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Immunostimulatory Activities of Mono- or Diglycosylated α-Galactosylceramides

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Abstract—We examined the effects of 2″- or 3″-monoglycosylated α-galactosylceramides (α-GalCers) and 2″,3″-diglycosylated α-GalCers on allogeneic MLR and the proliferation of murine spleen cells. It was found that their ceramide portions greatly affect their immunostimulatory activities, and that the 3″-hydroxyl group plays a more important role in the immunostimulatory effects of α-GalCer derivatives than the 2″-hydroxyl group. © 1997 Elsevier Science Ltd.

Introduction

We previously examined the immunostimulatory and antitumor activities of various kinds of α- and β-D-monopyranosylceramides (α- and β-D-MonoCers) having different sugar moieties and the same ceramide portions, and found that the α-anomeric configuration of D-MonoCers plays an essential role in the manifestation of their activities[1] and that α-galactosylceramide (α-GalCer) has the strongest potencies among α-D-MonoCers with the same ceramide portion.[1] Based on this finding, we studied the structure–antitumor activity relationship of α-GalCers with different ceramide structures, and selected an α-GalCer named KRN7000 as a candidate for clinical application.[2] Further investigation demonstrated that KRN7000 shows marked antitumor activities through a unique immunostimulatory mechanism.[3,4] Thus, α-GalCers are interesting glycolipids.

In the course of our screening for novel antitumor agents from Okinawan marine sponge, we isolated three kinds of 2″-monoglycosylated α-GalCers (α-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→1)-ceramide) which have been reported by Cafieri et al.,[5] three kinds of 3″-monoglycosylated α-GalCers (β-D-glucofuranosyl-(1→3)-α-D-galactopyranosyl-(1→1)-ceramide) which have been isolated by Cafieri et al.,[6] and two kinds of 2″,3″-diglycosylated α-GalCers (α-D-*N*-acethylgalactosaminyl-(1→3)-[α-D-glucopyranosyl-(1→2)]-α-D-galactopyranosyl-(1→1)-ceramide) which were reported by Costantino et al.[7] Costantino et al. have reported immunostimulatory effects of 2″- or 3″-monoglycosylated α-GalCers and 2″,3″-diglycosylated α-GalCers, but they evaluated their effects using mixtures with different long-chain base (LCB) moieties and fatty acid (FA) moieties.[8] Since we succeeded in obtaining eight kinds of pure α-GalCer derivatives as mentioned above, we examined their individual immunostimulatory effects.

In this paper, we describe the isolation of 2″- or 3″-monoglycosylated α-GalCers and 2″,3″-diglycosylated α-GalCers and their effects on allogeneic mixed leukocyte reaction (MLR), and the proliferation of murine spleen cells. In addition, we present different effects of 2″-monoglycosylated α-GalCers on the proliferation of spleen cells and lymph node cells.

Chemicals

Isolation and identification of mono- or diglycosylated α-GalCers

The sponges *Agelas axisera* (for 719-1, -3, and -7), *Stylissa frabeliformis* (for STL-4 and -8), and an unidentified sponge (for S1140B-1, -5 and -9) were collected in Okinawa, Japan. Mono- or diglycosylated α-GalCers were isolated essentially according to our previous methods for isolating α-GalCers from the sponge *Agelas maiuritianus*.[9] Briefly, each sample was lyophilized to give a dry powder which was extracted with 1:1 chloroform:methanol followed by hot methanol. All of the extracts were concentrated in vacuo, and the residues were then distributed between organic solvent and water. The organic layers were dried over anhydrous sodium sulfate and evaporated in vacuo. The residues were partitioned between 10% aqueous methanol and *n*-hexane. Alcoholic portions were evaporated in vacuo and repeatedly chromatographed on silica gel to give biologically active fractions. These fractions were further purified by reversed-phase HPLC using an ODS resin column eluting with 0–3% aqueous methanol.

The purified glycosphingolipids were acetylated using acetic anhydride and pyridine, and their NMR spectra

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measured. The representative ^{1}H and ^{13}C NMR data of saccharide regions of acetates are summarized in Table 1. The structures of each glycosphingolipid were identified by comparing ^{1}H and ^{13}C NMR spectral data with reported data,[5-7] with the exception of the areas around terminal methyl regions of LCBs and FAs. By comparing the FDMS spectral data of the methyl esters of the FAs and the tetraacetates of the obtained LCBs from each compound (measured by a previous method) with the authentic data,[10] we elucidated each carbon chain length of the FAs and LCBs. The chemical structures of α-GalCer derivatives which were used in this study are shown in Figure 1.

Results and Discussion

We first examined the effects of three kinds of 2″-monoglycosylated α-GalCers (S1140B-1, S1140B-5, and S1140B-9) on allogeneic MLR. As shown in Table 2, all 2″-monoglycosylated α-GalCers concentration-dependently stimulated the MLR response. The immunostimulatory potencies of S1140B-5 and S1140B-9 were nearly equal, but S1140B-1, having an FA which is only two carbon atoms less than those of S1140B-5 and S1140B-9, had a much lower potency than S1140B-5 and S1140B-9. To confirm this marked difference among them, we evaluated the effects of the above 2″-monoglycosylated α-GalCers on the proliferation of spleen cells. As shown in Table 3, S1140B-1 showed much weaker spleen cell proliferation stimulatory activity than S1140B-5 and S1140B-9. These results strongly suggest that the carbon chain lengths of FAs in the ceramide moiety greatly affect the immunostimulatory effect of 2″-monoglycosylated α-GalCers. In addition, the stimulatory effect of S1140B-9 on spleen cell proliferation was stronger than that of S1140B-5 (Table 3). The result is believed to indicate that the carbon chain lengths of LCBs, as well as FAs, greatly affect the immunostimulatory effects of 2″-glycosylated α-GalCers, i.e. the longer carbon chains in the ceramide portion show the stronger immunostimulatory effects.

We then examined allogeneic MLR stimulatory effects of 719-1, 719-3, and 719-7 (3″-monoglycosylated α-GalCers) which have the same ceramide portions as those of S1140B-1, S1140B-5, and S1140B-9, respectively. As Table 2 shows, these 3″-monoglycosylated α-GalCers also stimulated the MLR response in a concentration-dependent manner, and 719-7 and 719-1 showed the strongest and the weakest effect, respectively. These compounds also stimulated the proliferation of spleen cells, and their potencies were parallel to those on allogeneic MLR (Table 3). These results strongly suggest that the longer FA and LCB chains in the ceramide portion induce the stronger immunostimulatory effects in 3″-monoglycosylated α-GalCers as well as 2″-monoglycosylated α-GalCers.

Table 1. ^{1}H and ^{13}C NMR spectral data of the saccharide region

Compd	719 series		STL series		S1140B series	
Position	δH (mult., J [Hz])	δC	δH (mult., J [Hz])	δC	δH (mult., J [Hz])	δC
1″	4.91 (d, 3.7)	97.0	4.97 (d, 3.4)	97.6	4.87 (d, 3.4)	98.0
2″	5.03 (dd, 3.7, 10.5)	69.9	4.03 (dd, 3.4, 10.7)	69.6	3.88 (dd, 3.4, 10.6)	73.9
3″	4.05	72.1	4.28	66.4	5.29 (dd, 3.4, 10.7)	70.5
4″	5.41	69.8	5.52 (broad d)	65.5	5.41 (broad d, 3.4)	68.7
5″	4.06	67.2	4.20	67.8	4.19 (broad t, 6.8)	67.3
6″ a	4.01 (dd,5.1, 9.0)	62.1	4.07 (dd, 6.0, 10.7)	61.0	4.11	61.6
b	4.09		4.14		4.11	
1‴	5.23 (s)	107.2	5.26 (d, 3.4)	92.1	5.17 (d, 3.4)	96.9
2‴	4.91 (broad s)	81.2	4.87 (dd, 3.4, 10.0)	71.4	5.12	74.1
3‴	4.96 (broad d, 5.4)	76.6	5.29 (t, 10.0)	69.3	5.31 (dd, 3.4, 9.0)	61.7
4‴	4.40 (dd, 3.2, 5.4)	80.7	5.08 (t, 10.0)	69.3	5.45 (broad d, 3.2)	68.7
5‴	5.41	69.3	4.27	69.0	4.40 (broad t, 6.6)	67.3
6‴a	4.22 (dd, 7.1, 11.2)	62.4	4.32	63.2	4.00 (dd, 6.8, 11.2)	61.6
b	4.35 (dd, 5.1, 11.2)			4.10		
1⁗			5.19 (d, 3.7)	94.1		
2⁗			4.70 (dt,3.7, 11.0)	47.3		
2⁗NH			5.98 (d, 9.8)			
3⁗			5.14 (dd, 3.7, 11.0)	68.0		
4⁗			5.62 (d,3.7)	67.6		
5⁗			4.48 (t, 5.6)	66.9		
6⁗a			4.21	62.1		
b			4.27			

δ (ppm), in $CDCl_3$, ^{1}H NMR: 500 MHz/^{13}C NMR: 125 MHz, 26 °C.

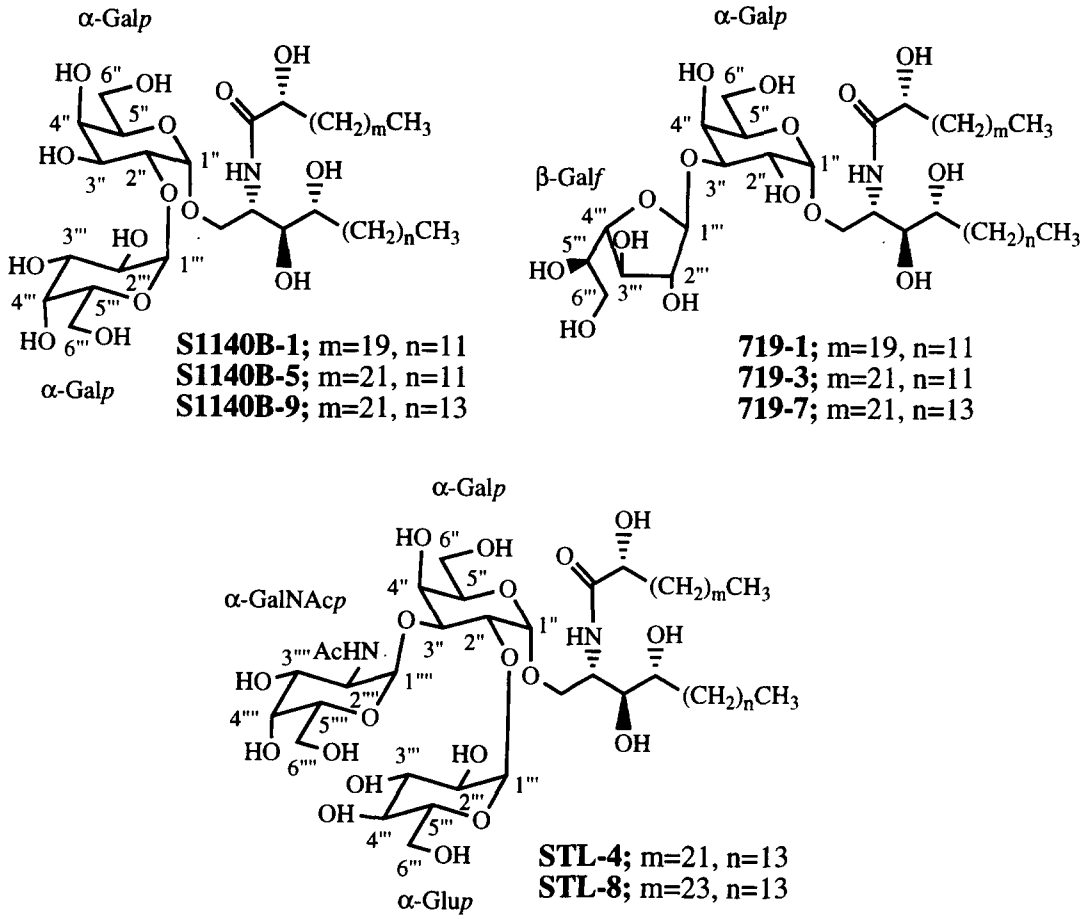

Figure 1. Structures of α-galactosylceramide derivatives.

Furthermore, we examined the immunostimulatory effects of 2″,3″-diglycosylated α-GalCers (STL-4 and STL-8). Both compounds significantly stimulated the allogeneic MLR response and the proliferation of spleen cells, and the potency of STL-4 was weaker than that of STL-8 (Tables 2 and 3). These results are considered to suggest that the carbon chain lengths in the ceramide moiety also greatly affect the immunostimulatory effects of 2″,3″-diglycosylated α-GalCers.

When we compare the immunostimulatory potencies between 2″-monoglycosylated α-GalCers and 3″-monoglycosylated α-GalCers which have the same ceramide portions, it appears that the potencies of 2″-monoglycosylated α-GalCers are stronger than those of 3″-monoglycosylated α-GalCers. This result is considered to suggest that the free 3″-hydroxyl group plays a more important role in the immunostimulatory effects of α-GalCer derivatives than the 2″-hydroxyl group. In

Table 2. Effects of α-galactosylceramide derivatives on allogeneic MLR

	^{3}H-TdR incorporation (cpm, mean±SD)		
Sample	**1 (ng/mL)**	**10 (ng/mL)**	**100 (ng/mL)**
Vehicle	3335±241	3713±609	3506±217
S1140B-1	3887±309	5445±1043	7157±833[b]
S1140B-5	10,183±712[c]	16,083±1839[c]	15,935±535[c]
S1140B-9	11,036±1455[c]	16,100±381[c]	16,866±363[c]
719-1	4242±257[a]	5504±692[a]	5409±340[b]
719-3	4231±514	5699±199[b]	7895±634[c]
719-7	7547±899[b]	10,848±502[c]	10,110±2182[b]
STL-4	5062±265[b]	5339±491[a]	6895±637[c]
STL-8	5661±1060[a]	6660±663[b]	8638±498[c]

The same volume (1×10^5 cells/50 μL/well) of responder cells (spleen cells from BALB/c mice) and stimulator cells (MMC-treated spleen cells from C57BL/6 mice) were placed in a 96-well plate in triplicate and cultured with 1, 10, and 100 ng/mL of α-GalCer derivatives at 37 °C in 95% air, 5% CO_2 for two days. ^{3}H-TdR were then pulsed for an additional 7 h. ^{3}H-TdR uptake into cells was counted using a liquid scintillation counter. Statistical analysis was performed by a two-sided unpaired Student's *t*-test. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$ (compared with vehicle-treated group).

Table 3. Effects of α-galactosylceramide derivatives on the proliferation of spleen cells

Sample	^{3}H-TdR incorporation (cpm, mean±SD)		
	1 (ng/mL)	10 (ng/mL)	100 (ng/mL)
Vehicle	3134±183	3000±372	2589±143
S1140B-1	2884±308	3892±670	9778±360[c]
S1140B-5	12,802±961[c]	28,214±1235[c]	32,354±198[c]
S1140B-9	17,406±1959[c]	36,153±1578[c]	36,952±2179[c]
719-1	2804±191	3519±228	8010±1079[c]
719-3	3079±336	3979±332[a]	10,692±142[c]
719-7	6900±1094[b]	17,794±544[c]	17,797±1245[c]
STL-4	3578±966	4147±851	7942±662[c]
STL-8	5391±389[c]	7265±419[c]	12,941±2042[c]

Spleen cells (2.5×10^5 cells/100 μL/well) from BALB/c mice were placed in a 96-well plate in triplicate. Other procedures were performed according to the methods described in Table 2. Statistical analysis was performed by a two-sided unpaired Student's *t*-test. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$ (compared with vehicle-treated group).

addition, STL-4 has the same ceramide structure as S1140B-9 and 719-7, but the immunostimulatory potency of STL-4 was much weaker than that of S1140B-9 and 719-7. This result suggests that increased sugar binding to α-GalCers causes the depression of immunostimulatory activities of α-GalCer derivatives.

Recently, Constantino et al. examined the immunostimulatory effects of 2″- or 3″-monoglycosylated α-GalCer mixtures[5,6] and 2″,3″-diglycosylated α-GalCer mixtures[7] on the proliferation of lymph node cells in vitro, and suggested that the free 2″-hydroxyl group plays an essential role in the immunostimulatory effects of monoglycosylated α-GalCers based on the result that only 2″-monoglycosylated α-GalCers did not show stimulatory effects.[8] Since Constantino et al.'s 2″-monoglycosylated α-GalCer mixture contains S1140B-5, S1140B-9, and other derivatives having a longer FA than that of S1140B-9 which showed or will show strong immunostimulatory effects in our system using spleen cells, their suggestion seems to be contrary to our present results. As a consequence of these contrary results, it was considered that 2″-monoglycosylated α-GalCers show different effects on spleen cells and on lymph node cells. We therefore examined the effects of 2″-monoglycosylated α-GalCers on spleen cells and on lymph node cells. As Table 4 shows, these compounds markedly stimulated the proliferation of spleen cells as same as the result shown in Table 3. In contrast, S1140B-1 even at the concentration of 100 ng/mL did not stimulate the proliferation of lymph node cells. Furthermore, S1140B-5 and S1140B-9 at the concentration of 100 ng/mL significantly augmented the proliferation of lymph node cells, but their potencies were surprisingly much weaker than those on the proliferation of spleen cells. It is of interest that the immunostimulatory effects of 2″-monoglycosylated α-GalCers on spleen cells and on lymph node cells were quite different. The result seems to suggest that spleen cells are a better source for the investigation of the structure–immunostimulatory activity relationships of α-GalCer derivatives.

In summary, the following suggestions were obtained in this study using murine spleen cells: (1) the 3″-hydroxyl group of α-GalCers plays a more important role in the immunostimulatory effects of α-GalCer derivatives than the 2″-hydroxyl group; (2) carbon chain lengths of

Table 4. Effects of 2″-monoglycosylated α-galactosylceramides on the proliferation of spleen cells and lymph node cells

Sample	^{3}H-TdR incorporation (cpm, mean±SD)			
	Spleen cells		Lymph node cells	
	10 (ng/mL)	100 (ng/mL)	10 (ng/mL)	100 (ng/mL)
Vehicle	2712±681	2581±809	2365±108	2201±311
S1140B-1	5472±722[a]	19894±1481[b]	2486±229	3005±408
S1140B-5	45590±2029[b]	48282±6815[b]	2844±424	3498±722[a]
S1140B-9	50975±5305[b]	49201±6650[b]	3865±328[b]	6399±1339[a]

Spleen cells (2.5×10^5 cells/100 μL/well) or lymph node cells (2.5×10^5 cells/100 μL/well) prepared from BALB/c mice were plated in 96-well plate in triplicate and cultured with 2″-monoglycosylated α-GalCers (1, 10, and 100 ng/mL) at 37 °C in 95% air, 5% CO_2 for three days. Other procedures were performed according to the methods described in Table 2. Statistical analysis was performed by a two-sided unpaired Student's *t*-test. [a]$p < 0.01$; [b]$p < 0.001$ (compared with vehicle-treated group).

LCBs and FAs in the ceramide portion greatly affect the immunostimulatory effects of α-GalCer derivatives such as 2″- or 3″-monoglycosylated α-GalCers, and 2″, 3″-diglycosylated α-GalCers.

Experimental

Chemical methods

Preparation of S1140Bs. The unidentified sponge was collected at the depth of 15–25 m in Kume Shima, Okinawa, Japan. A sample was lyophilized to give a dry powder (447.1 g) which was extracted with chloroform–methanol (1:1). The extract was then concentrated in vacuo to give 51.28 g of residue. The residue was distributed between ethyl acetate and water. The upper and middle layers were dried over anhydrous sodium sulfate, then evaporated in vacuo to give 18.37 g and 9.44 g of residue, respectively. The upper residue was partitioned between 10% aqueous methanol and *n*-hexane. The alcoholic portion was evaporated in vacuo. The solids obtained from the middle layer and alcoholic portion were combined and applied to silica gel columns (Wako Gel C-200), and eluted with step gradients of chloroform:methanol:water (9:1:0.1→8.5:1.5:0.15→8:2:0.2) repeatedly to give an active fraction (169.9 mg). This fraction was further purified by reversed-phase HPLC using an ODS-AM column (YMC Ltd 250 × 20 mm ID) eluting with methanol at the flow rate of 9 mL/min to afford S1140B-1 (3.0 mg, retention time 14.1 min), S1140B-5 (15.2 mg, 21.6 min) and S1140B-9 (10.2 mg, 30.3 min).

Preparation of 719s. The sponge *Agelas axisera* was collected at the depth of 15–25 m in Kume Shima, Okinawa, Japan. A sample was lyophilized to give a dry powder (950.9 g) which was extracted with chloroform:methanol (1:1). The extract was then concentrated in vacuo to give 232.25 g of residue. The residue was distributed between ethyl acetate and water. The upper and middle layers were dried over anhydrous sodium sulfate, then evaporated in vacuo to give 65.03 g and 27.75 g of residue, respectively. The upper residue was partitioned between 10% aqueous methanol and *n*-hexane. The alcoholic portion was evaporated in vacuo. The solids obtained from the middle layer and alcoholic portion were combined and applied to silica gel columns (Wako Gel C-200) and eluted with step gradients of chloroform:methanol:water (9:1:0.1→8.5:1.5:0.15→8:2:0.2→7:3:0.3) repeatedly to give an active fraction (2.76 g). This fraction was further purified by reversed-phase HPLC using a D-ODS-5 column (YMC Co., Ltd 250 × 20 mm ID) eluting with methanol at the flow rate of 10 mL/min to afford 719-1 (30.3 mg, retention time 21.6 min), 719-3 (245.7 mg, 30.3 min), and 719-7 (55.0 mg, 41.4 min).

Preparation of STLs. The sponge *Stylissa frabeliformis* was collected at the depth of 15–25 m in Miyako Shima, Okinawa, Japan. A sample was lyophilized to give a dry powder (420.3 g) which was extracted with chloroform:methanol (1:1). The extract was then concentrated in vacuo to give 59.1 g of residue. The residue was distributed between chloroform and water. The lower layer was dried over anhydrous sodium sulfate and the upper aqueous layer was extracted with 1-butanol. The chloroform and 1-butanol layers were combined and concentrated in vacuo. The solid (23.25 g) obtained above was applied to silica gel columns (Wako Gel C-200) and eluted with step gradients of chloroform:methanol:water (9:1:0.1→8:2:0.2) repeatedly to give a crude solid (1.39 g). This crude solid was chromatographed on a Toyopearl HW-40 column (chloroform:methanol 1:1) to give an active fraction (1.16 g). This fraction was further purified by reversed-phase HPLC using a CAPCELL PAK C18 column (Shiseido Co., Ltd 250 × 15 mm ID) eluting with 3% aq methanol at the flow rate of 5 mL/min to afford STL-4 (73.1 mg, retention time 48.7 min) and STL-8 (11.6 mg, 67.5 min).

Acetylation of purified glycosphingolipids. Purified glycosphingolipids were dissolved in pyridine and allowed to react with acetic anhydride (more than 20 equiv) (ca. 20 h at room temparature). The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was concentrated and chromatographed on silica gel.

Methanolysis of purified glycosphingolipids and acetylation of obtained LCB. Each compound was treated with 0.9N HCl in 82% aq methanol (1–3 mL) for 18 h at 80 °C. The reaction mixture was extracted with *n*-hexane and the hexane layer was concentrated and chromatographed on silica gel (*n*-hexane:ethyl acetate 7:3) to give a methyl ester of FA. The aq methanol layer was neutralized and chromatographed using Amberlite CG-400 column eluting with methanol to give an LCB and 1-*O*-methylated sugar units. The LCB fraction was dried and heated with acetic anhydride:pyridine (1:1) for 1.5 h at 70 °C. The reaction mixture was diluted with water and extracted with ethyl acetate. The residue of the ethyl acetate layer was chromatographed on silica gel (*n*-hexane:ethyl acetate 3:2) to give a peracetate of each LCB. Structures of FA methyl ester and LCB peracetate were identified by comparing the authentic data.[10] The results are summarized in Table 5.

Table 5

Compd	FA ester	LCB acetate
S1140B-1	C22	C16
S1140B-5	C24	C16
S1140B-9	C24	C18
719-1	C22	C16
719-3	C24	C16
719-7	C24	C18
STL-4	C24	C18
STL-8	C26	C18

FA ester C22: Methyl-2-(*R*)-hydroxydocosanoate.
C24: Methyl-2-(*R*)-hydroxytetracosanoate.
C26: Methyl-2-(*R*)-hydroxyhexacosanoate.
LCB acetate C16: 2-Acetoamino-1,3,4-triacetoxyhexadecane.
C18: 2-Acetoamino-1,3,4-triacetoxyoctadecane.

Biological methods

Animals. Female C57BL/6 or BALB/c mice, five to 10 weeks old, purchased from Nippon SLC Co., Ltd. were used in this study. Mice were maintained under our standard laboratory conditions.

Preparation of spleen cells. The mice were killed, and the spleens were resected. The spleens were dissociated in 10% fetal calf serum (FCS, Gibco) RPMI 1640 (Gibco) medium, and RBC were lysed with Tris–NH_4Cl. The cells were washed three times using phosphate buffer saline (Nissui Pharmaceutical, Ltd), and viable cells were counted and resuspended in 10% FCS RPMI 1640 medium.

Spleen cell-proliferation assay. Spleen cells (2.5×10^5 cells/100 μL/well) suspended in 10% FCS RPMI 1640 medium and 1, 10, and 100 ng/mL of α-GalCer derivatives (10 μL/well) were placed in a 96-well round-bottomed plate (Nunclon) and cultured at 37 °C in 95% air, 5% CO_2. Two days (Table 3) or three days (Table 4) later, 0.5 μCi/well of tritium–thymidine (^{3}H-TdR, Du Pont/NEN Research Products) were added into each well and the plate was incubated for an additional 7 h. ^{3}H-TdR uptake into cells was measured using a liquid scintillation counter.

Allogeneic MRL assay. Spleen cells obtained from BALB/c mice were used as responder cells, and MMC-treated (50 μg/mL, 30 min) spleen cells from C57BL/6 mice were used as stimulator cells in an allogeneic MLR assay. The same volume (1×10^5 cells/50 μL/mL) of responder cells and stimulator cells suspended in 10% FCS RPMI 1640 medium with the above samples was placed in a 96-well plate and the plate was cultured at 37 °C in 95% air, 5% CO_2. Two days later, 0.5 μCi/well of ^{3}H-TdR were added into each well and the plate was incubated for an additional 7 h. ^{3}H-TdR uptake into cells was measured using a liquid scintillation counter.

Lymph node cell-proliferation assay. Lymph node cells (2.5×10^5 cells/100 μL/well) suspended in 10% FCS RPMI 1640 medium and 1, 10, and 100 ng/mL of 2″-monoglycosylated α-GalCers (10 μL/well) were placed in a 96-well round-bottomed plate (Nunclon) and cultured at 37 °C in 95% air, 5% CO_2. Three days later, 0.5 μCi/well of ^{3}H-TdR were added into each well and the plate was incubated for an additional 7 h. ^{3}H-TdR uptake into cells was measured using a liquid scintillation counter.

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