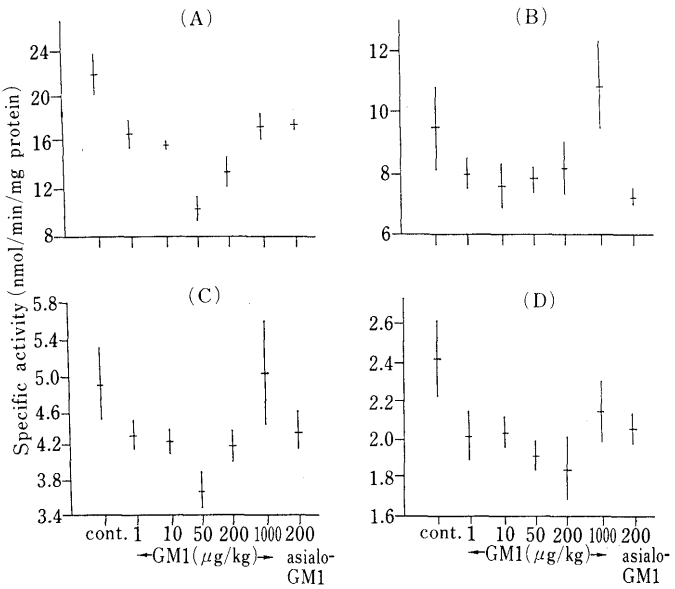

Fig. 2. The Activity Changes of DPP-I (A), DPP-II (B), DPP-III (C), and DPP-IV (D) Caused by the Administration of Various Doses of GM1 and Asialo-GM1

and DPP-III, whereas at 10 μg/kg for DPP-II and at 200 μg/kg for DPP-IV.

Discussion

The present study showed the dose dependent enzymatic suppression in spleen by GM1. One of the reasons we chose this organ as the object of the study was the possible relationship of GM1 to immunological phenomena in the body. Kasai *et al.*[25] and Young *et al.*[26] reported that asialo-GM1 is a marker of natural killer cells. Later, this substance was also found to exist in other immune competent cells, such as macrophages and bone-marrow cells.[27,28] Although the level of maximal effects was around 50 μg/kg, it varied depending on the enzyme tested. Judging from the fact that the effects did not simply increase with the dose it is unlikely that the present findings were due to toxic effects of the exogenously administered GM1.

We presume that GM1 did not act directly on enzymes but acted indirectly by modifying the cell function in general. It is to be noted that asialo-GM1 was less effective than GM1 in the present study. This may suggest that the presence of sialic acid at the terminal of gangliosides plays an important role at least in its enzyme modulating action, although this may not be generally applicable to the physiologic actions of gangliosides.

In normal people, the gangliosides are continually synthesized and degraded. In Tay-Sachs disease, however, plasma membranes of the brain cells accumulate ganglioside GM2 because of the lack of β-*N*-hexosaminidase A, a key enzyme in the normal turnover of the ganglioside. Although the excess GM2 is believed to be responsible for all the symptoms of the disease,[29] there have been no investigations except ours[10] reporting the relation of this factor to metabolic abnormality.

These result indicating the relationship between the amount of ganglioside in the body and enzyme actions may not only offer some clues to the pathologic physiology of the ganglioside accumulation diseases, but also may suggest the significance of gangliosides in the physiological regulation of the metabolism in the body.

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