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ACS chemical biology

Alpha Anomers of iGb3 and Gb3 Stimulate Cytokine Production by Natural Killer T Cells

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ABSTRACT Natural killer T cells (NKT cells) respond to presentation of specific glycolipids with release of a variety of proinflammatory and immunomodulatory cytokines. The repertoire of glycolipid antigens for these cells includes α-glycosylceramides, α-glycosyldiacylglycerols, and the triglycosylceramide iGb3. Two features of iGb3 set it apart from these other antigens: (i) three sugars are required for stimulation and (ii) the glycosidic bond between ceramide and the proximal sugar is beta in iGb3, whereas it is alpha in other antigens. We have synthesized the alpha versions of iGb3 and Gb3 and demonstrate that they are effective antigens for NKT cells and that they do not require lysosomal processing to the monoglycosylceramides for stimulation. These triglycosylceramides constitute a new class of antigen that stimulates NKT cells comparably to monoglycosylceramides.

Natural killer T cells (NKT cells) are a subpopulation of T cells that control multiple immune responses including autoimmune, antitumor, and allergic reactions (*1*). In response to presentation of specific types of glycolipids, NKT cells are capable of rapidly producing a variety of cytokines that in turn trigger proinflammatory and/or immunomodulatory immune responses (*1, 2*). A key step in the stimulation of NKT cells involves interactions among three molecules: (i) a T cell receptor on NKT cells, (ii) a glycolipid, and (iii) an antigen presentation protein, termed CD1d, found on antigen-presenting cells.

As a result of the impact that NKT cells have on human health, there has been considerable interest in understanding the types of glycolipids that can stimulate cytokine release from NKT cells (*1, 3*). Stimulation of NKT cells has been studied extensively in the context of α-galactosylceramides first isolated from a marine sponge (*e.g.*, **1** in Figure 1) (*4*), and recently α-glycosylceramides (**2**) (*5*–*7*) and α-glycosyldiacylglycerols (**3**) (*8*) from bacterial sources (*9*) have been identified as ligands for NKT cells. A feature common to these glycolipids is the alpha linkage of the sugar bonded to the lipid portion of the molecules.

iGb3 (Figure 2) is a triglycosylceramide that has been identified as an antigen for NKT cells (*10*), and controversy remains regarding its role as an endogenous ligand for NKT cells (*11, 12*). Notably, Gb3 (Figure 2), an isomer of iGb3, is not an antigen for NKT cells. A feature of iGb3 that distinguishes it from other known NKT cell antigens is the linkage between ceramide and the proximal sugar. In iGb3, this linkage is beta, in contrast to the antigens in Figure 1. In addition, bacterial antigens for NKT cells are generally considered to be monoglycosylceramides and monodiacylglycerols (*7*), whereas iGb3 contains three sugars.

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Received for review November 9, 2008 and accepted January 26, 2009.

Published online January 28, 2009

10.1021/cb800277n CCC: $40.75

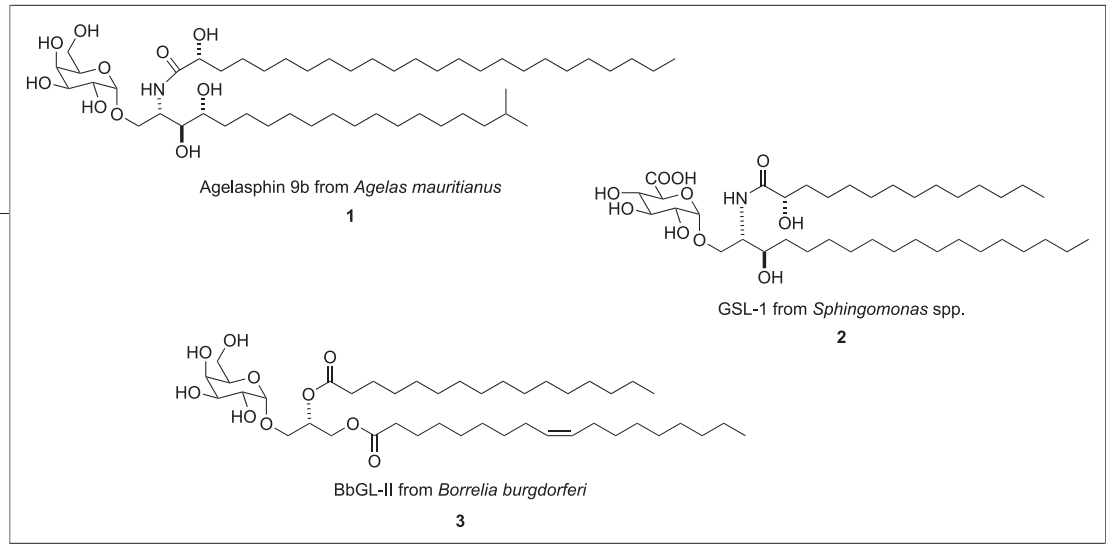


Figure 1. Naturally occurring glycolipids that stimulate cytokine release from NKT cells.

An important aspect of glycolipid presentation to NKT cells is the processing that can occur in the lysosomes of antigen-presenting cells (*1*). Glycolipids are trafficked to lysosomes, and within these organelles a variety of glycosidases are present that can truncate oligoglycosylceramides to monoglycosylceramides prior to presentation to NKT cells. Others (*13, 14*) and we (*6, 7*) have shown that most diglycosylceramides require lysosomal processing to monoglycosylceramides before they can cause NKT cell stimulation. However, in the case of iGb3, it is clear that all three sugars are necessary; β-lactosylceramide, the diglycosylceramide derived from iGb3 *via* truncation of the terminal galactose, does not stimulate NKT cells (*10*).

While alpha and beta anomers of monoglycosylceramides have been prepared and tested for NKT cell stimulatory activity, only variants of iGb3 with beta linkages between the proximal sugar and the ceramide have been prepared and tested as NKT cell antigens (*10, 15*). To determine if alpha anomers of iGb3 and related glycolipids are antigens for NKT cells, we prepared αiGb3 and αGb3 (Figure 2). It was anticipated that these triglycosylceramides would require truncation to α-glucosylceramide in lysosomes for effective stimulation of NKT cells. In early structure activity studies of glycosylceramides with NKT cells, α-glucosylceramide was identified as an antigen for NKT cells (*16*), indicating that truncation of αiGb3 would yield a stimulatory antigen. Wang and co-workers (*14*) showed that α-lactosylceramide stimulates NKT cells and that glycosidase-mediated truncation was required for stimulation. That is, the terminal galactose had to be removed to give α-glucosylceramide for stimulation to occur.

RESULTS AND DISCUSSION

Syntheses of Gb3 and iGb3 have been reported (*17, 18*); however, αGb3, and αiGb3 have not been reported and required a modified protecting group strategy, as

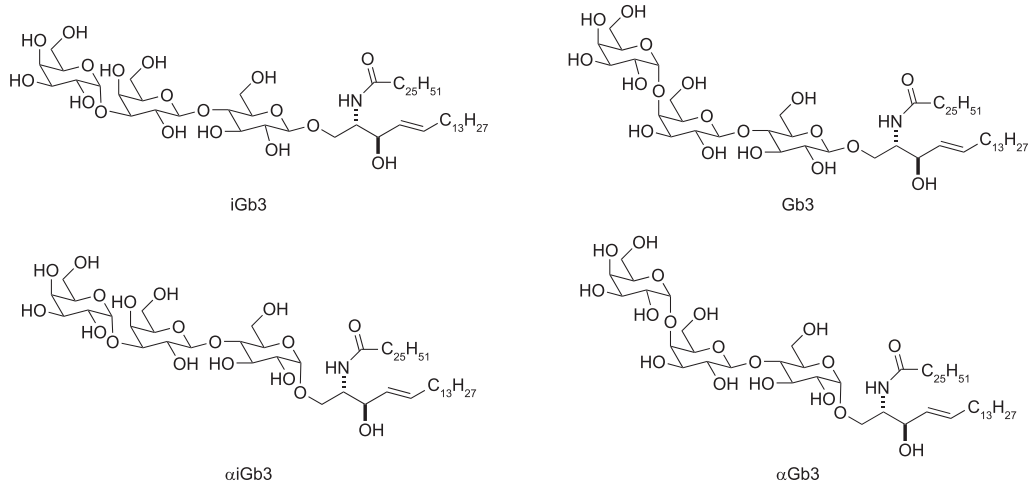
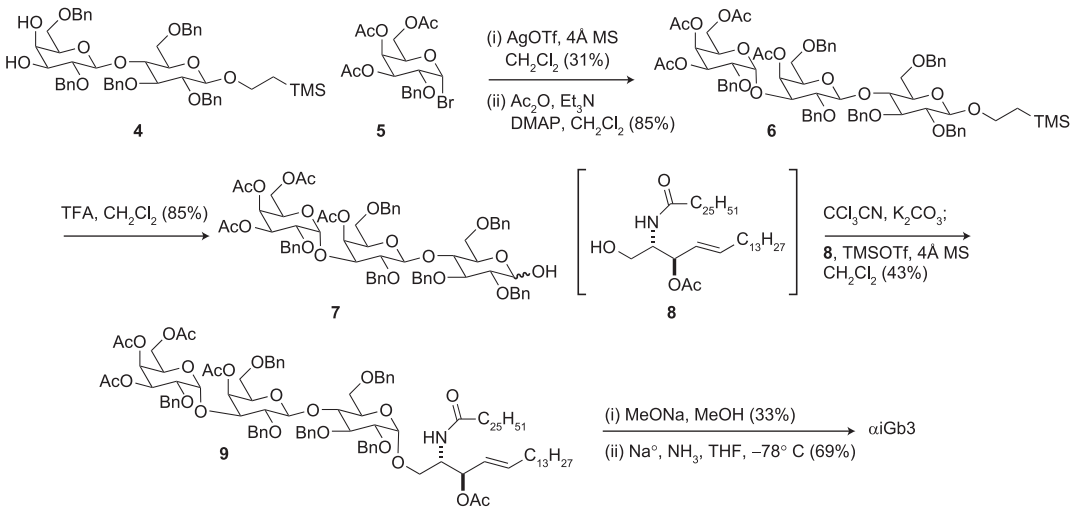


Figure 2. Structures of triglycosylceramides used in the present study.

SCHEME 1. Synthesis of αiGb3 (yields in parentheses)



compared to the syntheses of Gb3 and iGb3, to allow formation of the α-carbohydrate-ceramide bond. A key aspect in the syntheses of these glycolipids was generation of appropriately protected lactose derivatives. We found that **4** (*19*) (Scheme 1) was appropriate for our syntheses of αGb3 and αiGb3.

Synthesis of αiGb3 (Scheme 1) began with coupling of **4** and acetobromogalactose (**5**) followed by protection of the remaining alcohol, generating **6**. Liberation of the C1 hydroxyl group gave **7**, and coupling with **8** gave a mixture of anomers (predominately α). The anomers were difficult to separate at this stage, so the ester groups were removed, and the anomers were separated effectively. Reductive deprotection gave αiGb3.

A closely related process was used in the synthesis of αGb3 (Scheme 2). Selective protection of the hydroxyl group at C3′ in **4** gave **10**. Coupling with **5** provided the protected Gb3 trisaccharide (**11**). The anomeric position of the reducing sugar was revealed, and the trisaccharide was coupled with **8** to give **12**. Sequential deprotection yielded αGb3.

Measurement of the abilities of glycolipids to stimulate NKT cells requires a source of CD1d, typically expressed by antigen-presenting cells, and NKT cells. Dose–response experiments with these glycolipids were performed with dendritic cells as antigen-presenting cells in an assay measuring IL-2 release by a mouse DN32.D3 NKT cell hydridoma (*20*). As expected, results indicated that cytokine release (IL-2) is dependent on iGb3 concentration (Figure 3). Notably, Gb3 did not stimulate, confirming that the CD1d-NKT cell recognition of glycolipids is specific for the substitution pattern offered by iGb3. In contrast, αiGb3 and αGb3 stimulated cytokine production with this NKT cell hybridoma. To confirm that this observation was not unique to mouse NKT cells, we determined stimulatory activity of αiGb3 with a human NKT cell line and found it

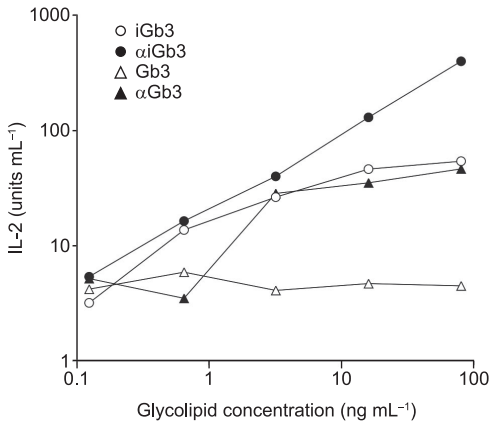
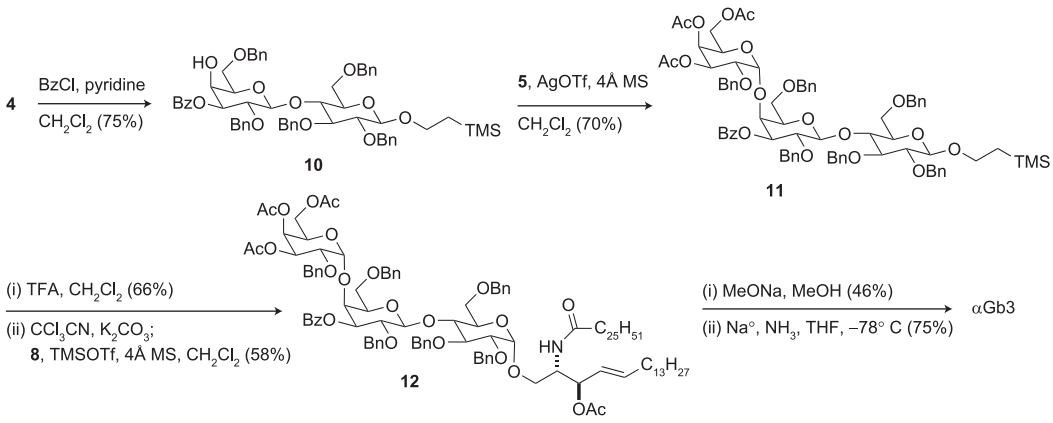


Figure 3. Dose response (IL-2 production) of NKT cells (mouse hybridoma DN32.D3) to glycolipids.

Scheme 2. Synthesis of αGb3



to be a very potent stimulator (data not shown). However, from these results it was not clear if αiGb3 and αGb3 directly stimulated NKT cells or if they required truncation to α-glucosylceramide to become stimulatory.

Previous work demonstrated that α-galactosylceramides substituted at the C4 position on the carbohydrate with a second sugar required processing for stimulation of NKT cells (*10, 13*). Both αiGb3 and αGb3 fit this pattern of substitution; therefore, it was expected that they would require truncation. Use of antigen-presenting cells that express tail-deleted CD1d (TD-CD1d) allows observation of the influences of lysosomal processing and the influence of lipid transfer proteins on NKT cell stimulation. TD-CD1d does not cycle to lysosomal compartments and is therefore not loaded with processed glycolipids (*21*). Attempts to stimulate NKT cells using TD-CD1d and iGb3, αiGb3, and αGb3 resulted in minimal cytokine release (data not shown), indicating that protein-assisted loading and/or glycolipid processing was required for stimulation of NKT cells.

To determine the requirements for lipid transfer protein-assisted loading of CD1d, we used a gel-shift assay in which CD1d was first loaded with a charged glycolipid (trisialoganglioside GT1b) (*22*). The complex was treated with αiGb3 or αGb3 in the presence or absence of saposin B, a lipid transfer protein (*23*). Displacement of GT1b from CD1d with either of these neutral glycolipids resulted in a decrease in electrophoretic mobility (Figure 4). Without saposin B, no displacement of GT1b was observed, but efficient loading, as indicated by the shift in gel mobility, was observed with saposin B. This result indicated that without processing (*i.e.*, loss of carbohydrate groups), these glycolipids were bound by CD1d and that a lipid transfer protein was required for loading.

The remaining issue was whether these glycolipids, loaded into CD1d, were capable of stimulating NKT cells without truncation of the oligosaccharide. To address this issue and to avoid any participation by glycosidases, we used CD1d immobilized in plastic plates rather than antigen-presenting cells in observing NKT cell stimulation (*22*). As with the CD1d loading experiments, NKT cell stimulation was performed in the presence and absence of saposin B. Stimulation was ob-

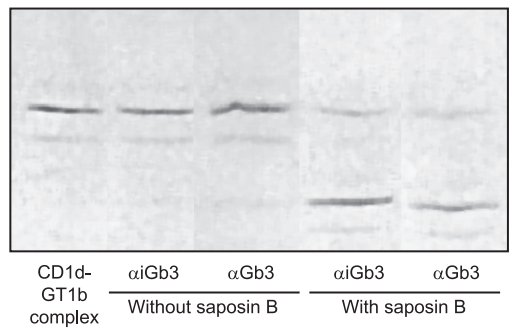


Figure 4. Gel-shift assays of GT1b displacement by αiGb3 and αGb3. CD1d-GT1b complex, 2 μM; glycolipids, 26 μM; saposin B, 2 μM.

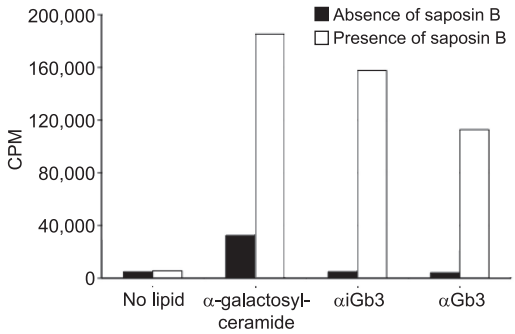


Figure 5. IL-2 release in the culture supernatant determined *via* tritiated thymidine incorporation by the IL-2 indicator cell line CTLL-2.

served as a function of IL-2 release from NKT cells (mouse DN32.D3 hybridomas), which was in turn measured by proliferation (incorporation of radiolabeled thymidine) of an IL-2 responsive cytotoxic T cell line (CTLL). As expected, in the absence of saposin B, no stimulation was observed (Figure 5). However, in the presence of the lipid transfer protein, NKT cell stimulation was observed with both αiGb3 and αGb3. IL-2 production stimulated by these glycolipids was compared to that from an α-galactosylceramide closely related to **1** (Figure 1). The α-galactosylceramide used contained ceramide derived from a C_{18} phytosphingosine chain and a nervonic acid acyl chain. We have shown that α-galactosylceramides based on this ceramide are highly stimulatory antigens of NKT cells (*24*).

Tetramer staining of NKT cells by glycolipid-loaded CD1d tetramers is a method commonly used to study association of glycolipids with T cell receptors on NKT

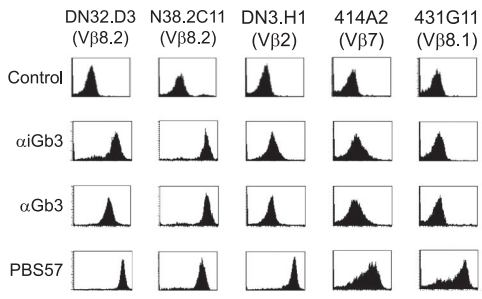


Figure 6. NKT cell hybridomas (DN32.D3) expressing different Vβ chains in their T cell receptors were stained by CD1d tetramers loaded with the indicated glycolipids. The control used was α-galactosylcholesterol.

cells (*25, 26*). NKT cell receptors are composed of Vα14 and Vα24 chains in mice and humans, respectively, and a limited repertoire of Vβ chains (*1*). It has been observed that iGb3 recognition is strongest with NKT cell receptors with Vβ7 chains (*27*), whereas α-galactosylceramides, such as PBS57 (*24*), are recognized by NKT cell receptors with variety of Vβ chains (*i.e.*, Vβ8.2, 8.1, 7, and 2). Staining of NKT cells with CD1d tetramers loaded with αiGb3 and αGb3 (Figure 6) indicated that only NKT cells with receptors with Vβ8.2 chains were significantly stained by tetramers loaded with these glycolipids, demonstrating the specific recognition of these trisaccharides.

Conclusion. With the discovery of glycolipids that stimulate cytokine release by NKT cells have come efforts to determine the structural features of glycolipids required for recognition by CD1d and T cell receptors. Both the ceramide and carbohydrate portions of monoglycosylceramides have been the focus of multiple studies, and the alpha anomeric configuration of the sugar bonded to ceramide has been identified as a key structural element in providing stimulatory properties. Because iGb3 has a beta anomeric configuration at the ceramide bond, it is distinct from other stimulatory glycolipids, and the fact that closely related glycolipids, such as Gb3 (an isomer of iGb3), are not stimulatory demonstrates the specificity of the protein−carbohydrate interactions.

Synthesis of αiGb3 and αGb3 has allowed observation of the effects of alterations in the anomeric configuration at the sugar-ceramide bond. In a fashion similar to iGb3, these glycolipids require lipid transfer proteins for loading into CD1d. However, it was unexpected that αiGb3 and αGb3 would not require lysosomal processing to become stimulatory. Glycolipids from *Sphingomonas* spp. include a tetrasaccharide linked to ceramide through an α-glycosidic bond, and this glycolipid, without lysosomal processing, has been described as weakly stimulatory if the ceramide portion of the molecule is sufficiently hydrophobic (*28*). Its recognition may be similar to that of αiGb3 and αGb3.

Differentiation of iGb3 and Gb3 is presumably due to protein−carbohydrate interactions. Because similar differentiation is not observed with αiGb3 and αGb3, it is likely that the terminal sugars in these glycolipids are not intimately involved in interactions with the T cell receptor and may be solvent-exposed in the complex. In the crystal structure of the complex of CD1d loaded with

an α-galactosylceramide bound to the T-cell receptor of an NKT cell, the receptor docked at the end of the CD1d binding cleft exposing a portion of the glycolipid to solvent (*29*). If αiGb3 and αGb3 interact similarly with CD1d and with the T cell receptor, it is likely that the two terminal sugars are tolerated in this solvent-exposed portion of the complex.

Because αiGb3 and αGb3 stimulate NKT cells, great care must be taken to ensure that β-glycosylceramides such as iGb3 are not contaminated with alpha anomers. The greatest danger comes from false positives, that is, the misinterpretation of trace contamination with a strong stimulator as weak stimulation by another glycolipid. In our earlier studies of iGb3 stimulation of NKT cells, we used iGb3 from three different sources, including glycolipid isolated from a natural source, to verify that stimulation was coming from iGb3.

As the structural requirements of exogenous and endogenous antigens for NKT cells are better understood, it will become easier to manipulate the responses of these cells. Control of NKT cell responses may lead to improved treatments to a wide range of disease states, including infection and autoimmunity. The discovery that αiGb3and αGb3 are antigens for NKT cells increases the number and understanding of stimulatory glycolipids.

Acknowledgment: Financial support from the National Institutes of Health (NIAID, P01 AI053725) is gratefully acknowledged. D.Z. and J.M. were supported by Cancer Research Institute (New York) Fellowship grants.

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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