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Structure–Activity Relationship and Conformational Analysis of Monoglycosylceramides on the Syngeneic Mixed Leukocyte Reaction

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Abstract—We examined effects of α-, β-galactosylceramides (CalCers) and α-, β-glucosylceramides (GlcCers) on the syngeneic mixed leukocyte reaction (MLR) using spleen cells (responder cells) and dendritic cells (DC, stimulator cells). The DC pretreated with these α-monoglycosylceramides markedly stimulated the proliferation of spleen cells, in contrast to the little stimulatory effects produced by the DC pretreated with the corresponding β-anomers. In addition, when we compared the effects of α-GalCer derivatives on the syngeneic MLR, it appeared that the 2″- and 3-hydroxyl groups in α-GalCers play a critical role in their stimulation of the MLR response. Based on these results, we performed a computer-aided molecular modeling study, and found that the orientations of the 2″-, 4″- and 3-hydroxyl groups common to α-GalCer and α-GlcCer are not accessible to those of inactive monoglycosyleeramides such as β-GalCer. These results suggest that there might be a receptor-like site for α-monoglycosylceramides on the cells which are involved in the MLR response.

Introduction

Monoglycosylceramides such as galactosylceramides (GalCers) are important surface molecules found in virtually all cells, and glycosphingolipids related to GalCer and their metabolites play important roles in promoting the regulation of nerve cells,[1] and in regulating protein kinase C activity.[2] Thus GalCers are considered to be important components for various organisms. Several kinds of β-GalCers (galactose binds to ceramide in a β-configuration) have been isolated from marine organisms[3,4] and organ tissues,[5,6] although any marked biological activities of β-GalCers have not been reported.

By contrast, we previously reported that α-GalCers (galactose binds to ceramide in an α-configuration) have significant immunostimulatory activities, including the stimulation of murine spleen cell proliferation, and antitumor effects.[7,8] It was also found that α-glucosylceramides (α-GlcCers) as well as α-GalCers markedly stimulate proliferative response of spleen cells, but their corresponding β-anomers have little stimulatory effects.[9] Furthermore, we found that an α-GalCer, **KRN7000**, activates the immune system via the enhancement of the antigen-presenting cell (APC) function of dendritic cells (DC),[10] which play a critical role as professional APCs in the primary immune response.[11] These results led us to hypothesize that the different effects of α- and β-monoglycosylceramides on the proliferation of spleen cell originate in their different activities on APC function of DC. In order to test this hypothesis, we studied the structure-activity relationship of synthetic monoglycosylceramide on syngeneic mixed leukocyte reaction (MLR) using spleen cells (responder cells) and DC pretreated with these monoglycosylceramides (stimulator cells).

The compilation of the structure–activity relationship data enabled us to speculate that a receptor-like site

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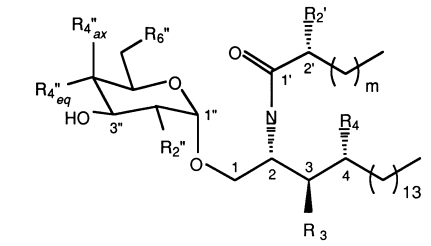
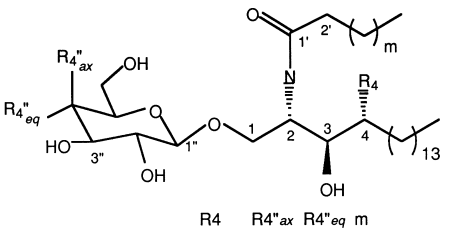
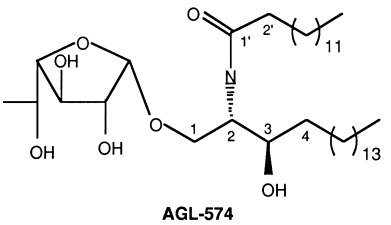


	R3	R4	R2'	R2"	R4"*ax*	R4"*eq*	R6"	m
KRN7000	OH	OH	H	OH	OH	H	OH	23
AGL-517	OH	H	H	OH	OH	H	OH	11
AGL-563	OH	H	H	OH	H	OH	OH	11
AGL-571	OH	H	H	OH	OH	H	H	11
AGL-575	OH	H	H	H	OH	H	OH	11
AGL-512	OH	OH	OH	OH	OH	H	OH	21
AGL-525	OH	OH	H	OH	OH	H	OH	21
AGL-506	OH	H	OH	OH	OH	H	OH	21
AGL-514	OH	H	H	OH	OH	H	OH	21
AGL-535	H	H	H	OH	OH	H	OH	21



	R4	R4"*ax*	R4"*eq*	m
AGL-583	OH	OH	H	23
AGL-564	H	OH	H	11
AGL-562	H	H	OH	11



AGL-574

Figure 1. Structures of monoglycosylceramides used in this study.

might exist on the cells, which were involved in the MLR response, and that these cells recognize the molecular property of α- and β-monoglycosylceramide through the receptor-like site. In order to examine the possibility, we carried out the conformational analysis of these monoglycosylceramides using a computer-aided molecular modeling system.

Chemistry

The monoglycosylceramides used in the present study were synthesized in our laboratory,[7,12,13] and are classified into the following three groups.

1. α-Glycopyranosylceramides; **KRN7000**,[7] **AGL-517**,[7] **AGL-563**,[13] **AGL-571**,[12] **AGL-575**,[12] **AGL-512**,[7] **AGL-525**,[7] **AGL-506**,[7] **AGL-514**,[7] and **AGL-535**.[7]
2. β-Glycopryranosyleeramides; **AGL-583**,[13] **AGL-564**,[13] and **AGL-562**.[13]
3. α-Glycofuranosylceramide; **AGL-574**[12]

The chemical structures of these monoglycosylceramides are shown in Figure 1.

Results and Discussion

We previously reported that an α-GalCer, **KRN7000**, markedly stimulates the proliferation of spleen cells, but its β-anomer, **AGL-583** (β-GalCer), did not augment the response.[9] Considering our previous finding that **KRN7000** enhances the antigen-presenting cell function of DC,[10] we speculated that the difference between **KRN7000** and **AGL-583** on the proliferative response of spleen cells originates in their different enhancing effects on the APC function of DC. To test this speculation, we carried out the syngeneic MLR assay using spleen (responder) cells and DC pretreated with **KRN7000** or **AGL-583** as stimulator cells. As Figure 2 shows, although the **KRN7000**-pretreated DC markedly stimulated the proliferation of spleen cells in a manner dependent on the number of DC, the DC pretreated

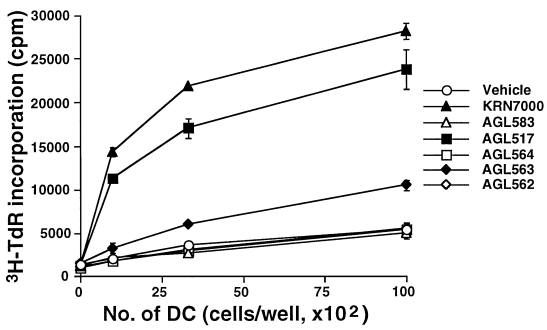


Figure 2. Effects of GalCers and GlcCers on the syngeneic MLR-DC pretreated with vehicle or monoglycosylceramides (100 ng/mlL were applied in graded amounts to syngeneic spleen cells (2.5×10^5 cells/well) in 96-well flat-bottomed plates, and the plates were then cultured at 37 °C in 95% air, 5% CO_2 for 3 days. The proliferation of spleen cells was measured by the 8h-^{3}H-TdR pulse method. Each value shows the mean with SD.

with **AGL-583** did not enhance the MLR response, in comparison with the vehicle-pretreated DC, even in the presence of 10^4 cells. To further study the relationship among α- and β-monoglycosylceramides, we examined effects of **AGL-517** (α-GalCer), **AGL-564** (β-GalCer), **AGL-563** (α-GlcCer) and **AGL-562** (β-GlcCer), all of which have the same ceramide moiety, on the syngeneic MLR system. As shown in Figure 2, the **AGL-517**- and **AGL-563**-pretreated DC markedly stimulated the proliferation of spleen cells in a manner dependent on the number of DC, but the MLR stimulatory activity of **AGL-563** was significantly weaker than that of **AGL-517**. By contrast, the DC pretreated with **AGL-564** or **AGL-562** did not stimulate the proliferation of spleen cells. These results are consistent with our speculation mentioned above. These results also demonstrated that the α-configuration of the glycosidic linkage between sugar (galactose or glucose) and ceramide plays an important role in the enhancement of APC function of DC, and that the α-GalCer isomers in the 4″-position can activate DC.

In order to test the role of hydroxyl group(s) of ceramide portion in the enhancement of the APC function of DC by α-GalCers, we then evaluated the effects of five kinds of α-GalCers, **AGL-512** (3,4,2′-OH), **AGL525** (3,4-OH), **AGL-506** (3,2′-OH), **AGL-514** (3-OH), and **AGL-535** (no hydroxyl groups in ceramide portion), which have the same length of fatty acid chain and long chain base, on the syngeneic MLR. As shown in Figure 3(A), the DC pretreated with **AGL-512**, **AGL-525**, **AGL-506** or **AGL-514** significantly stimulated the proliferation of spleen cells, but the DC pretreated with **AGL-535** did not augment the syngeneic MLR response. This result demonstrated that the 3-hydroxyl group in the ceramide moiety plays an essential role in the enhancement of APC function by α-GalCers.

Furthermore, the structural requirement for sugar moiety on the syngeneic MLR assay was examined by employing a series of **AGL-517** analogues; **AGL-571** (6″-des-OH), **AGL-575** (2″-des-OH) or **AGL-574** (the furanose form). As Figure 3(B) shows, although the DC pretreated with **AGL-571** had the similar capability to stimulate the proliferation of spleen cells to that of the **AGL-517**-pretreated DC, the DC pretreated with **AGL-575** or **AGL-574** did not enhance the MLR response. This result demonstrated that the existence of the 2″-hydroxyl group and the pyranose form of sugar moiety is essential in the enhancement of the APC function of DC by α-GalCers.

As mentioned above, the enhancement of the APC function of DC induced by monoglycosylceramides was so restricted depending on their structural property, that we speculated that a receptor-like site for monoglycosylceramides might exist on the cells such as DC, which were involved in the syngeneic MLR, and that these cells might recognize the molecular shape of monoglycosylceramides through a receptor-like site. We therefore carried out a conformational analysis in order to examine whether common orientations of the above-mentioned hydroxyl groups (O3, O″2 and O″4) and the sugar rings exist among active monoglycosylceramides such as α-GalCer and α-GleCer.

Molecular models of **AGL-517** (α-GalCer) and **AGL-563** (α-GlcCer) were built using standard bond length and angles. The sterically allowed conformations of **AGL-517** were generated systematically varying the torsion angles of the six rotatable bonds as indicated in Figure 4. Angle increment of 10° were applied to the bonds no. 1–4 and 30° to no. 5 and 6. The long alkyl chains (C5-C18 and C2′-C14′) were substituted by methyl groups. The rigid body approximation was used with a set of van der Waals radii.[14] The atomic coordinates of the 2″- and 4″-oxygens were recorded as vectors (O2″-O4″) for all possible conformers, with the position of C2, C3, O3 and C4 fixed as a frame of reference. The oxygen atoms, O3 and O2″, and O″4 were selected as

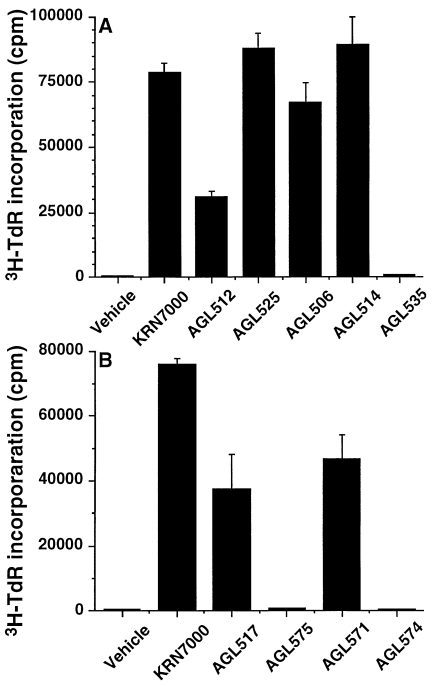


Figure 3. Effects of α-GalCer derivatives on syngeneic MLR. DC (1×10^4 cells/well) pretreated with vehicle or α-GalCer derivatives (100 ng/mL) were applied to syngeneic spleen cells (2.5×10^5 cells/well) in 96-well flat-bottomed plates, and the plates were then cultured at 37 °C in 95% air, 5% CO_2 for 3 days. The proliferation of spleen cells was measured by the 8h-^{3}H-TdR pulse method. Each value shows the mean with SD.

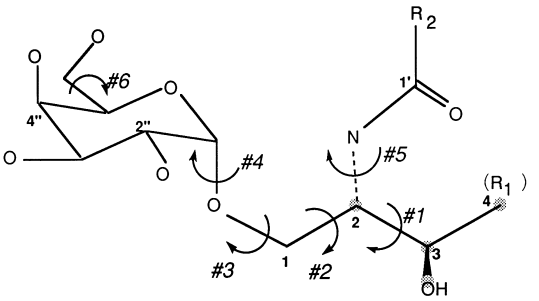


Figure 4. Molecular model of **AGL-517**. The fixed atoms (C2, C3, O3 and C4) are hashed. For the conformational analysis calculation, the long chain base (R_1) and the fatty acid chain (R_2) were replaced by methyl groups. $R_1=(CH_2)_{12}CH_3$, $R_2=(CH_2)_{13}CH_3$.

essential atoms and as a reference atom of sugar ring orientation, respectively, because the 2″- and 3-hydroxyl groups were shown necessary for the immunostimulatory effects (Fig. 3), and both configurations of the 4″-hydroxyl group were accepted in the immunostimulatory activities (Fig. 2). Using the same orientation of the reference atoms in **AGL-563**, the O2″–O4″ vectors were recorded. The vectors were then compared to determine which vectors were common to **AGL-517** and **AGL-563**. A vector was assumed to correspond if both O2″ and O4″ atoms were within 0.4 Å of the coordinates of another vector. The same operations were applied to the inactive analogues such as **AGL-562** (β-GlcCer) and **AGL-564** (β-GalCer), in order to examine whether they can access the common topology between **AGL-517** and **AGL-563**.

Table 1. The orientation of O2″ and O4″ common in **AGL-517** and **AGL-563**. The coordinates of the reference atoms were also given at the bottom. The coordinates are of orthogonal system in angstromes

	O2″			O4″		
Orientation	x	y	z	x	y	z
No. 1	2.57	2.76	5.75	2.26	−1.36	7.69
No. 2	3.00	3.46	4.89	−0.49	2.14	7.51
No. 3	0.30	3.04	5.92	4.01	0.50	6.68
No. 4	1.30	5.16	1.68	1.14	6.18	−2.75
No. 5	4.05	2.86	4.56	3.02	7.17	3.52
No. 6	3.21	3.00	−2.18	7.73	2.44	−2.07
No. 7	6.26	0.91	−0.27	7.02	5.22	1.01

Fixed atoms	x	y	z
O3	0.00	0.00	0.00
C3	1.44	0.00	0.00
C4	1.97	−1.46	0.00
C2	1.93	0.77	1.25

This computer-aided molecular modeling demonstrated that seven orientations were common in α-GalCer and α-GlcCer (Table 1). Figure 5 shows an example of such conformation of **AGL-517** and **AGL-563** which have a

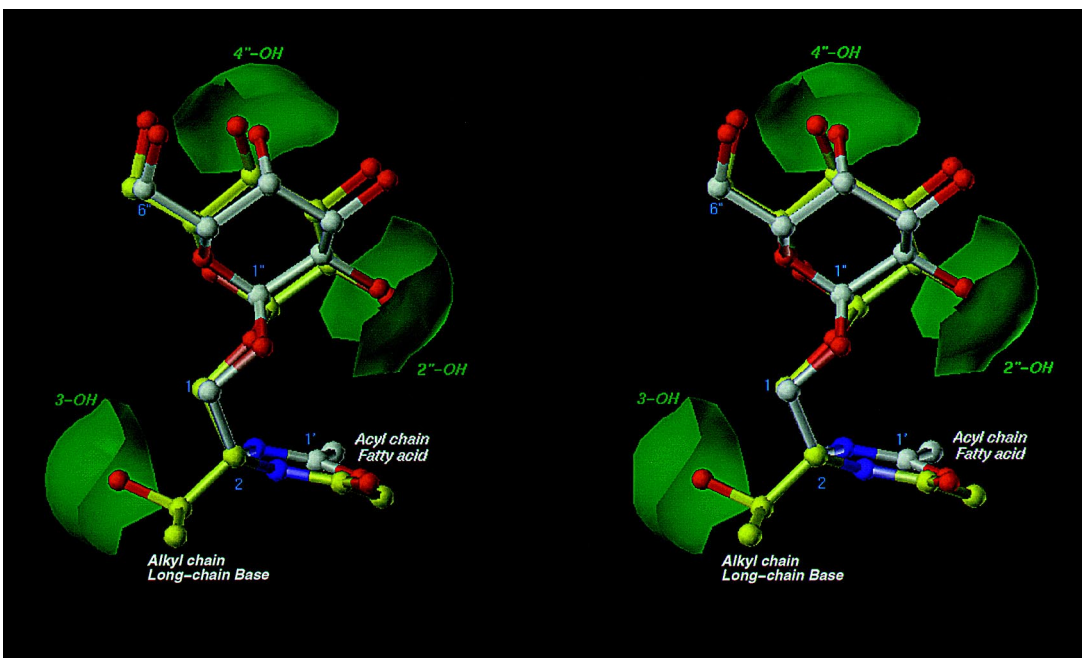


Figure 5. A stereoview representation of common orientation of **AGL-517** (white carbons) and **AGL-563** (yellow carbons). The orientation shown in this figure corresponds to the orientation no. 2 in Table 1. The green spheres indicate the position of the oxygen atoms (O3, O″2 and O″4) used as reference atoms to extract the common orientations.

common orientation (no. 2 in Table 1). Not only the 2″-, 4″- and 3-hydroxyl groups, but also the 3″-hydroxyl group, and the pyranose moiety overlaps well in terms of atomic positions and molecular volume. The orientations were compatible with other active monoglycosylceramides such as **AGL-571** (6″-des-OH **AGL-517**). By contrast, the orientations were not accessible to the inactive monoglycosylceramides such as **AGL-562** (β-GlcCer), **AGL-564** (β-GalCer), **AGL-575** (2″-des-OH **AGL-517**), or **AGL-574** (the furanose form). This molecular modeling study supports the involvement of a receptor-like mechanism that the cells, which were involved in the syngeneic MLR, recognize the molecular shape and structural property of monoglycosylceramides through a receptor-like site.

Kawano et al. demonstrated that Vα14 natural killer T (Vα14 NKT) cells, a novel T cell lineage, and DC play an essential role in stimulation of the initial immune response, such as syngeneic MLR, induced by **KRN7000**, and that the Vα14 NKT cell proliferation by the DC pretreated with **KRN7000** was selectively inhibited by anti-CD1d and anti-T cell receptor (TCR) monoclonal antibodies.[15] Furthermore, they suggested that **KRN7000** may bind to CD1d molecule.[15] Take these findings into consideration, our present data seem to suggest that Vα14 NKT cells and DC play an important role in our syngeneic MLR response induced by monoglycosylceramide, and that, if so, a receptor-like site for monoglycosylceramides, which is presumed by the conformational analysis, may exist in CD1d molecule on DC and/or TCR molecule on Vα14 NKT cells. However, further study is necessary to identify the binding site for α-GalCers and α-GlcCers.

Experimental

Computer aided molecular modeling

The computer aided molecular modeling was carried out using SYBYL software package (Tripos Associate, St Louis, MO, USA).

Biological methods

Animals. Female BALB/c mice, 5–10 weeks old, purchased from Nippon SLC Co., Ltd. (Shizuoka, Japan) were used in this study. Mice were maintained under our standard laboratory conditions.

Preparation of spleen cells. Mice were sacrificed, and the spleens were resected. The spleens were dissociated in 10% fetal calf serum (FCS, Gibco, Grand Island, NY, USA) RPM1 1640 (Gibco) medium, and RBC were lysed with Tris–NH_4Cl. The cells were washed three times using phosphate buffer saline (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), and viable cells were counted and resuspended in 10% FCS RPM1 1640 medium.

Preparation of DC from spleens. DC from spleens were prepared according to the methods of Crowley et al.[16] Briefly, spleens were digested with collagenase type III (Worthington Biochemical Corp., Freehold, NJ, USA) and the remaining tissues were further disrupted on a metal screen. Cells were suspended in dense bovine serum albumin (Pentex Path-O-Cyte 4, Bayer Corp., Kankakee, IL, USA), and centrifuged to equilibrium, and the low-density population was applied to plastic culture dishes for 1.5 h. After nonadherent cells were dislodged, low-density adherent cells were cultured overnight in 10% FCS RPM1 1640 medium with vehicle and samples (100 ng/ml). The nonadherent cells were recovered, washed three times with medium, and used as DC.

Syngeneic MRL assay. DC (stimulator cells) were applied in graded amounts to syngeneic spleen cells (2.5×10^5 cells/well) in 96-well flat-bottomed plates, and the plates were then cultured at 37 °C in 95% air, 5% CO_2. Three days later, 0.5 μCi/well of tritium-thymidine (^{3}H-TdR, Du Pont/NEN Research Products, Boston, MA, USA) was added into each well, and the plates were incubated for an additional 8 h. ^{3}H-TdR uptake into cells was measured by a liquid scintillation counter.

References

1. Ledeen, R. W.; *J. Neurosci. Res.* **1984**, *12*, 147–159.
2. Hannun, Y. A.; Bell, R. M. *Science* **1989**, *243*, 500–507.
3. Higuchi, R.; Kagoshima, M.; Komori, T. *Liegigs Ann. Chem.* **1990**, 659–663.
4. Hirsch, S.; Kashman, Y. *Tetrahedron* **1989**, *45*, 3897–3906.
5. Svennerholm, L.; Bruce, A.; Mansson, J. E.; Rynmark, B. M.; Vanier, M. T. *Biochim. Biophys. Acta* **1972**, *280*, 626–636.
6. Karlsson, K. A.; Samuelsson, B. E.; Steen, G. O. *Biochim. Biophys. Acta* **1973**, *316*, 317–335.
7. Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Kobayashi, E.; Fukushima, H.; Koezuka, Y. *J. Med. Chem.* **1995**, *38*, 2176–2187.
8. Kobayashi, E.; Motoki, K.; Uchida, T.; Fukushima, H.; Koezuka, Y. *Oncology Res.* **1995**, *7*, 529–534.
9. Uchimura, A.; Shimizu, T.; Morita, M.; Ueno, H.; Motoki, K.; Fukushima, H.; Natori, T.; Koezuka, Y. *Bioorg. Med. Chem.* **1997**, *5*, 2245–2249.
10. Yamaguchi, Y.; Motoki, K.; Ueno, H.; Maeda, K.; Kobayashi, E.; Inoue, H.; Fukushima, H.; Koezuka, Y. *Oncology Res.* **1996**, *8*, 399–407.
11. Nussenzweig, M. C.; Steinman, R. M. *J. Exp. Med.* **1980**, *151*, 1196–1212.
12. Motoki, K.; Morita, M.; Kobayashi, E.; Uchida, T.; Akimoto, K.; Fukushima, H.; Koezuka, Y. *Biol. Pharm. Bull.* **1995**, *18*, 1487–1491.

13. Morita, M.; Natori, T.; Akimoto, K.; Osawa, T.; Fukushima, H.; Koezuka, Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 699–704.

14. Iijima, H.; Dunbar, J. B.; Marshall, G. R. *Proteins: Struct. Funct. Genet.* **1987**, *2*, 330–339.

15. Kawano, T.; Cui, J.; Koezuka, Y.; Toura, I.; Kaneko, Y.; Motoki, K.; Ueno, H.; Nakagawa, R.; Sato, H.; Kondo, E.; Koseki, H.; Taniguchi, M. *Science* **1997**, *278*, 1626–1629.

16. Crowley, M.; Inaba, K.; Witmer-Pack, M. D.; Steinman, R. M. *Cell Immunol.* **1989**, *118*, 108–125.