Comparison of Glycolipid Compositions of NKT Cell Lines with those of NK and T Cells

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ABSTRACT

Glycolipid compositions of NK1.1⁺TCR $\alpha\beta$ ⁺ T (NKT) cell lines were analyzed to predict the cell lineage of NKT cells. NKT cell hybridomas produced by fusion of mouse thymic NKT cells with thymoma, BW5147, either with or without V α 24-J α 281 rearrangement in their T cell receptor genes were metabolically labeled with [¹⁴C]-galactose and -glucosamine hydrochloride and their glycolipid compositions were examined by high performance thin layer chromatography. Several glycolipid components that were helpful for diagnosis were found in the NKT cell lines. They expressed components specific for natural killer (NK) and/or cytotoxic T cells. Expression of asialogangliotetraosylceramide (AsGM₁), a typical NK cell marker, was also helpful for the diagnosis. As the expression of AsGM₁ in the NKT cell hybrids were not enough for the quantitative analysis by TLC, mouse liver NKT, conventional T and NK cells *ex vivo* were analyzed for the comparison of AsGM₁ level by means of immunofluorescence flow cytometry. The average of AsGM₁ expression in the NKT cells was much less than that in the classical NK cells but was slightly higher than that in the conventional T cells. Taken together, glycolipids remarkable in both T and NK cells were observed in the NKT cell lines, suggesting that NKT cells diverge between NK and T cells during ontogeny as predicted by the analyses of their cell surface markers so far reported.

NK1.1⁺TCR $\alpha\beta$ ⁺ T (NKT) cells are a newly characterized subset of mature cells that co-express marker molecules for both T and NK (natural killer) cells including each antigen receptor (4, 6, 19, 31). NKT cells express a very limited TCR repertoire with an invariant α -chain (V α 14-J α 281) (18, 21) and three frequently used V β domains (V β 8, 7 and 2) (2), and this

restricted TCR repertoire is considered to recognize a conserved ligand composed of the nonpolymorphic CD1 (5). On the other hand NKT cells are capable of being induced to kill tumor cells in the same manner as classical NK cells after exposure to cytokines such as IL-2, IL-4 and IL-12 *in vitro* (3, 15, 30). NKT cells exhibit distinct responses toward stimulation through T and NK cell receptors; when their TCRs are cross-linked by anti-CD3 monoclonal antibody (mAb), they secrete both IL-4 and IFN- γ , whereas upon exposure to anti-NK1 antibodies, they produce IFN- γ but no IL-4 (1). This particular feature of them provide a hypothesis that NKT cells may play important roles in immune regulation such as Th1, Th2 cell differentiation. Collectively, these findings on NKT cells suggest that they possess characteristics for both T

Abbreviations: NK, natural killer; NKT, NK1.1⁺TCR $\alpha\beta$ ⁺T; AsGM₁, asialogangliotetraosylceramide (Gg₄ Cer); CTL, cytotoxic T lymphocyte; NK, natural killer; γ_c , common cytokine receptor γ ; mAb, monoclonal antibody; HPTLC, high performance thin layer chromatography

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and NK cells in addition to those of their own. However, the cell lineage of NKT cells is not clearly understood.

Glycoconjugates are one of the major cell membrane components and have been implicated in a number of functions involved in cell-cell interaction, cell proliferation and cell recognition (11, 31). Moreover, several glycolipids have been reported to be a convenient marker to predict a lineage of lymphoid cells, for example, asialoganglioside (AsGM₁) for murine NK cells (14, 33), GM_{1b} for T cell blast (22), GD_{1a} and GD_{1c} for Th1 cells (9, 23), and GD_{1a} for Th2 cells (9).

In this study, the glycolipid compositions of cloned T-T hybrids derived from NKT cells (26) were analyzed and were compared with those of T and NK cells to predict the cell lineage of NKT cells.

MATERIALS AND METHODS

Mice.

C57BL/6 and *scid/scid* on BALB/c background mice were purchased from Japan SLC Inc. (Japan).

Cells.

Thymic NKT cell hybridomas, produced by fusion with BW5147 thymoma (7) as previously described (26), were given by Drs H. R. MacDonald and T. Ohteki (Ludwig Institute for Cancer Research, Lausanne Branch). A cytotoxic T cell clone, OE4 (28), and a thymoma, EL4 (10), derived from C57BL/6 mice were presented by Dr H. Takayama (Mitsubishi Kasei Institute of Life Sciences). NK cells were prepared from fetal liver of BALB/c (*scid/scid*) mice as described by Orihuela *et al.* (25). Briefly, nylon wool nonadherent fetal liver mononuclear cells were cultured in the medium supplemented with the culture supernatant of PMA-stimulated EL-4 cells (10%) for 3 days. The cells strongly adhered to the plastic substratum were removed by panning and the resultant cells were cultured for 3 more days and used for metabolic labeling.

Metabolic labeling and fractionation of cells.

Cells of several lines and NK cells in the primary culture (1×10^7) were labeled with [$1\text{-}^{14}\text{C}$]-Gal (1.9 GBq/mmol, Du Pont NEN, USA) and [$1\text{-}^{14}\text{C}$]-

GlcNH₂-HCl (1.9 GBq/mmol, Du Pont NEN) at a concentration of 37 kBq/mL in a glucose-free DMEM (Gibco BRL, USA) supplemented with 10% FCS for 18h. For the labeling of OE4 and NK cells, the culture supernatant of EL4 (10%) was also added to the medium. Cells were washed twice with Hanks' balanced salt solution, once with PBS, then extracted with chloroform/methanol (2:1 and 1:2) sequentially as described previously (27). Glycolipids in the extracts were analyzed by high-performance thin-layer chromatography (HPTLC) (Kiesel gel 60, Merck, Germany) after removal of neutral lipids by wash with n-hexane. Immunostaining of TLC plates were done as reported (13). The residues were used to analyze the glycoprotein compositions by SDS-polyacrylamide gel electrophoresis (PAGE) under the conditions of Laemmli (16).

IL-4 assay. Cells (10^4) were placed on a 96 well plastic plate precoated with anti-CD3 antibodies (2C11, 0.1 and 1 $\mu\text{g/mL}$) (19) and cultured for 48 h. Culture supernatants were analyzed for the production of IL-4 using an ELISA kit (Endogen, USA).

Immunofluorescence staining of liver cells and flow cytometric analysis.

C57BL/6 liver lymphoid cell fraction was prepared as previously described (24). Briefly, livers were pressed through stainless steel mesh and suspended in DMEM. Cells were washed once with DMEM, then fractionated by discontinuous (40 and 80%) Percoll gradient centrifugation for 10 min at 900g. The interface was harvested and washed with DMEM including 5% FCS and use for immunofluorescence staining. Cells were immunostained on ice in Hanks' balanced salt solution supplemented with 3% FCS and 0.025% sodium azide. Flow cytometric analysis was conducted with a FACScan and the CELL Quest program (Becton Dickinson, USA). The following mAbs were used: H57-597-PE (anti-TCR C β ; Caltag Laboratories, USA), PK136-biotin (anti-NK1.1; Pharmingen), rabbit anti-AsGM₁ antiserum (Wako Pure Chemical Industries, Japan) and goat anti-rabbit IgG-FITC (Vector Laboratories, USA). Biotin-labeled antibodies were developed by streptavidin-labeled Tri-color (Caltag Laboratories).

RESULTS

It is of great interest to determine the cell lineage of NKT cells, belonging to a newly discovered T cell repertoire characterized by the expression of both T and NK cell surface markers (4, 6, 19, 31). To address further this question, cell lines derived from NKT cells were analyzed with regard to their glycoconjugate compositions to compare them with those of T and NK cells. In Table I are summarized the cell lines used for the present study. Six representative hybridomas derived from thymocytes with a phenotype of NK1.1^+ , $\text{TCR } \alpha\beta^+$, were used, all of them except for RT8

have a TCR α chain with a canonical sequence ($\text{V}\alpha 14\text{-J}\alpha 281$) (18, 21) characteristic for NKT cells, although their $\text{V}\beta$ usage is different ($\text{V}\beta 8$ or $\text{V}\beta 2$), whereas RT8 has a TCR α chain with a rearrangement other than $\text{V}\alpha 14$ ((26) and this study). Although they have different TCRs, all possess a potency to secrete IL-4 following stimulation to their TCR as shown in Fig. 1, thus suggesting that a functional feature characteristic for NKT cells is preserved in the most of the hybridomas. RT21 is less responsive to stimulation through TCR in spite of its possession of a TCR α chain with $\text{V}\alpha 14\text{-J}\alpha 281$ rearrangement, but its transcription was less than others in the

Table I Cell lines used in this study

	Cell lines	V α 14-J α 281 ^(a)	V β ^(b)	Reference
RT2	thymic NKT cell hybridoma	+	8	26
RT5	thymic NKT cell hybridoma	+	8	26
RT8	thymic NKT cell hybridoma	—	8	26
RT21	thymic NKT cell hybridoma	+	8	26
RT23	thymic NKT cell hybridoma	+	8	26
RT24	thymic NKT cell hybridoma	+	2	26
BW5147	thymic T lymphoma	—	—	7
OE4	cytotoxic T cell clone	—	8	28
EL4	thymic T lymphoma	—	nd	10

a) TCR gene $\text{V}\alpha 14\text{-J}\alpha 281$ rearrangement

b) Surface expression of TCR $\text{V}\beta$, —, absence of surface expression of TCR molecules
nd, not determined

Fig. 1 IL-4 production of NKT cell hybridomas following stimulation with anti-CD3 mAb (2C11). Cells were cultured for 48h on a plastic plate pre-coated with anti-CD3 mAb (0.1 and 1 $\mu\text{g/mL}$), and the culture supernatants of the cells were assayed for IL-4 by ELISA. Recombinant IL-4 was used for the standard.

analysis by RT-PCR method (data not shown). OE4 and EL4 cells were used as references for T cells, and primarily cultured NK cells isolated from lymphocyte-deficient mice (scid mice) were used for the reference of NK cells since no appropriate NK cell lines were available.

These cells were extracted with the mixture of chloroform and methanol (2 : 1 and 1 : 2) and the extracts were used for the analysis of their glycolipid compositions. Fig. 2 shows HPTLC analyses of glycolipids expressed by the NKT cell hybridomas and their referential cell lines. Several components (numbered in the figure) that were absent in the parental cell line, BW5147, were helpful for diagnosis of the cell lineage of the original NKT cells fused with BW5147, although identification of these glycolipid species were difficult in some cases because of the discord of their Rf values to those of the references isolated from bovine brain (for gangliosides) and porcine blood cells (for neutral glycolipids) on the HPTLC analysis. The expression of the component 1 (just lower than Gb4) and 3 (near to GM1) was remarkable in the NK cells, and both components were shared by all the NKT cell lines. The component 4 that was augmented in a cytotoxic T cell clone, OE4, was expressed by RT5, 21, 23 and 24, but was slightly found in RT2 and 8. On the other hand the distribution of the component 5 characteristic for a T lymphoma, EL4, was reciprocal to that of the component 4. These observations suggest that NK T cells are apparently heterogeneous in terms of not only TCR V usage but also of glycolipid expression.

An attention was then paid to examine the expression of AsGM₁ in these NKT cell lines since it is known that this neutral glycosphingolipid is constitutively expressed in a large amount in NK cells (14, 33) but not so much in T cells except for in those in an activated state (29). Expression of AsGM₁ analyzed by a immunostaining of a TLC plate was observed in the NK and OE4 cells that had been activated with IL-2, but its expression was only detectable after longer exposure of the TLC plate in the NKT cell lines and also even in a LGL-like NK clone, SPB2.4 (12) (data not shown). To get more information on the expression of AsGM₁ in NKT cells, they were freshly prepared from C57BL/6 adult livers and were analyzed for the expression of the glycolipid by flow cytometry. Fig. 3 shows the expression of AsGM₁ in classical NK (TCR $\alpha\beta$ ⁻ NK1.1⁺), NKT (TCR $\alpha\beta$ ⁺ NK1.1⁺), conven-

tional T (TCR $\alpha\beta$ ⁺ NK1.1⁻) and TCR $\alpha\beta$ ⁻ NK1.1⁻ cells. Most NKT and conventional T cells were dull positive and only a part of these cells were brightly stained as NK cells were, although the average level of AsGM₁ expression in NKT cells was slightly higher than that in conventional T cells, suggesting that NKT cells are near to conventional T cells in terms of the expression of AsGM₁.

The residues obtained during the chloroform-methanol extraction of the cells labeled with [¹⁴C]-Gal and -GlcNH₂-HCl were used for the analysis of glycoproteins by SDS-PAGE, and it was found that the expression patterns of glycoproteins in the NKT cell hybridomas were apparently similar to each other, and the components found in them were almost shared by BW5147, indicating that their glycoprotein compositions were less informative than their glycolipid compositions (data not shown).

DISCUSSION

Although biological functions of glycoconjugates are not fully understood, it has been known that alteration of glycoconjugate compositions sometimes well reflect physiological changes or progress of cell differentiation. Particularly, compositional changes of glycolipid rather than glycoprotein are facile to detect because glycoproteins highly associated to certain physiological function are generally minor components and changes in their amounts are masked by other large amount of house-keeping components. Glycoconjugate compositions of NKT cell hybridomas (26) were analyzed in this study since this strategy is only available at present to obtain some informations on the compositions of such a newly characterized T cell repertoire, NKT cell, in a clonal level. As shown in Figure 2 (d), the NKT cell lines contain components remarkable to NK and/or T cells, thus suggesting that NKT cells are developmentally divided from a different between T and NK lineage. In addition, component 2 and 6 were found to be uniquely expressed by the NKT cell lines, implying that NKT cells were located at their own status in the lymphocyte lineage.

There seemed to be no apparent relationship between glycolipid composition and either TCR structure (Table I), CD4 expression (RT2, 23 and 24 are CD4 negative and RT5, 8 and 21 are CD4 positive (26)) or preserved potency to secrete IL-4

Fig. 2 Glycolipid composition of NKT cell hybridomas. Glycolipids metabolically labeled with [14 C]-Gal and GlcNH $_2$ -HCl were analyzed by HPTLC (Kieselgel 60). The HPTLC plate was developed in the mixture of chloroform, methanol and water including 0.02% CaCl $_2$ ((a) 65 : 35 : 8, (b) 50 : 45 : 10, (c) 55 : 40 : 10, and the components were visualized by an image analyzer (Bas 2,000, Fuji Film Co., Japan). NKT cell hybrid cell lines, RT2, RT5, RT8, RT21, RT23, RT24, their fusion partner, BW5147, NK cells in the primary culture, thymoma, EL4 and CTL clone, OE4, were analyzed. Abbreviations: CMH, ceramide monohexoside; PE, phosphatidylethanolamine; CDH, ceramide dihexoside; Sul, sulfatide; PC, phosphatidylcholine; CTH, ceramide trihexoside; Gb4, globotetraoside ceramide. (d) The expression of diagnostic components in the NKT cell lines are summarized. Abbreviations: OE4, EL4, or NK, glycolipids that are expressed in OE4, EL4 or NK cells but are absent in BW5147; U, components unique for the NKT cell lines. Note that not only glycolipids but also phospholipids were labeled with [14 C]-Gal, -GlcNH $_2$ and their metabolites under the present labeling conditions.

(d) Expression of diagnostic glycolipid components

Diagnostic components	RT2	RT5	RT8	RT21	RT23	RT24
1 NK	+	+	+	+	+	+
2 U	+	+	+	+	+	+
3 NK	+	+	+	+	+	+
4 OE4		+		+	+	+
5 EL4	+		+			
6 U		+		+	+	+

in the NKT cell lines. One possible interpretation of these data may be that thymic NKT cells in the criteria of the phenotype (TCR $\alpha\beta^{\text{low}}$, NK1.1 $^{+}$) and the responsiveness to IL-7 are positively selected by a common ligand (CD1 + an antigen) and originally possessed a common glycolipid composition irrespective of their TCR V- (D) -J usage and CD4 expression, and after fusion with BW5147, some chromosomes have been lost in RT2 and 8 on which several genes for glycosylation enzymes are coded.

Another key to predict the cell lineage of NKT cells was given by the analysis of the expression of AsGM $_1$ in NKT cells prepared from mouse livers (Fig. 3). NKT cells *ex vivo* showed much less constitutive expression of AsGM $_1$ than classical NK cells did but on an average they expressed slightly more AsGM $_1$ (peaked at the 120th channel of the fluorescence intensity in the FACS analysis) than most conventional T cells (peaked at the 70th) did except for the cells in a small peak at about the 600th channel (these cells were presumably in an activated state (29)). This observation also implies that NKT cells are characterized as cells in a lineage between T and NK cells. AsGM $_1$ has long been known as a cell surface marker for NK cells (14, 33), although its biological significance is not clear. Chen *et al.* reported that the activation of a cell membrane T cell specific neu-1 sialidase is required for the generation of IL-4 in spleen T cells following TCR-mediated signaling which results in the increase of the amount of membrane AsGM $_1$ (8). It may thus be possible to speculate that high expression of AsGM $_1$ in NK cells is maintained by a constitutive activation of a NK cell specific

membrane sialidase so far unidentified. A slight increase of the basal level of AsGM $_1$ observed in the liver NK T cells might be due to the limited function of such a NK cell specific sialidase.

Recently, Lantz *et al.* found a new thymic T cell population in mice deficient for the common cytokine receptor γ (γ_c). These T cells expressed the V α 14-J α 281 invariant α chain and were able to secrete IL-4 following TCR engagement but did not co-express the NK markers such as NK1.1 and Ly49. The presence of this population suggests that the development of NKT cells occurs in a step-wise manner. These cells would get matured and finally possess features of both T and NK cells suppose that they could be given a signal through γ_c (17). It seems of great interest to analyze glycolipid compositions of these V α 14 $^{+}$, NK1.1 $^{-}$ thymic T cells in such mutant mice, presumably their components are more T cell-like than the compositions of mature NKT cells are.

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Fig. 3 Expression of AsGM₁ in C57BL/6 mouse liver lymphoid cells revealed by a 3 color FACS analysis. Liver lymphoid cells enriched by Percoll gradient were separated into NK cells (Region 1), NKT cells (Region 2), conventional T cells (Region 3) and non NK, non T cells (Region 4) in terms of the expression of NK1.1 and TCR $\alpha\beta$. The AsGM₁ expression in the cells of each region is shown in the histograms. The negative control level determined by the cells stained only with the FITC-conjugated second antibodies is shown with a broken line in each histogram.

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