Isoglobotriaosylceramide (iGb3) is a stimulatory antigen for a unique type of T cell, Natural Killer T cells. Produced in the lysosomal compartment by mammalian antigen-presenting cells, iGb3 is one of the few clearly identified carbohydrate ligands for biological receptors. A major source of glycoconjugate structural diversity arises from the possibility of forming different linkages between the same monosaccharide units. Globotriaosylceramide (Gb3) exists as a natural isomer for iGb3, and both isomers are frequently found together in mixtures of glycosphingolipids extracted from mammalian cell membranes. Discriminating these isomers has been feasible using monoclonal antibodies raised against specific carbohydrate epitopes, or by unambiguous structural characterization, which requires relatively large amounts of pure compounds isolated from grams, or tens of grams, of biological samples. However, the precise detection of iGb3 from small amounts of biological samples, where it may be mixed with Gb3 present in much higher abundance, is a prerequisite for answering further important biological questions such as stimulation of NKT cells. Here we describe a specific and sensitive method based on ion trap mass spectrometry to discriminate iGb3 from Gb3. We also demonstrate its application to quantifying the amount of iGb3 in a prototype antigen-presenting cell, rat RBL-CD1d cells, using a chemically synthesized short N-acyl chain iGb3 as internal standard. This methodology may have wide implications for functional glycosphingolipidomics of immune cells and glycosphingolipid biomarker analysis.

Keywords: CD1d/globotriaosylceramide/glycosphingolipid/NKT cell/thymocyte

Introduction

Natural Killer T (NKT) cells are a lineage of T cells involved in innate immunity that regulate the outcomes of adaptive immunity (Bendelac and Fearon 1997). NKT cells are a hybrid of NK cells and T cells (Kronenberg 2005; Pear et al. 2004; Sugita et al. 2004), but are only activated by recognizing an antigenic stimulation through T cell receptor signaling. NKT antigens are presented by a nonpolymorphic antigen-presenting molecule, CD1d (Jayawardena-Wolf and Bendelac 2001; Joyce and Van Kaer 2003; Park and Bendelac 2000; Forcelli and Modlin 1999; Sugita et al. 2004). In contrast to conventional antigen-presenting molecules (MHC Class I and MHC Class II), CD1d molecules present lipid and glycolipid antigens. In professional antigen-presenting cells, the CD1d pathway processes and surveys microbial lipids and glycolipids from pathogenic bacteria (Kinjo et al. 2005, 2006; Mattner et al. 2005; Sriram et al. 2005). Furthermore, the CD1d molecule presents self-lipids to NKT cells in disease settings such as asthma, allergy (Akbari et al. 2003; Lisbonne et al. 2003; Meyer et al. 2007), and cancer (Ambrosino et al. 2007; Moodycliffe et al. 2000; Smyth et al. 2000, 2002). The identities of these self-lipid ligands are not yet known, in spite of extensive searches by several laboratories. The biochemical fractionation of natural stimulatory antigens may be hampered by the low abundance of these natural ligands in the starting materials used for biochemical purification. It has been known for a long time that one copy of the peptide/MHC complex from an antigen-presenting cell is sufficient for stimulating a T cell (Brower et al. 1994; Christinck et al. 1991; Davis et al. 2003; Lisbonne et al. 2003; Harding and Unanue 1990; Reay et al. 2000; Sykulev et al. 1996). Very recently, by using a combined genetic screening and candidate ligand testing, a lysosomal glycosphingolipid (GLS), isoglobotriaosylceramide (iGb3), was found to be a stimulatory antigen to both mouse and human NKT cells (Zhou et al. 2004). Expression of iGb3 in peripheral tissues may be involved in controlling NKT cell responses to infections, malignancy, and autoimmune (Zhou 2006).

Urgently needed for the study of GLS-mediated NKT cell functions is the development of sensitive methods to detect iGb3 in small amounts of biological samples, typically a few million antigen-presenting cells. This is one of the major challenges for cellular immunologists who want to study this important GLS antigen. For example, in a recently published paper about a knockout mouse which is deficient of iGb3 synthase, the authors had no method capable of detecting thymic iGb3 in wild-type mice; therefore, it is impossible to prove that iGb3 is indeed missing in the thymus of iGb3 synthase knockout mice (Porubsky et al. 2007).

Mass spectrometry (MS) has been applied to GLS characterization and quantitation for decades (Adams and Ann 1993; Costello and Vath 1990; Egge 1978; Egge and Peter-Katalinic 1987; Levery 2005; Peter-Katalinic and Egge 1990; Samuelsson et al. 1990). The sensitivity of this technique has revolutionized since the application of electrospray
ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), in conjunction with tandem collision-induced dissociation (MS/CID-MS), time of flight (TOF), and quadrupole ion trap (QIT) techniques (Costello 1999; Dell and Morris 2001; Harvey 1999; Levery 2005; Reinhold et al. 1995; Reinhold and Sheeley 1998). However, a major barrier persists, namely, glycans with the same molecular mass (isobars) can have different (isomeric) glycosidic linkages, even between identical monosaccharide residues. In the case of detecting iGb3, it was until recently impossible to discriminate iGb3 (Galα3Galβ4Glcβ1Cer) from Gb3 (Galα4Galβ4Glcβ1Cer) by MS methods (excluding linkage analysis, which requires labor-intensive derivatization and GC/MS). Recently, MS methods based on multistep fragmentation of permethylated glycans in ion traps have shown great promise for distinguishing isobaric glycan structures, such as Galα3Gal and Galα4Gal (Ashline et al. 2005; Lapadula et al. 2005; Levery, Ashline, Singh, et al. 2005; Levery, Ashline, Hanneman, et al. 2005; Zhang et al. 2005). In this study, we have applied this finding to isoglobaltriosylceramides and globotriaosylceramides in the cultured rat RBL-CD1d cell line, and developed a quantitative approach for iGb3 detection.

**Results**

**Discrimination of iGb3 from Gb3 by permethylation and multistep ion trap MS**

Previous studies found that the terminal Galα4Gal disaccharide from the sodium adduct of permethylated globotriositol (Galα4Galβ4Glcβ1-tol) produces an ion trap MS3 spectrum readily distinguishable from that of the terminal Galα3Gal disaccharide of a similarly treated and analyzed 'xenotransplantation antigen' trisaccharitol (Galα3Galβ4Glcβ1ac-tol) (Ashline et al. 2005). Consistent with this, it was observed that the terminal disaccharides from the GSLs Gb3 and iGb3 similarly yielded analogous, distinguishable mass spectra at the MS4 level (Levery, Ashline, Singh, et al. 2005; Levery, Ashline, Hanneman, et al. 2005). Compared with the reduced trisaccharides previously analyzed, an extra fragmentation step is required to isolate the trisaccharide fragment \( m/z \) 667 from the sodium-adducted permethylated GSLs (MS2); the nonreducing terminal disaccharide-1-ene fragment \( m/z \) 445 is obtained as a product of \( m/z \) 667 (MS3), and the product ions of \( m/z \) 445 are then acquired in the final step (MS4) (see Scheme 1; note that all reported fragments will be denoted by nominal, monoisotopic \( m/z \) values).

Shown in Figure 1 are examples of MS4 spectra (\( m/z \) 1355→667→445→) thus obtained from isobaric synthetic standards of d18:1/26:0 Gb3 and iGb3, respectively (for permethylated sodium adducts, expected nominal, monoisotopic \( m/z \) 1354; calculated monoisotopic \( m/z \) 1355.02). Product ions of \( m/z \) 445 from pure Gb3 are reproducibly obtained at \( m/z \) 227, 241, 257, 259, 315, 329, 413, and 415 (with a highly abundant \( m/z \) 329 ion as base peak, and the remaining ions at 5–15% relative abundance), while the isobaric disaccharide-1-ene fragment from pure iGb3 yields highly abundant products at \( m/z \) 211, 257, 259, 371, and 413, ions of intermediate abundance at \( m/z \) 241, 315, 343, and 383, and ions in the 5–15% relative abundance range at \( m/z \) 227, 339, 341, and 415. These are consistent with typical neutral losses, along with specific glycosidic and cross-ring cleavages shown in Scheme 1. It is notable that several ions in the \( m/z \) 445 product spectrum from iGb3, including two highly abundant fragments \( m/z \) 371 and 211, are almost absent (\( \leq 0.4\% \) and \( \leq 0.2\% \) for these ions, respectively) in the corresponding spectrum from Gb3. Conversely, the most abundant ion in the \( m/z \) 445 product spectrum from Gb3, \( m/z \) 329.18, is not observed in the corresponding spectrum from iGb3. A third observation of interest is that the abundance ratio of two fragments, \( m/z \) 413 and 415, is 1:1 in the MS4 spectrum of Gb3, while it increases to almost 7:1 in the corresponding iGb3 spectrum. These can be proposed, therefore, to form not only a basis for discriminating the two isobaric terminal disaccharide ions, but for detecting and quantitating them in binary mixtures containing isobaric Gb3/iGb3 molecular species (lipofoms). This could be done provided that there are no confounding isobaric ions from other terminal disaccharide precursor species, i.e., with alternate anomeric configurations and/or linkages present in the spectrum. In the case at hand, the likelihood of observing all of the ions characteristic of the Galα3Gal linkage of iGb3, in the same relative abundance ratios, from another linkage is vanishingly small.

**Scheme 1.** Characteristic decomposition pathways for positive mode ion trap mass spectrometry of permethylated GSLs Gb3Cer and iGb3Cer. Gb3 and iGb3 are clearly discriminated by the fragmentation patterns of disaccharide-1-ene ions \( (m/z \) 445) derived from their nonreducing termini. The differences in linkage (Gb3Gal versus Galα3Gal; lower-left and right-hand structures, respectively) are reflected in the MS4 product ions consistent with the isobaric \( m/z \) 445 precursors.
Detection and quantitation of iGb3 in an isobaric mixture of Gb3 and iGb3 standards

We found that product ions m/z 371 and 211 were reliably observed as markers of iGb3 in MS4 spectra of artificial mixtures of isobaric Gb3 and iGb3 lipoforms, detectable below the 1% range (Figure 2). Calibration plots were constructed from the abundance (A) ratios A(iGb3)211/[A(iGb3)211 + A(Gb3)329] and A(iGb3)371/[A(iGb3)371 + A(Gb3)329] versus the relative concentration of iGb3 in a mixture with Gb3 (Figure 3, Panels A and B, respectively). Since m/z 371 and m/z 329 are the base peaks in the spectra of the pure isomeric iGb3 and Gb3, respectively, the ratio A(iGb3)371/[A(iGb3)371 + A(Gb3)329] would be expected to fit a first-order linear expression in the range of 1–100% iGb3, if their ion yields relative to the absolute amounts of respective GSLs present are similar. In this case the line was indeed constrained with high correlation ($R^2 = 0.9971$). On the other hand, the ratio A((Gb3)211/[A(iGb3)211 + A(Gb3)329] would be expected to deviate from linearity, because the maximum abundance of m/z 211 is less than 100%. These results were therefore allowed to fit a second-order equation such that, e.g., the value could be <0.5 at 50% iGb3; the least-squares fit correlated well ($R^2 = 0.9997$). Note that this function could be linearized if the values of A((Gb3)211 were corrected by normalizing them to the maximum value in pure iGb3, i.e., if A((Gb3)211 (corr) = A((Gb3)211/A((Gb3)329 (max) is substituted for A((Gb3)211.

Interestingly, it was also observed that abundance ratios of m/z 413 to m/z 415 significantly above 1 correlated with the
Resolution and quantitation of isomeric glycosphingolipids

Fig. 2. Positive ion ESI-LIT-MS4 spectra (m/z 1355→667→445→) of permethylated mixtures of isobaric Gb3 and iGb3 standards. Pure iGb3 and Gb3 of identical molecular weight (chemically synthesized as described), mixed to percentages of iGb3 of 94.3, 14.3, 1.8, and 0.87% (Panels A through D, respectively), were permethylated, and analyzed by ESI-LIT-MS as described.

The presence of even small amounts of iGb3 in the mixture; a plot of the ratio A(iGb3 + Gb3)413/A(iGb3 + Gb3)415 versus relative concentration of iGb3 was also constrained to linearity in the same range (Figure 3, Panel C); the calculated y-intercept was 0.904 ($R^2 = 0.996$). In the end, we did not use this function explicitly for calculations of % iGb3, but it could be useful as confirmatory evidence for its presence, especially in higher proportions.

A very good linear correlation ($R^2 = 0.9992$) for % iGb3 was established by combining abundances of m/z 211, m/z 371, and m/z 329, into a single average function, substituting A(iGb3)211 (corr) for A(iGb3)211, i.e., [A(iGb3)211 (corr) + A(iGb3)371]/[A(iGb3)211 (corr) + A(iGb3)371 + 2 × A(Gb3)329] (Figure 3, Panel D). With this combined function, the limit of quantitation (LOQ) for iGb3 in an isobaric mixture of iGb3 and Gb3 is roughly estimated to be ∼1%. The characteristic m/z 211 and 371 ions could actually be detected at much lower levels. However, we sometimes observed them in low abundance in spectra of pure Gb3; based on the relative abundance of these background signals, the LOQ for iGb3 is conservatively estimated not lower than 1%.

With respect to the amount of iGb3 theoretically quantifiable, this can be estimated from the total amount of GSL in the artificial mixtures, and the percentage of each consumed to obtain a usable spectrum. The standard mixtures were each 1 µg; following permethylation, these were dissolved in 200 µL, of which a minimum of ∼200 nL was required to obtain a high-quality MS4 spectrum (the exemplary spectra shown in Figure 2 were acquired with consumption of 1 µL, but in later work much less was used, with little apparent sacrifice of reliability). This works out to 1 ng total GSL per spectrum and, for the standard containing ∼1% of the target antigen, would yield a conservative LOQ of 10 pg (∼9 fmol) of iGb3. The LOQ reported here is of the same order of magnitude as the limit of detection (LOD) previously reported for an HPLC-based assay (Neville et al. 2004). We estimate that the absolute amount of iGb3 theoretically detectable by our MS4 method is ultimately lower. An additional advantage of the MS4 method is that the iGb3 content of individual lipofoms can be estimated, which cannot be done using the HPLC-based method, because the ceramide is first enzymatically released (Neville et al. 2004). As shown in the example below, this is important information.

Quantitative analysis of iGb3/Gb3 trihexosylceramides in RBL-CD1d cells

In order to quantitate the iGb3 content of a biological sample, it is necessary to determine (i) the total content of trihexosylceramide (Gb3 + iGb3) and (ii) the relative content of iGb3 in each iGb3/Gb3 isobar observed. From these values the absolute
amount of iGb3 in the sample can be calculated. The latter value can be derived from the MS3 protocol proposed above; the former value would be best obtained by the use of a nonnatural internal standard similar in structure and ion yield to the intended analytes. In this case, we used a synthetic iGb3 standard having 8:0 fatty-N-acylation, which is not found significantly in mammalian GSLs.

The potential use of this method is demonstrated by the following analysis of neutral GSLs purified from $5 \times 10^7$ RBL-CD1d cells. In an ESI-LIT-MS$^4$ profile spectrum of the permethylated RBL-CD1d neutral GSL fraction (Figure 4, Panel A), a number of potential GSL molecular ions (as Na$^+$ adducts) were observed (structure determination of species containing more than three monosaccharide residues ["Higher Glycosylceramides"] is currently in progress). MS$^2$ spectra were systematically acquired for every molecular precursor ion $m/z$ in the MS$^1$ spectrum from $m/z$ 900 to 1400 (covering the range of trihexosylceramides with ceramide compositions having d18:1 sphingosine and C16 to C26 fatty acid). This was done because multiple molecular ions for iGb3/Gb3 are often detected, due to the presence of a variety of lipoforms having different fatty acid and sphingosine chain lengths (and other possible modifications, such as hydroxylation); we have further observed that the relative content of iGb3 in different lipoforms can vary considerably. Therefore, in order to determine the total amount of iGb3 in a sample, it is necessary either to subject every potential molecular ion detectable in the MS$^1$ spectrum, in the $m/z$ range appropriate for mammalian iGb3/Gb3, to MS$^2$ analysis, looking for the trisaccharide product ion $m/z$ 667, or, if appropriate capabilities are available, to perform a precursor ion scan looking for all molecular precursors of $m/z$ 667. (The latter option was not available in our facility; the search for a given product ion, such as $m/z$ 667, can be automated on the LTQ, yielding results superficially similar to that of a precursor ion scan, but with no significant gain in sensitivity. In practice we found this method convenient, and frequently employed it for these studies.)

Among all ions in this range, only the $m/z$ 1327.0 precursor yielded ions characteristic for iGb3 along with those for Gb3 (Figure 4, Panel B); the $m/z$ 1325 precursor yielded a product spectrum consistent only with pure Gb3 (spectrum not shown).
Resolution and quantitation of isomeric glycosphingolipids

Fig. 4. ESI-LIT-MS of neutral GSLs extracted from RBL-CD1d cell. MS1 molecular ion profile (Panel A) displays a single set of Na+ adducted triglycosylceramide ions at m/z 1325.0 and 1327.0, consistent with Gb3/iGb3 having d18:1 sphingoid with 24:1 and 24:0 fatty-N-acylation (nominal, monoisotopic m/z 1324 and 1326 [brackets]; calculated m/z 1324.95 and 1326.97, respectively). Using MSn analysis, remaining peaks were found to be either lactosylceramide (LacCer; m/z 1120.9, 1122.9), higher glycosylceramides (m/z 1529.1, 1531.1, 1733.2, 1735.2, 1774.2, 1776.2, 1937.4, 1939.4, 1978.4, 1980.4), or non-GSL impurities (unmarked). Of the two triglycosylceramide adducts, the ion at m/z 1325.0 yielded an MS4 spectrum consistent with pure Gb3; the one at m/z 1327.0 yielded MS4 product ions consistent with a mixture of both Gb3 and iGb3 (m/z 1327→667→445→; Panel B). Inset: section of MS1 molecular ion profile (m/z 1080–1340) with internal standard added (iGb3 with 8:0 fatty-N-acylation; 1.75 × 10⁻³ µg/200 µL).

Quantitation was carried out by analysis of an aliquot of the same sample to which the synthetic d18:1/8:0 iGb3 was added as an internal standard. This was titrated into the sample at several levels to (i) verify that the response was linear, and (ii) make sure that a signal with S/N comparable to that of the iGb3/Gb3 adducts was present in the spectrum (as shown in Figure 4, inset). The permethylated sodium adduct of the standard was observed at m/z 1102.9 (calculated monoisotopic m/z 1102.7); for standard added at a level of 1.75 × 10⁻³ µg (1.9 pmol)/200 µL, the abundance ratios were 1.23:1 relative to the m/z 1325 peak, and 1.48:1 relative to the m/z 1327 peak (after subtraction of the contribution of M+2+Na of the unsaturated lipoform). This aliquot represented 25% of the extract from 5 × 10⁷ cells.

Using the equation of the least-squares calibration line given in Figure 3, Panel D (relating MS4 fragments m/z 211, 329, 371),
the relative quantity of iGb3 in the molecular precursor $m/z$ 1327 was determined to be $\sim 52 \pm 3\%$. Thus, the absolute amount of iGb3 in the sample is determined to be 52% of 0.10 amol/cell, or roughly $3.2 \times 10^4$ copies per cell. Counting the remaining contribution of Gb3 to the $m/z$ 1327 peak, plus 100% of the $m/z$ 1325 peak, yields $\sim 10^5$ copies of Gb3 per cell. For 50 million cells, the total yield of iGb3 is on the order of 3 ng. The overall ratio of Gb3:iGb3 appears to be $\sim 3:1$ but, more importantly, is nowhere near the same in the two lipoforms represented by $m/z$ 1325 and 1327, being $\sim 100\%$ of the former and $\sim 50\%$ of the latter. This may point to different origins for the Gb3 and iGb3 species, with the former being synthesized from a ceramide precursor having two fatty-N-acyl lipoforms (24:1 and 24:0), and the latter from a single lipoform (24:0).

**Approximate limit of detection for cellular iGb3/Gb3**

Given that the sample was not, in fact, extremely challenging, it was of some interest to determine what the LOD would be if much less sample were available, or if iGb3/Gb3 isobars represented a considerably smaller proportion of the total GSL. The sample was therefore serially diluted until the iGb3/Gb3 signal could no longer be observed above the noise level. This LOD was reached at a dilution such that $\sim 2$ fmol was consumed to obtain an MS$^1$ spectrum (Figure 5, inset). This is somewhat lower than the LOQ for iGb3 reported above with the standard iGb3/Gb3 mixtures; however, detection and approximate quantitation of iGb3 could still be carried out at this level, provided it makes up a significant proportion of the mixture. As shown in Figure 5, an MS$^4$ spectrum could be obtained as before; some variation in relative abundances could be observed compared to the spectrum previously obtained (Figure 4, Panel B), but ions characteristic for iGb3 were clearly present and could be used to give a rough estimate of its percentage in the mixture.

**Discussion**

There is a general need for the development of specific and sensitive methods for the detection of potential GSL biomarkers in small scale biological samples, such as a few hundred µL of bodily fluid, or a few million antigen-presenting cells. In the current study, which addresses the urgent need to understand crucial details of GSL-mediated functions in NKT cells, the problem of specificity is especially acute, because the target antigen (iGb3) is to be expected to be an extremely minor, even elusive component that co-purifies with an isobaric/isomeric GSL (Gb3) representing 99% or more of the trihexosylceramide fraction in the cells of interest. Herein, we have described an analytical method, based on permethylation and ion trap MS$^4$, for specific detection and quantitation of iGb3 in limited biological samples.

The selectivity of our method is based on distinguishing characteristic fragmentations of the nonreducing terminal disaccharide of permethylated iGb3, which differ considerably from those of isomeric Gb3 when isolated and analyzed by ion trap MS at the MS$^3$ level. Relative proportions of iGb3 and Gb3...
could thus be determined from the abundance ratios of selected characteristic fragmentation products of the isomeric terminal disaccharide MS$^3$ products, Galz3Gal-1-ene and Galz4Gal-1-ene. The abundance ratios for the selected ions were correlated with iGb$_3$/Gb$_3$ ratios by calibration curves obtained from mixtures of chemically synthesized pure iGb$_3$ and Gb$_3$ standards in known ratios. The method was tested by application to neutral GSLs of the RBL-CD1d cell line, extracted from samples in the range of 50 million cells.

RBL-CD1d cell line is a rat basophil leukemia cell line transfected by a mouse CD1d molecule. This cell line was chosen because it has been used in several studies on glycolipid antigen presentation, including the critical finding that natural ligands are dependent on lysosomal processing (Chiu et al. 1999; Zhou et al. 2004). On the other hand, among all of our cell lines transfected by the mouse CD1d, the RBL-CD1d cell line gives the highest stimulative activity toward canonical NKT cell hybridomas, although the response of NKT cell hybridomas can be influenced by not only the amount of antigenic ligands, but also other factors such as co-stimulatory molecules of the antigen-presenting cells. In this study, the absolute amount of iGb$_3$ in the neutral lipid extract from 50 million RBL-CD1d cells was determined to be ~3.2×10$^4$ copies per cell. It is, in addition, highly significant that it was possible to detect iGb$_3$ specifically in one of two lipofoms differing by only 2 mass units. This has two important implications with respect to methods utilizing release of ceramide prior to the assay (Neville et al. 2004): (i) important information about GSL lipoforms, with possible implications for differential processing, and possibly even cellular origins, may be lost and (ii) incorporating iGb$_3$ occurring in one or a few lipofoms into a larger pool of Gb$_3$ encompassing a broader range of lipofoms, by removing the ceramide moieties, could actually serve to dilute the overall iGb$_3$ signal rather than enhance it.

The sensitivity of the method was somewhat limited by a number of factors which allow considerable room for future improvement: (i) the standard protocols used purify GSLs from cells that require ion-exchange chromatography and multiple steps of rotary evaporation, and the subsequent permethylation procedure could be streamlined and downscaled (some down-scaled approaches to permethylation have been described (Kang et al. 2005) and (ii) application of a true precursor ion scanning function, which cannot be implemented in a single ion trap analyzer configuration, would improve considerably the detection of GSL molecular ion species, especially when multiple lipofoms are present (e.g., all isobaric trihexosylceramide could be detected selectively as precursors of m/z 667).

For a specific detection of iGb$_3$ that does not require quantification, we only need to identify the characteristic MS$^4$ fragments (m/z 211, 371, possibly along with m/z 413 in excess over m/z 415). For this purpose, we have been able to demonstrate the presence of iGb$_3$ for human monocyte derived dendritic cells in as few as 4 million of cells (Teneberg et al. in preparation). Incorporation of novel approaches for small-scale purification and permethylation of glycolipids (e.g., Kang et al. 2005) would significantly improve the overall sensitivity of our method, and therefore reduce the amount of cells required for the assay.

Our method can be established in conventional chemistry laboratories with access to ion trap or hybrid quadruple-ion trap instruments, for multistep MS$^n$ assays. In addition to the high specificity and high sensitivity, this method might provide further important information on the structural features of iGb$_3$ antigen, such as the fatty acid and sphingosine chain lengths, which are indeterminate in protocols where the ceramide moiety is removed. We are also working on extending these methods to detection of interconversion products/precursors of iGb$_3$ antigen (e.g., iGb$_3$ as both glycosyltransferase product and glycosidase substrate), in order to follow in more detail the metabolic fate of this elusive NKT cell antigen. This method may be among the first few that enable the bridging of functional studies with lipidomics/glycosphingolipidomics assays (Han and Gross 2005; Lavery 2005; Maceyka et al. 2005; Merrill et al. 2005; Peterson and Cummings 2006; Sommer et al. 2006; Tan et al. 2006; van Meer 2005; Watson 2006; Woods and Jackson 2006). It is probable that many biologically critical GSL structures are of extremely low abundance among total cellular lipids, and their detection may depend on assays targeting ‘signature’ MS$^2$ ions for the required specificity and sensitivity. Thus, only lipidomics/glycosphingolipidomics methods with strong capabilities for structure discrimination can meet the standard for immunological studies on CD1d ligands. Since the same qualities of low abundance and co-expression of multiple isomeric lipofoms can also apply to aberrant, disease-associated components, this methodology should also have wider implications for GSL biomarker analysis.

**Experimental methods**

**GSL samples**

Isobaric iGb$_3$ and Gb$_3$ with d18:1 sphingoid and 26:0 fatty-N-acylation (C$_{62}$H$_{117}$NO$_{18}$, monoisotopic MW 1163.82) were chemically synthesized as described (Zhou et al. 2004; Xia et al. 2006). They were stored in chloroform–methanol 1:1 (v/v) and further diluted to obtain standards of concentration as indicated. A synthetic iGb$_3$ with short chain saturated fatty-N-acylation (d18:1/8:0; C$_{44}$H$_{81}$NO$_{18}$, monoisotopic MW 911.54) was also prepared for use as a nonnatural internal standard (the synthesis will be published elsewhere).

**Total lipid extraction from RBL-CD1d cells**

Cultured RBL-CD1d cells (5×10$^7$ cell aliquots) were stored in 16×100 mm glass tubes at −80°C. Lipids were extracted by extensive sonication four times (1 mL each) with mixed polarity solvents. The first and last solvent used was chloroform–methanol 1:1 (v/v). The second and third solvent used was isopropanol–hexane–water 55:25:20 (v/v/v, upper phase removed by aspiration before use). Supernatants removed after centrifugation were pooled and dried under nitrogen stream at 40°C.

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3Note that we don’t claim that everyone will get absolutely identical spectra from different instruments, or even the same instrument operated in different labs, or that there won’t be some changes over time with a given instrument in a given lab. We did observe that they don’t vary much in the short term when sufficient numbers of scans are acquired and averaged under the same conditions. Thus, as is generally the case, an individual lab trying to apply this method should make its own standard curves, and ideally these should be updated periodically.
Separation of neutral and acidic lipids from RBL-CD1d cells
Neutral and acidic lipids were fractionated by anion-exchange chromatography on a small column (5 × 40 mm) of DEAE Sephadex A-25 (Yu and Ledeen 1972) in chloroform–methanol–water 30:60:8 (v/v/v); neutral lipids were eluted with 5 column volumes of this solvent, while the acidic lipid fraction was eluted with 0.8 sodium acetate in methanol. Both neutral and acidic fractions were dried; the latter was desalted by dialysis and dried by rotary evaporation.

Florisil fractionation of neutral GSLs from CHO cells
The method of Saito and Hakomori (1971) was employed for removing non-GSL impurities from the neutral GSL fraction. The DEAE Sephadex A-25 pass-through fraction was dried under vacuum over P2O5 and peracetylated with 4 mL pyridine and 2 mL acetic anhydride in the dark at room temperature overnight. The peracetylated material was dried by Speed-Vac (Thermo-Savant), with the addition of 2 mL toluene three times to ensure complete evaporation. A Florisil column (30–60 mesh, 10 × 80 mm) was equilibrated in 1,2-dichloroethane–hexane 4:1 (v/v), and the peracetylated sample was applied in this solvent; the column was then washed with 100 mL of the same solvent, followed by 100 mL 1,2-dichloroethane. Neutral peracetylated GSLs were eluted with 200 mL 1,2-dichloroethane–acetone 1:1 (v/v), dried by rotary evaporation, and deacetylated with 5 mL 0.5 M sodium methoxide in 10 mL methanol for 3 h at room temperature. The mixture was neutralized with methanolic acid, dried by Speed-Vac, desalted by dialysis, and permethylated as described below.4

Per-N,O-methylation of neutral GSLs
A modification of the method of Ciucanu and Kerek (Ciucanu and Costello 2003; Ciucanu and Kerek 1984) was employed for per-N,O-methylation of GSLs. GSLs (1–20 µg) were introduced into a conical-glass vial, and dimethyl sulfoxide (150 µL) was added without using special drying conditions or inert gas atmosphere. Powdered sodium hydroxide (40–60 mg), ground with an agate mortar and pestle, dried at 100°C in an oven, and measured out approximately on the end of a microspatula, was added to the sample solution, and was stirred at room temperature until completely dissolved. Iodomethane (80 µL) was added with a syringe, and the mixture was vortexed at room temperature for 1 h. The methylation reaction was quenched with 2 mL water. The permethylated products were extracted 3 × by addition of dichloromethane (2 mL); the combined dichloromethane extracts were then washed 3 × with water (2 mL). Following the final wash the supernatant was transferred to a new tube and dried under nitrogen stream at 35–40°C.

Electrospray ionization-ion trap-mass spectrometry (ESI-IT-MSn) of permethylated neutral GSLs
Electrospray ionization mass spectrometry (MS and MSn) was carried out in positive ion mode on a linear ion trap mass spectrometer (LTQ, ThermoFinnigan, San Jose, CA), using a nanoelectrospray source (in later experiments, samples were measured out approximately on the end of a microspatula, was added to the sample solution, and was stirred at room temperature until completely dissolved. Iodomethane (80 µL) was added with a syringe, and the mixture was vortexed at room temperature for 1 h. The methylation reaction was quenched with 2 mL water. The permethylated products were extracted 3 × by addition of dichloromethane (2 mL); the combined dichloromethane extracts were then washed 3 × with water (2 mL). Following the final wash the supernatant was transferred to a new tube and dried under nitrogen stream at 35–40°C.

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4The GSL contents of all three fractions were compared by HPTLC (E. Merck, Silica Gel 60, chloroform-methanol-water 60:35:8 [v/v/v], detection by Bial’s orcinol reagent) before permethylation; GSLs were always observed to be in the third fraction.

Semiquantitative analysis of iGb3 in the iGb3/Gb3 mixture
Mixtures of isobaric d18:1/26:0 iGb3 and Gb3 were prepared at different percentages of Gb3, including 0.00, 0.87, 1.8, 5.3, 14.3, 33.3, 60.0, 94.3, 100% iGb3. These mixtures (1 µg each) were permethylated and subjected to ESI-IT-MS analysis as described above (see Figure 2). The relative abundances of major product ions appearing in the MS4 spectra (1355 → 667 → 445→) were plotted with respect to the percentage of iGb3 in these mixtures. Specifically, plots were made using abundance ratios of the ions m/z 211, 371, 329, 413, and 415, as follows: A(iGb3)211/[A(iGb3)211 + A(Gb3)329]; A(iGb3)371/[A(iGb3)371 + A(Gb3)329]; A(iGb3)311/[A(iGb3)311 + A(Gb3)311]; and A(iGb3)415/[A(iGb3)415 + A(Gb3)415]. The calibration line for the MS1 profile spectrum, compared to that of a known amount of the d18:1/8:0 iGb3 internal standard with S/N in the same range. It is assumed that iGb3 and Gb3 will not differ significantly in ion yield after permethylation, and the contribution of the fatty-N-acyl chain length will be relatively minor (see the Discussion section). Note that for molecular adducts differing interface for direct infusion of samples dissolved in methanol, with a flow rate of 0.30 µL/min and at capillary temperature 230°C, with injection time 100.00 ms, activation time 30 ms, activation Q-value, 0.250, isolation width m/z 1.5, acquisition time 3 min. Normalized collision energies were set to leave a minimal residual abundance of precursor ion; in this case 30% was used for all product ion scans. All ions were detected as sodium adducts. To obtain MS4 spectra specifically from the characteristic nonreducing Galα3/4Gal-1-en disaccharides, each iGb3/Gb3 molecular species (or isomer, observed at, e.g., m/z X), was subjected to multistep fragmentation via the glycan fragment m/z 667 and the terminal disaccharide 1-enone ion m/z 445 (i.e., the MS3 pathway corresponding to Scheme 1, X→667→445→). The patterns of MS4 product ions from sodiated molecular ions of pure permethylated Gb3 and iGb3 standards were acquired first (Figure 1, Panels A and B, respectively), followed by standard mixtures of isobaric Gb3 and iGb3.

Quantitation of iGb3 in the iGb3/Gb3 mixture
Mixtures of isobaric d18:1/26:0 iGb3 and Gb3 were prepared at different percentages of Gb3, including 0.00, 0.87, 1.8, 5.3, 14.3, 33.3, 60.0, 94.3, 100% iGb3. These mixtures (1 µg each) were permethylated and subjected to ESI-IT-MS analysis as described above (see Figure 2). The relative abundances of major product ions appearing in the MS4 spectra (1355 → 667 → 445→) were plotted with respect to the percentage of iGb3 in these mixtures. Specifically, plots were made using abundance ratios of the ions m/z 211, 371, 329, 413, and 415, as follows: A(iGb3)211/[A(iGb3)211 + A(Gb3)329]; A(iGb3)371/[A(iGb3)371 + A(Gb3)329]; A(iGb3)311/[A(iGb3)311 + A(Gb3)311]; and A(iGb3)415/[A(iGb3)415 + A(Gb3)415]. The calibration line for the MS1 profile spectrum, compared to that of a known amount of the d18:1/8:0 iGb3 internal standard with S/N in the same range. It is assumed that iGb3 and Gb3 will not differ significantly in ion yield after permethylation, and the contribution of the fatty-N-acyl chain length will be relatively minor (see the Discussion section). Note that for molecular adducts differing

The amount of triglycosylceramide (essentially composed of iGb3/Gb3) in the neutral fraction was calculated from the total ion abundance of all identified iGb3/Gb3 molecular species in the MS1 profile spectrum, compared to that of a known amount of the d18:1/8:0 iGb3 internal standard with S/N in the same range. It is assumed that iGb3 and Gb3 will not differ significantly in ion yield after permethylation, and the contribution of the fatty-N-acyl chain length will be relatively minor (see the Discussion section). Note that for molecular adducts differing

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by m/z 2 (e.g., a pair of lipofoms differing with respect to unsaturation/saturation of the fatty-N-acyl group), the calculated contribution of the M+2+Na isotope peak of the unsaturated lipofom had to be subtracted from the apparent signal M+Na of the saturated lipofom to get the true abundance of the latter (e.g., for molecules with ~60 carbon atoms, the relative abundance of M+2 peaks is ~20% that of M). The approximate percentage of iGb3 in each triglycosylceramide species was then determined separately from analysis of MS4 product ion spectra as described above. The total percent iGb3 in the sample was obtained by summation of the percent abundance of individual triglycosylceramide molecular species (out of the total GSL signal) multiplied by the percentage of iGb3 found in each. It was essential to consider individual isobaric triglycosylceramide species individually, since the percentage of iGb3 in each cannot be assumed to be identical (it was observed that, in contrast, significant differences could be found). The LOD is here defined as the lowest amount of iGb3/Gb3 giving rise to a molecular ion signal visible above noise. The LOQ is defined as the lowest percentage of iGb3 in any iGb3/Gb3 isobar that can be reliably determined.

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Conflict of interest statement

None declared.

Abbreviations

GSL, glycosphingolipid; Gb3, globotriaosylceramide; iGb3, isoglobotriaosylceramide; LOD, limit of detection; LOQ, limit of quantitation; MALDI, matrix-assisted laser desorption ionization; MS/CID-MS, tandem collision-induced dissociation; NKT, natural killer T cells; QIT, quadrupole ion trap; TOF, time of flight.

References


