Chemistry & Biology, Vol. 12, 677–683, June, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.04.014

Synthetic α-Mannosyl Ceramide as a Potent Stimulant for an NKT Cell Repertoire Bearing the Invariant Vα19-Jα26 TCR α Chain

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Summary

A NKT cell repertoire is characterized by the expression of the Vα19-Jα26 invariant TCR α chain (Vα19 NKT cell). This repertoire, as well as a well-established Vα14-Jα281 invariant TCR α$^+$ NKT cell subset (Vα14 NKT cell), has been suggested to have important roles in the regulation of the immune system and, thus, is a major therapeutic target. Here, we attempted to find specific antigens for Vα19 NKT cells. Vα19 as well as Vα14 NKT cells exhibited reactivity to α-galactosyl ceramide (α-GalCer). Thus, a series of monoglycosyl ceramides with an axially oriented glycosidic linkage between the sugar and ceramide moiety were synthesized and their antigenicity to Vα19 NKT cells was determined by measuring their immune responses in culture with glycolipids. Comprehensive examinations revealed substantial antigenic activity for Vα19 NKT cells by α-mannosyl ceramide.

Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a member of the NKR-P1 gene family, and the TCR-CD3 complex [1, 2]. The major component of NKT cells bearing the invariant Vα14-Jα18 TCR α chain (Vα14 NKT cells) [3, 4] is positively selected by the nonpolymorphic MHC class-I-like CD1d molecule in association with β2-microglobulin (β2m) [5–9]. Vα14 NKT cells are shown to be reactive to certain glycosphingolipids (GSLs) such as α-galactosyl ceramide (α-GalCer) in the context of CD1d [10]. An important feature of NKT cells is their ability to secrete both proinflammatory (Th1) cytokines such as IFN-γ and antiinflammatory (Th2) cytokines such as IL-4 and IL-10 upon stimulation through the invariant TCR [11–14], suggesting a pivotal role in immunoregulatory functions including tumor immunity [15, 16].

Recently, we demonstrated the presence of a novel NKT cell repertoire (designated the Vα19 NKT cell) that was characterized by the expression of the Vα19-Jα26 (AV19-AJ33) invariant TCR α chain [17]. This invariant TCR was previously found in the peripheral blood of human, bovine, and mouse deficient in transporter associated with antigen-processing (Tap)-1 by PCR-based techniques [18]. Positive selection of the invariant Vα19-Jα26 TCR-bearing cells by the nonclassical MHC class I molecule MR1 has also been shown [19]. Similar to Vα14 NKT cells, the cells of this repertoire also produce large amounts of immunoregulatory cytokines in response to the engagement of the invariant TCR and are considered to have important roles in the regulation of the immune system (M. Shimamura et al., submitted). In addition, they are suggested to participate in the control of IgA production in intestine [19]. Thus, the search for specific antigens for Vα19NKT cells is of extreme importance in developing new therapies for immunoregulatory disorders utilizing the function of the repertoire.

GSLs are one of the principal components in mammalian cell membranes [20, 21]. Most glycosphingolipids share a common β-linked glucosyl or galactosyl ceramide as a skeletal structure. A discovery of α-GalCer from marine sponges [22], which is stimulus to the mammalian immune system by specifically activating Vα14 NKT cells, prompted us to investigate unnatural GSLs as agents for stimulating Vα19 NKT cells.

In the current study, we focused on GSLs related to α-GalCer as potential stimulants for Vα19 NKT cells because Vα19 as well as Vα14 NKT cells are suggested to recognize antigens presented by nonclassical MHC class I molecules, presumably MR1. Therefore, an efficient method of producing α-GalCer-related GSLs was addressed. We synthesized GSLs by coupling an activated monosaccharide with a sphingosine precursor whose amino functionality was masked with an azide group. The present method enabled us to efficiently obtain a series of naturally occurring and related GSLs. As a result of comprehensive examinations, immunological activities toward Vα19 NKT cells were found in α-mannosyl ceramide (ManCer).

Results and Discussion

Synthesis of GLSs

Several approaches have been reported for the synthesis of azidosphingosine, which is particularly useful as a glycosylation acceptor [23–28]. However, these methods are relatively time consuming and technically difficult. Here, a means of producing azidosphingosine with fewer steps and easier operations was established (Figure 1). Herold reported an efficient method of synthesizing sphingosine [29] starting with Garner's aldehyde **1** [30] as a precurser. Addition of 1-pentadecynyllithium to **1** afforded amino alcohol **2** in 72% yield as a mixture of diastereomers. The ratio of diastereomers was determined by ^{1}H NMR spectroscopy (51% yield for the anti product). Although these isomers could be separated at this point by repeating the chromatographic purification, it was easier to separate them after protection of the hydroxyl group with benzyl ether in the case of large-scale preparation. After the 3-OH group had been protected by the benzylation, a desired *anti* isomer of **2** was exposed to HCl-MeOH to give **3**.

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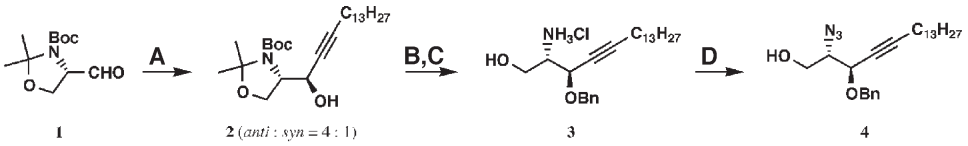


Figure 1. Synthesis of Azidosphingosine **4**

Reagents and conditions. (A) 1-pentadecyne, *n*-butyllithium, hexamethylphosphoramide (HMPA), THF, –78°C, 72%. (B) NaH, BnBr, DMF, 0°C to rt. (C) 5% HCl/MeOH, 0°C to rt. (D) TfN_3, NaOMe, DMAP, MeOH, room temperature, 81% yield from **2**.

The amino alcohol **3** was then treated with TfN_3 (0.4 M in CH_2Cl_2) [31, 32], NaOMe, and DMAP in MeOH at room temperature to afford compound **4** in 81% yield from *anti*-**2**. As a result, the sphingosine building block **4** was prepared in 4 steps starting from Garner's aldehyde **1** in an overall yield of 40%.

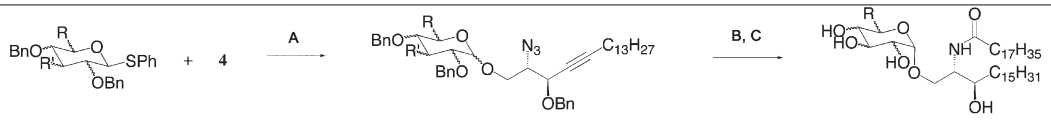
Next, the appropriate glycosyl donors to be coupled with **4** were considered. We decided to use 2-*O*-benzylated glycosyl donors for coupling a monosaccharides with a 2-OH group and 2-azido-sugars for coupling glucosamine or galactosamine to preferentially obtain α-glycosides while taking into account the anomeric effect. To select these protective groups was advantageous to the simple deprotection of the final products. The thioether was chosen as a selective protecting group for the anomeric center.

The glycosylation of neutral sugars (glucose, galactose, mannose, xylose, fucose, talose, and altrose) with **4** was carried out by using DMTST (Dimethyl(methylthio) sulfonium trifluoromethane sulfonate) (Table 1). The α and β glycosides were separated by chromatography with a silica-gel column eluted with n-hexane-ethyl acetate.

In the case of glucosyl and xylosyl donor, 6-*O*-acetyl-2, 3, 4-tri-*O*-benzyl thioglucoside **5** and 3-*O*-acetyl-2,4-*O*-dibenzyl thioxyloside **8** were selected because inseparable glycosylation products were obtained when a perbenzylated donor was used. The azide group in **12–18** was converted to a stearoyl amide group by hydrogenation with Lindlar's catalyst in the presence of stearic anhydride followed by hydrogenation with Perlmann's catalyst to afford the desired neutral-sugar-based GLSs (**19–25**) in good yield (Table 1). Although the altrosylceramide **25** obtained at this step was a mixture of anomers, the components were easily separable after peracetylation.

Glucuronic acid containing glycolipid was prepared from **12**α (Figure 2). After transformation of the azide in **12**α to a stearoyl amide group, Jones oxidation of the alcohol in **26** afforded carboxylic acid, which afforded **27** upon hydrogenation with Pearlman's catalyst in 69% yield from **12**α.

Table 1. Synthesis of Neutral Sugar-Based α-Glycosyl Ceramides



entry	substrate				products			isolated yield (%)		α-GLCs		yield (%)
			R	R'		R	R'	α	β		R	
1	Glc	**5**	CH_2OAc	OBn	**12**[a]	CH_2OH	OBn	77	19	**19**	CH_2OH	**84**
2	Gal	**6**	CH_2OBn	OBn	**13**	CH_2OBn	OBn	51	36	**20**	CH_2OH	**84**
3	Man	**7**	CH_2OBn	OBn	**14**	CH_2OBn	OBn	73	14	**21**	CH_2OH	**62**
4	Xyl	**8**	H	OAc	**15**[a]	H	OH	35	36	**22**	H	**55**
5	L-Fuc	**9**	CH_3	OBn	**16**	CH_3	OBn	52	17	**23**	CH_3	**99**
6	Tal	**10**	CH_2OBn	OBn	**17**	CH_2OBn	OBn	68	11	**24**	CH_2OH	**70**
7	Alt	**11**	CH_2OBn	OBn	**18**	CH_2OBn	OBn	76 (α : β = 1 : 2)[b, c]		**25**	CH_2OH	**70**[c]

Reagents and conditions. (A) DMTST, MS 4 Å, CH_2Cl_2, 0°C to room temperature. (B) H_2, Pd-$CaCO_3$, stearic anhydride, AcOEt, room temperature. (C) H_2, $Pd(OH)_2$, MeOH/AcOEt (2/1), room temperature.

[a]After deacetylation with NaOMe/MeOH.

[b]At this step, α and β anomers could not be separated. These anomers were separated after peracetylation of altrosylceramide. The ratio of anomers was determined by ^1H NMR of peracetylated altrosylceramide.

[c]Yield for a mixture of anomers.

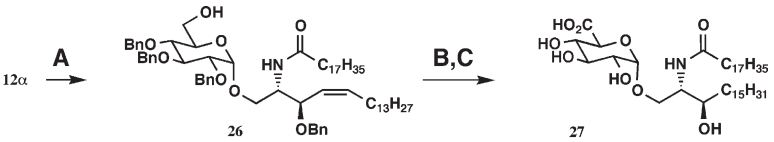


Figure 2. Synthesis of α-Glycosylceramide Containing Glucuronic Acid

Reagents and conditions. (A) H_2, Pd-$CaCO_3$, stearic anhydride, AcOEt, rt. (B) Jones reagent, acetone, 60°C. (C) H_2, $Pd(OH)_2$, MeOH:AcOEt=2:1, rt, 69% yield from **12α**.

For the sialylation of **4** (Figure 3), thiophenyl sialoside **28a** showed no reactivity to **4** with DMTST or NIS-TfOH as a promoter probably because of the bulkiness of the anomeric position being a quarternally carbon center and the weak nucleophilicity of the thiophenyl group. Hence, a less bulky and more nucleophilic ethyl thiosialoside was selected as a donor. Using NIS-TfOH but not DMTST for coupling, this donor gave the sialoside **29b** in a dramatically improved yield in a shorter reaction time. The glycosylation reaction was completed within 2 hr, judging from a TLC analysis (n-hexane-ethyl acetate), and the combined yield of α and β anomers was 84% (α/β = 2.3/1). The desired β-sialoside **29b**-β was converted to sialylceramide **32** in 3 steps with 82% yield (amide formation using hydrogenation with Lindlar's catalyst in the presence of stearic anhydride followed by hydrolysis of acetyl and methyl ester with lithium hydroxide and hydrogenation with Pearlman's catalyst).

Selective manipulation of 2-amino group in the sugar residue and the 2-amide group in the sphingosine moiety is required to synthesize glycosaminyl and galactosaminyl ceramide via the glycosylation of **4**. Because of the difficulty in controlling the reactivity of these two functional groups, we chose ceramide **33** as a glycosyl acceptor in this particular case. Glycosyl imidates were chosen as more reactive glycosyl donors in order to compensate for the weak nucleophilicity of the acceptor. As a result (Figure 4), a glycoside **35** was obtained in 48% yield as a mixture of anomers upon the reaction of **33** and **34** with TESOTf as a promoter. Each isomer was separated by silica-gel column chromatography. The coupling of **36** and **33** afforded **37** in 29% yield with $BF_3 \cdot Et_2O$ as a promoter. In this case, α-anomer was formed as the sole product. **35**α and **37** were hydrogenated with Pearlman's catalyst in the presence of 0.1 N aqueous HCl to afford **38** and **39** in a quantitative yield, respectively.

Immunological Activities of the Synthesized GSLs

Alpha-GalCer, a specific activator for Vα14 NKT cells, was tested for its potential to activate Vα19 NKT cells in a continuous search for specific antigens for this novel NKT cell repertoire. Total MNCs were prepared from livers of invariant Vα19-Jα26 transgenic $(Tg)^+$ $TCR\alpha^{-/-}$ as well as C57BL/6 and $\beta 2m^{-/-}$ mice for the determination of the biological activity of GSLs. They were used as responders without further fractionation because (1) only Vα19 Tg^+ cells are generated as TCR^+ cells in Vα19 Tg^+ TCR $\alpha^{-/-}$ mice, (2) the liver is the organ with the largest proportion of NKT cells among MNCs (Vα19 Tg^+ $NK1.1^+$ cells are about 30%), and (3) liver MNCs include populations of dendritic cells, macrophages, and B cells, which have the potential to function as antigen-presenting cells (APCs). The liver MNCs were cultured in the presence of α-GalCer, and the immune responses were monitored by measuring cytokine secretion in the culture fluid and cell proliferation (Figure 5A). Similar to C57BL/6 cells, Vα19 Tg^+ cells seemed to have some responsiveness to α-GalCer. In contrast, both C57BL/6 and Vα19 Tg^+ cells showed no detectable responsiveness to β-GalCer. Vα19 NKT cells are the sole component of the NKT cell population in Vα19 Tg^+ $TCR\alpha^{-/-}$ mice, whereas Vα14 NKT cells are the major subset in C57BL/6 mice. Therefore, these findings strongly suggest that Vα19 NKT cells as well as Vα14 NKT cells [10] recognize α-glycosylated ceramides.

To test this hypothesis, we synthesized a series of α-glycosyl ceramides with a naturally occurring monosaccharide residue and examined their antigenic activity (Figure 5A). Vα19 $Tg^+TCR\alpha^{-/-}$ but not C57BL/6 cells

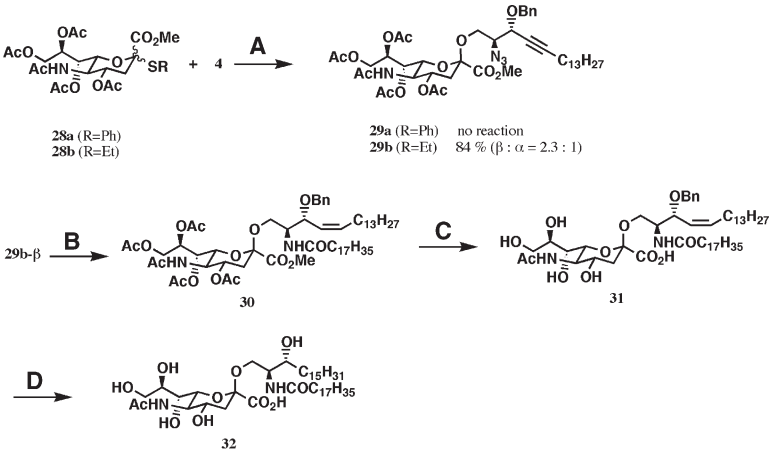


Figure 3. Synthesis of β-Sialyl Ceramide

Reagents and conditions. (A) NIS, TfOH, MS 4 Å, CH_2Cl_2:$(CH_2Cl)_2$=2:1, −40°C to −30°C. (B) H_2, Pd-$CaCO_3$, stearic, anhydride, AcOEt, rt. (C) LiOH, MeOH:H_2O=4:1, 0°C to rt. (D) H_2, $Pd(OH)_2$, MeOH, rt, 82% yield from **29b-β**.

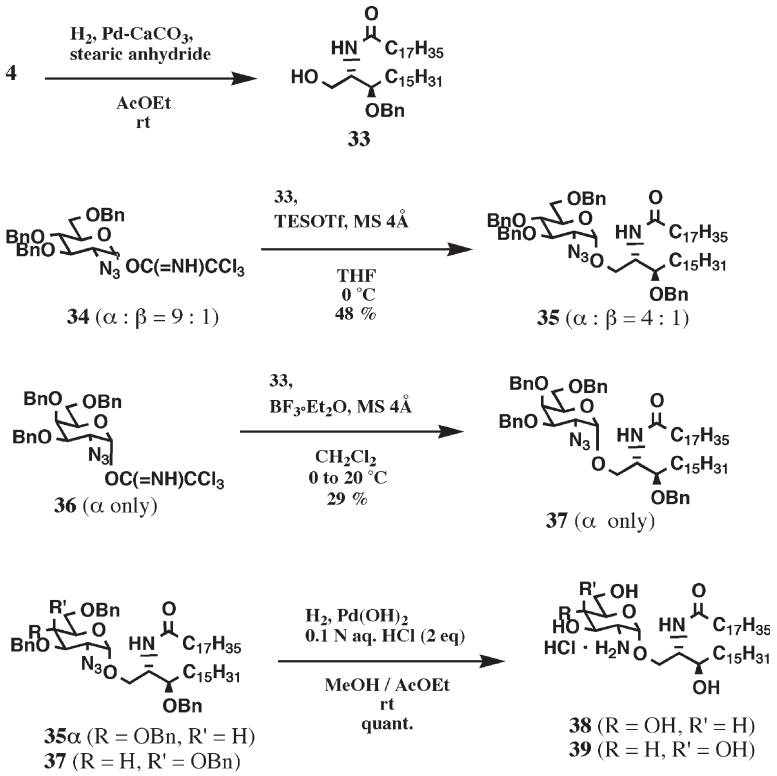


Figure 4. Synthesis of α-Glucosaminyl and α-Galactosaminyl Ceramides

For the synthesis of glucosaminyl and galactosaminyl ceramides, 2-azide-glycosyl donors (**34, 36**) were used. They were coupled with 3-*O*-benzyl ceramide. deprotection of the benzyl groups and conversion of the azide to the amino group in the adducts (**35, 37**) were performed in a successive procedure to afford the final products (**38, 39**).

were most efficiently induced to proliferate and produce IL-4 and IFN-γ by α-ManCer rather than α-GalCer. In contrast, C57BL/6 cells were responsive to α-GalCer and, to a certain extent, to α-glucosyl and glucuronyl ceramide (α-GlcCer and α-GlcUACer). Thus, these results suggest that Vα19 NKT cells recognize α-ManCer as a specific antigen in contrast to Vα14 NKT cells reactive to α-GalCer [10], α-GlcCer [10], and α-GlcUACer [33, 34]. Few NKT cells are generated in β2m$^{-/-}$ mice because the expression of β2m associated with nonclassical MHC class I molecules is required for the positive selection of these NKT cell repertoires. Less reactivity by β2m$^{-/-}$ cells to α-ManCer further supports that the immune responses to α-ManCer were conferred to Vα19 NKT cells in Vα19 Tg^{+} TCRα$^{-/-}$ mice.

The biological activity of α-ManCer toward Vα19 NKT cells is not apparently so significant compared with the activity of α-GalCer on Vα14 NKT cells. Thus, we performed a Student's t test to examine whether the immune responses of Vα19 Tg cells to α-ManCer were statistically significant. The results of the test indicated that the biological activity of α-ManCer to Vα19 Tg cells was more significant than the activity of controls (α-fucosyl ceramide [FucCer] or β-GalCer) with probability more than 96% (the α values were less than 0.04).

The immune responsiveness of Vα19 Tg cells to α-ManCer was dependent on the dose of the glycolipid. Vα19Tg cells but not C57BL/6 or β2m$^{-/-}$ cells responded to α-Mancer in a dose-dependent manner (Figure 5B). The immune responses reached a maximum with the concentration of α-ManCer more than 2 μg/ml, whereas α-FucCer did not induce any immune responses of Vα19 Tg cells even with such concentration. These observations further support that α-ManCer specifically induces immune responses of Vα19 NKT cells.

MR1-dependent development of invariant Vα19 TCR-bearing cells has been reported [19]. This report well corresponds to the present result that immune responses of Vα19 Tg cells to α-ManCer are β2m dependent. Vα19 Tg cells were stimulated with MR1 transfectants, and the immune responses were determined to examine whether the immune responsiveness of Vα19 NKT cells are MR1 dependent (Figure 6). Vα19 Tg cells but not C57BL/6 or β2m$^{-/-}$ cells were activated during coculture with MR1 transfectants. This activation was enhanced when the MR1 transfectants were loaded with α-ManCer before coculture. Thus, it is strongly suggested that invariant Vα19 TCR^{+} cells recognize and respond to α-ManCer presented by MR1.

We next focused on the antigenicity of α-glycosyl ceramides with an α-mannosyl-related sugar moiety. Assuming the importance of the 2-axial hydroxy group of the α-mannosyl residue, we tested for activity of α-altrosyl ceramide and α-talosyl ceramide (the 3-hydroxy group in α-altrose and 4-hydroxy group in α-talose are reversed from those in α-mannose). However, no significant immune responses of Vα19 NKT cells to these glycolipids were found (Figure 5A), suggesting a stringent recognition of the α-mannosyl residue by the invariant Vα19-Jα26 TCR. Because the natural occurrence of α-ManCer has not been reported yet, this glycolipid possibly mimics natural ligands for the NKT cells. We have recently found that 2,6-α-Man (α-Man)- and 6-α-Man-β-GlcNH$_2$-phosphatidylinositol

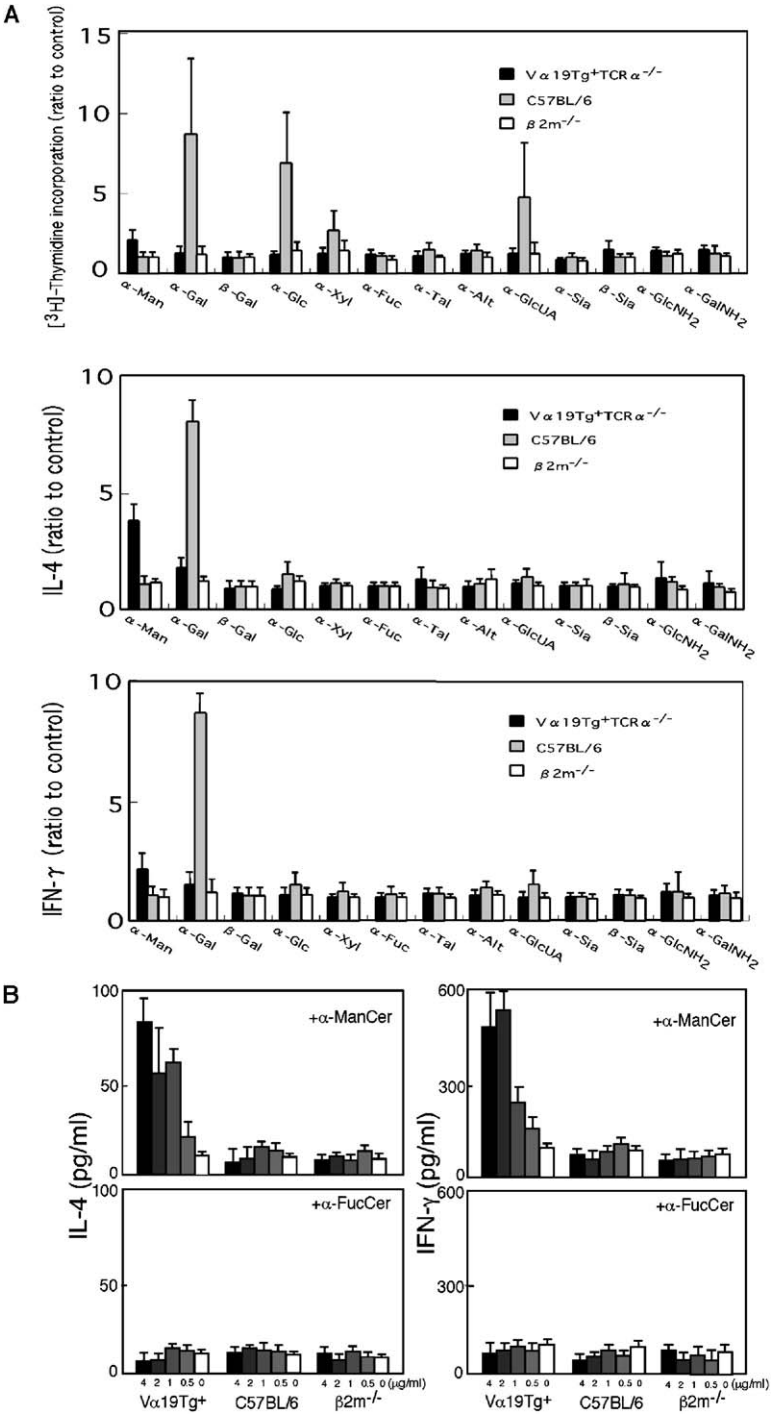


Figure 5. Activation of Vα19 Tg+ Cells with Glycolipid Antigens in Culture

(A) Liver MNCs prepared from Vα19 Tg+ TCRα−/−, C57BL/6, and β2m−/− mice were cultured in the presence (1 μg/ml) or absence of glycolipids. After 2 days, the immune responses were monitored by measuring cell proliferation ([3H]-thymidine incorporation for 5 hr) and IL-4 and IFN-γ secretion in the culture supernatants. Results are shown as the fold increase relative to the control cultures with vehicle (1/200 v/v DMSO). The error bars indicate the standard deviations. Abbreviations: α-Gly, α-glycosyl ceramide; β-Gal, β-galactosyl ceramide; Sia, D-N-acetyl neuraminyl; Tal, D-talosyl; and Alt, D-altrosyl.

(B) Dose-dependent activation of Vα19 Tg+ cells with α-ManCer in culture. Liver MNCs prepared from Vα19 Tg+ TCRα−/−, C57BL/6, and β2m−/− mice were cultured in the presence of the indicated dose of glycolipids. After 2 days, the immune responses were monitored by measuring cytokine secretion into the culture supernatants. One of two independent experiments giving similar results is demonstrated.

have a similar degree of antigenicity to Vα19 NKT cells as α-ManCer (M. Shimamura et al., submitted). These observations suggest the importance of the α-mannosyl residue in an appropriate location for the recognition by the invariant Vα19-Jα26 TCR when the antigenic glycolipid is presented by MR1. It is possible that glycolipids with nonreducing end α-mannosyl residue(s) occur in mammalian cells that are more immunocompetent toward Vα19 NKT cells than the glycolipids so far characterized. Another possibility is that modification of the lipid structure in α-mannosylated glycolipids will alter the interaction with MR1 and improve the recognition of the α-mannose residue by the invariant TCR.

Significance

In this study, we accomplished the efficient chemical synthesis of a series of GSLs with azide sphingosine as a glycosyl acceptor. The methods we used will be applicable to the synthesis of related glycolipids.

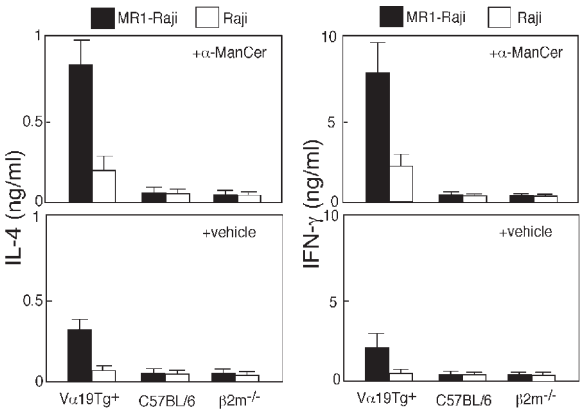


Figure 6. Stimulation of Vα19 Tg+ Cells with α-ManCer in the Context of MR1

MR1-transfected or nontransfected Raji cells were incubated with α-ManCer (2 μg/ml) or vehicle for 18 hr. They were irradiated (3,000 R), washed with medium, and then cultured with liver MNCs isolated from Vα19 Tg+ TCRα−/−, C57BL/6, or β2m−/− mice for 2 days. Cytokine production in the culture fluid was determined by ELISA. The error bars indicate the standard deviations. Experiments were repeated twice, and similar results were obtained.

The biological data on the GSLs demonstrate that α-ManCer is a potent stimulant for Vα19 NKT cells. The present study serves as the second example of the recognition of GSLs in the context of nonpolymorphic MHC class-I-like molecules by invariant TCRs. Such an immune recognition system, namely the specific recognition of α-ManCer with MR1 or α-GalCer with CD1 by the invariant Vα19 or Vα14 TCR, may be of fundamental importance for the regulation of the immune system presumably by linking the natural and adoptive immune systems.

Experimental Procedures

Synthesis of GSLs

Outlines of the methods used to synthesize GSLs are shown in Table 1 and the Supplemental Data available with this article online. For data on the products and the procedures, see the Supplemental Data.

Mice

C57BL/6 mice were purchased from Sankyo Service (Tokyo). Recombination activating gene (Rag)-2-deficient mice and β2m-deficient mice were obtained from Jackson Laboratory (Bar Harbor, Maine). TCR Cα-deficient mice with the C57BL/6 background [35] were provided by Drs. H. Ishikawa (Keio University) and M. Nanno (Yakult Co.). All experiments on mice were performed in accordance with the guidelines of Mitsubishi Kagaku Institute of Life Sciences.

Establishment of Vα19 Transgenic Mice

A Vα19-Jα26 (AV19-AJ33) transgene with the endogenous TCR α promoter and the enhancer was injected into TCR Cα-deficient fertilized eggs, and transgenic (Tg) mouse lines were established (M. Shimamura et al., submitted).

Cell Preparations

Mononuclear cells (MNCs) were prepared from single-cell suspension of mouse organs by density gradient centrifugation with Lymphosepar II (IBL, Gunma, Japan; *d* = 1.090) for spleen and bone marrow and Percoll (Pharmacia, Uppsala, Sweden) for liver-cell preparations as described previously [17].

MR1 Transfectants

Mouse MR1 A cDNA [36] was amplified from C57BL/6 spleen cells with the following PCR primers: 5′-MR1, 5′-ATGATGCTCCTGGT TACCTGG-3′; FLAG-3′-MR1, 5′-CTACTTGTCATCGTCATCCTTGTA GTC(FLAG)-AGAGGGAGAGCTTCCCTCAT-3′.

MR1 transfectants were established as described elsewhere (M. Shimamura et al., submitted). Briefly, the cloned PCR product was recombined into a eukaryotic expression vector, (pCXN) [37] (provided by Dr. J. Miyazaki, Osaka University), and the expression vector was transfected into a human Burkitt's B lymphoma, Raji [38] (obtained from ATCC). The transfectants were selected in the culture medium containing G418 (1 mg/ml) for 1 month. The expression of FLAG (Asp-Tyr-Lys-Asp-Asp-Asp)-MR1 in the transfectants was analyzed by Western blot with anti-FLAG antibody and HRP-labeled anti-mouse immunoglobulin (Sigma, St. Lewis).

Stimulation of Vα19 Tg+ Cells with MR1 Transfectants

MR1-transfectants or their parental cells (1×10^5 per well in DMEM, 10% FCS) were irradiated (3,000 R). They were incubated in the medium with glycolipids (2 μg/ml) for 18 hr and washed twice with DMEM. These cells were cocultured with liver MNCs (1×10^6 per well) from Vα19 Tg or non-Tg mice (2–4 months of age) in 200 μl DMEM (10% FCS) for 2 days. Immune responses by the liver MNCs in the mixed lymphocyte reactions were monitored by measuring cytokines in the culture fluid.

Antigen Assay

Liver MNCs (10^6) from indicated mouse strains (2 to 4 months of age) were cultured in 200 μl of DMEM (10% FCS) in the presence of glycolipids (1 μg/ml). In some experiments, concentration of glycolipids in culture was serially altered. Concentration of cytokines in the supernatants was determined by ELISA after 2 days. Cell proliferation was assessed at day 2 of culture by measuring the incorporation of [^{3}H]-thymidine (0.5 μCi/ml, Amersham, Buckinghamshire, United Kingdom) for 5 hr.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/6/677/DC1/.

Acknowledgments

The authors thank Drs. H. Ishikawa and M. Nanno for providing TCR Cα-deficient mice. They also thank Mr. S. Kamijo and his group for taking care of the mice. This work was supported by a grant from the Ministry of Health, Welfare, and Labor of Japan.

Received: December 24, 2004
Revised: April 12, 2005
Accepted: April 13, 2005
Published: June 24, 2005

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