Glycosphingolipids of Rat T Cells. Predominance of Asialo-GM1 and GD1c†

Keiko Nohara,*,‡,§ Hiromitsu Nakauchi,‖ and Sarah Spiegel⊥

Environmental Health Sciences Division, National Institute for Environmental Studies, Onogawa, Tsukuba, Ibaraki 305, Japan, Department of Immunology, Institute of Basic Medical Science, University of Tsukuba, Tsukuba, Ibaraki 305, Japan, and Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D.C. 20007

Received November 24, 1993; Revised Manuscript Received February 7, 1994®

ABSTRACT: Glycosphingolipids play an important role in the immune response, yet their compositions in T and B cells which mediate cellular and humoral immunity, respectively, have not been elucidated. In this study, characteristic features of glycosphingolipids in rat T lineage cells were revealed by comparing the gangliosides and neutral glycolipids of spleen T- and β-cell-enriched fractions and thymocytes. In T cells, GD1c(NeuGc,NeuGc), a unique ganglioside synthesized through asialo-GM1 (GA1), was the predominant ganglioside as previously found in thymocytes [Nohara, K., Suzuki, M., Inagaki, F., & Kaya, K. (1991) *J. Biochem. (Tokyo) 110*, 274–278], and the amount was much higher than in thymocytes. In addition, three other GA1-derived gangliosides were detected in T cells and identified as GM1b(NeuAc), GM1b(NeuGc), and GD1α(NeuAc,NeuAc). In contrast, GD1α(NeuAc,NeuAc) was not discernible in thymocytes, although gangliosides corresponding to GM1b(NeuAc) and GM1b(NeuGc) were detected. The neutral glycolipids of T cells contained almost exclusively GA1, while thymocytes contained much lower amounts. The predominance of these GA1-derived gangliosides was confirmed as a singular feature of T lineage cells by comparison with gangliosides of spleen B-cell-enriched fractions which mainly consisted of gangliosides synthesized through GM3 and GM1. Furthermore, the unique structures, which contain the GM1 core and the extended modification of the lacto series, αGal-LacNAc-GM1, αGal-$(LacNAc)_2$-GM1, and sialyl-LacNAc-GM1, were found in B-cell-enriched fractions. Unexpectedly, the neutral glycolipid composition of the thymocytes resembled that of the B-cell enriched fraction rather than that of the T cells.

Glycosphingolipids, ubiquitous constituents of the plasma membrane of all eukaryotic cells, may play a role in the regulation of the immune response. This concept of immunoregulatory properties of glycosphingolipids developed from the demonstrations that gangliosides suppressed the proliferation of a variety of T and B lymphocytes induced by lectins, antigens, and interleukin-2 (Miller & Esselman, 1975; Marcus, 1984; Chu & Sharom, 1991; Jackson et al., 1987), as well as inhibition of T helper cells and cytotoxic effector function (Offner et al., 1987). Studies from Miller and Esselman (1975) raised the possibility that glycosphingolipids shed by antigen-stimulated T cells modulated humoral immune responses (Miller & Esselman, 1975). The potent immunosuppressive activity of specific gangliosides together with the observation that high concentrations of gangliosides are present in patients with certain types of tumors led to the suggestion that cellular immunodeficiency and associated diminished T cell responses frequently associated with cancer are due to shedding of gangliosides by tumors [reviewed in Ladisch et al. (1992)]. Furthermore, ganglioside GM1 also modulated CD4 surface expression on T cells (Offner et al., 1987; Grassi et al., 1990; Morrison et al., 1993). Recently, it was also shown that GM1-induced CD4 down regulation (Repke et al., 1992) was accompanied by CD4 degradation, which was preceded by dissociation of $p56^{lck}$ from CD4, and via a protein-kinase-C-independent pathway (Saggioro et al., 1993).

To gain further insight into the functions of gangliosides in lymphocyte activation, ganglioside-binding proteins, such as toxins (Spiegel et al., 1985; Spiegel, 1989; Olivera & Spiegel, 1992) or antiganglioside antibodies (Hakomori, 1990; Hersey et al., 1989), which interact specifically with individual gangliosides on the cell surface, were used. We have previously extensively used the B subunit of cholera toxin (CTB),[1] which binds with very high affinity to ganglioside GM1, to accumulate important clues to the role of ganglioside GM1 in cellular proliferation and signal transduction (Spiegel et al., 1985; Spiegel, 1989; Dixon et al., 1988; Olivera & Spiegel, 1992). CTB was mitogenic for rat thymocytes (Spiegel et al., 1985; Dixon et al., 1987) and for resting human B lymphocytes (Dugas et al., 1991) and also has immunomodulatory properties as it potentiates the thymus-dependent antibody response (Campbell & Munson, 1987). In contrast, binding of CTB to murine leukemia cell lines inhibited their prolif-

† This study was supported in part by a Grant-in-Aid for Scientific Research (No. 04780192) from the Ministry of Education, Science and Culture of Japan to K.N. and (1 R 29 GM 39718) from the National Institutes of Health to S.S.

* To whom correspondence should be addressed.

‡ National Institute for Environmental Studies.

§ Present address: Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, 313 Basic Science Building, 3900 Reservoir Rd. NW, Washington, DC 20007. (202)687-1444.

‖ University of Tsukuba.

⊥ Georgetwon University Medical Center.

® Abstract published in *Advance ACS Abstracts*, March 15, 1994.

[1] Abbreviations: CTB, cholera toxin B subunit; mAB, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin. Abbreviations for glycosphingolipids: GA1, asialo-GM1; GA2, asialo-GM2; CDH, lactosylceramide; αGal,Fuc-GA1, Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; GM1b, sialylα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; GalNAc-GM1b, GalNAcβ1-4(sialylα2-3)Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; GD1α, sialylα2-3Galβ1-3(sialylα2-6)GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; GD1c, sialylα2-8sialylα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; LacNAc-GM1, Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcβ1-4(NeuGcα2-3)Galβ1-4Glcβ1-1′Cer; αGal-LacNAc-GM1, Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcβ1-4(NeuGcα2-3)-Galβ1-4Glcβ1-1′Cer; αGal-$(LacNAc)_2$-GM1, Galα1-3$(Galβ1-4GlcNAcβ1-3)_2$Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; sialyl-LacNAc-GM1, NeuGcα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GalNAcβ1-4(NeuGcα2-3)Galβ1-4Glcβ1-1′Cer; globoside, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer. Other gangliosides are designated according to Svennerholm (1963). Sialic acid species are indicated as (A) for NeuAc and (G) for NeuGc.

0006-2960/94/0433-4661$04.50/0

eration (Pessina et al., 1989) and also inhibited mitogen- and antigen-induced T cell proliferation and anti-IgM-induced B cell proliferation of mouse splenocytes (Woogen et al., 1987). Further studies revealed that while binding of recombinant CTB subunit inhibited mitogen-stimulated B cell proliferation, it enhanced the expression of MHC class II (Ia) molecules (Francis et al., 1992), which provides a potential explanation for its ability to act as an immunoadjuvant. Others have found that monoclonal antibodies (mAb) against GD3, a human melanoma-associated ganglioside, stimulate proliferation of T lymphocytes derived from peripheral blood and also potentiate lymphocyte proliferation in response to a variety of stimuli, such as phytohemagglutinin, interleukin-2, and and T3 antigen, amplifying the normal activation pathway (Hersey et al., 1989).

Although these studies illustrate the importance of glycosphingolipids in the immune response, little information on the actual compositions of glycosphingolipids in T and B lymphocytes is available, which has hindered clarification of the molecular basis of the mechanism of their functions. Murine lymphocyte gangliosides have been shown to have peculiar structural features. In mouse splenic T lymphoblasts, which were activated with ConA and metabolically labeled with radioactive carbohydrates, GM1b, GalNAc-GM1b, and GD1α were detected (Müthing et al., 1987, 1989). A peculiar feature of these three gangliosides is that they are synthesized through asialo-GM1 (GA1) and GM1b and not through gangliosides GM3 and GM1. Previously, GM1b had only been found in rat ascites hepatoma cells (Hirabayashi et al., 1979; Matsumoto et al., 1981) and mouse spleen (Nakamura et al., 1984) and GD1α in rat ascites hepatoma cells (Taki et al., 1986). Recently, another GM1b-derived ganglioside, GD1c, was found to be the predominant ganglioside of rat and mouse thymocytes (Nohara et al., 1991; Nakamura et al., 1991). We also found a novel group of gangliosides containing LacNAc-GM1 structures as the major gangliosides of rat spleen lymphocytes (Nohara et al., 1990a, 1992). Moreover, in rat thymocytes, the composition of gangliosides is greatly altered after cell activation, and the compositional changes depend on the nature of stimuli (Nohara et al., 1993). In the present study, we determined the compositions of gangliosides and neutral glycolipids in resting spleen T cells, B-cell-enriched fractions, and thymocytes. Characteristic features of glycosphingolipids in T cells were revealed.

MATERIALS AND METHODS

Materials. RPMI 1640 medium and Dulbecco's phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceutical Co. (Tokyo). Dynabeads M-450 were from Dynal Inc. (Great Neck, NY). *Vibrio cholera* sialidase (type V) was from Sigma (St. Louis, MO). *Arthrobacter ureafacience* sialidase was from Nacalai Tesque, Inc. (Kyoto). mAbs against rat thymocytes and T cells (MRC Ox-34) and against rat B cells (MRC Ox-33) were from Serotec (Oxford). Rabbit antiasialo-GM1 was from Iatron Lab. (Tokyo). Biotinylated goat antirabbit IgG was from Caltag Laboratories Inc. (South San Franciso, CA). CTB and goat anti-CTB were from List Biological Laboratories (Campbell, CA). Biotinylated rabbit antigoat IgG and Vectastain ABC Kit were from Vector Laboratories (Burlingame, CA). Precoated thin-layer plates (HPTLC silica gel 60) for standard analyses were purchased from Merck (Darmstadt), and those (Si-HPF TLC plate silica gel) for immunostaining were from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Standard Glycolipids. GD1α(NeuAc,NeuAc) from frog brain (Ohashi, 1981) was kindly provided by Prof. M. Ohashi (Ochanomizu University). Globoside from porcine erythrocytes was purchased from Wako Pure Chemical (Osaka). Lactosylceramide (CDH) and GA1 were prepared from GM3-(NeuAc) and GM1(NeuGc) of rat spleen (Nohara et al., 1990b) by mild acid hydrolysis (Svennerholm et al., 1973), respectively. Asialo-GM2 (GA2) and αGal,Fuc-GA1 were prepared by hydrolyses of GM2(NeuGc) and αGal,Fuc-GM1-(NeuGc) from rat spleen, respectively, with *A. ureafacience* sialidase (Nohara et al., 1990b; Sugano et al., 1978).

Fractionation of Spleen Lymphocytes. Cell suspensions were prepared from spleens of adult male Wistar rats by chopping in RPMI 1640 medium supplemented with 12 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (complete medium), and the lymphocytes were obtained by centrifugation on Ficoll-Paque gradient as described previously (Nohara et al., 1992). After three washes with complete medium, the lymphocytes were fractionated using a column packed with nylon wool (Wako Pure Chemical, Osaka) according to Julius et al. (1973). T cells were obtained in the column pass through and treated with ammonium chloride/EDTA solution to eliminate contaminating red blood cells (Nohara et al., 1991). The adherent cells were recovered from the column by gentle shaking in PBS and used as the B-cell-enriched fraction after removing contaminating T cells by immunomagnetic negative selection. Briefly, cells were incubated with mouse mAb directed against rat T cells (MRC Ox-34) and subsequently rosetted with Dynabeads M-450 (sheep antimouse IgG) (cell:beads = 1:4), according to the manufacturer's protocol. The rosetted cells were then isolated by applying a magnet on the wall of the tube. Purities of T- and B-cell-enriched fractions were assessed by immunofluorescent staining with mAbs specific for rat T and B cells followed by flow cytometric analysis using either FACS440 or FACStar^{PLUS} (Becton Dickinson, San Jose, CA). Morphologic examination of Wright-Giemsa stained cytospin preparations was also performed to identify monocytes and large granular lymphocytes, referring to Reynolds et al. (1981).

Preparation of Thymocytes. Thymocytes were prepared from thymuses of 6–9-week-old male Wistar rats as described previously (Nohara et al., 1991).

Preparation of Gangliosides. Total glycolipids were prepared from T cells, B-cell-enriched fractions, and thymocytes by extraction, partition, and mild alkali treatment and then fractionated by DEAE-Sephadex column chromatography into the four fractions of neutral glycolipids and mono-, di-, and tri- and tetrasialogangliosides, exactly as described previously (Nohara et al., 1991).

Detection of Gangliosides with GA1 Core Structure. Gangliosides were separated on silica gel plates (Si-HPF) using 1-propanol/28% NH_4OH/water (75:5:25). The plates were fixed with 0.1% poly(isobutyl methacrylate) in chloroform and air-dried (Nohara et al., 1993). Gangliosides were hydrolyzed for 3 h at room temperature in situ with *V. cholera* sialidase (40 milliunits/mL) and then washed once with PBS (Müthing & Mühlradt, 1988). After immersion in PBS containing 1% bovine serum albumin (BSA) for 20 min, the gangliosides were subsequently overlaid with rat anti-GA1 (1:250 dilution) for 1 h, biotinylated antirabbit IgG (1:250 dilution) for 1 h, and Vectastain ABC solution for 1 h, and then, the peroxidase activity was detected with 4-chloro-1-naphthol/H_2O_2 solution (Hawkes et al., 1982).

Detection of Gangliosides That Bind to CTB. In order to identify the minor CTB-binding monosialogangliosides, the sensitive overlay technique was used (Spiegel et al., 1985;

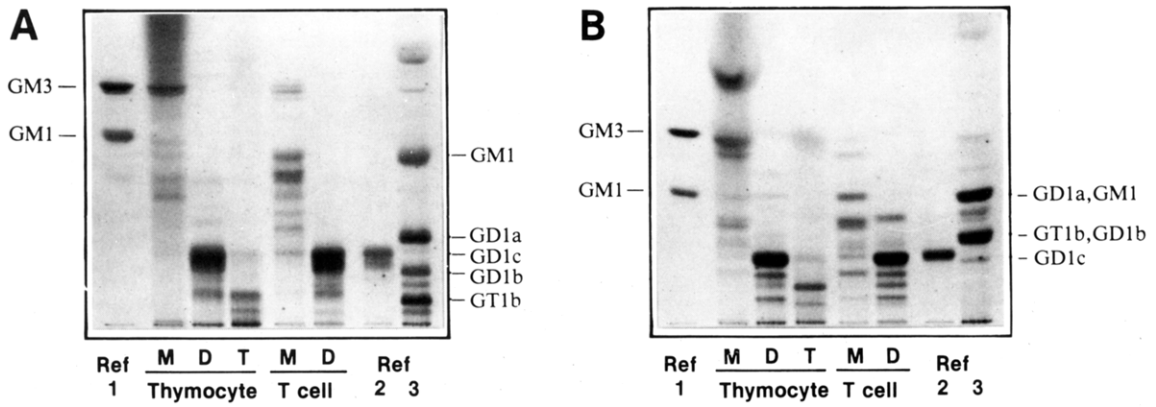

FIGURE 1: TLC profiles of gangliosides from thymocytes and T cells. The gangliosides from thymocytes and T cells were fractionated on a DEAE-Sephadex column into mono- (M), di- (D), and tri- and tetrasialogangliosides (T). Each fraction from thymocytes (8×10^8 cells) and T cells (8×10^7 cells) was applied to HPTLC silica gel 60 plates, developed with chloroform/methanol/0.2% $CaCl_2$ (60:40:9) (A) or 1-propanol/28% NH4OH/water (75:5:25) (B), and visualized with resorcinol reagent. Ref (reference glycolipids): 1, GM3(NeuAc) and GM1(NeuAc); 2, GD1c(NeuGc,NeuGc); 3, total gangliosides from rat brain.

Nohara, et al., 1993). Briefly, after gangliosides were chromatographed on Si·HPF TLC, the chromatogram was treated with poly(isobutyl methacrylate), overlaid with 1% bovine serum albumin in PBS for 20 min, CTB (1:500 dilution) for 1 h, goat anti-CTB (1:500 dilution) for 1 h, and then with biotinylated antigoat IgG (1:250 dilution) for 1 h. The bound toxin was detected by Vectastain ABC solution and 4-chloro-1-naphthol/H_2O_2 solution as mentioned above.

RESULTS

Isolation of Lymphocytes. The recoveries of the T cells after the nylon wool column procedure ranged from 20% to 40% of total splenic lymphocytes applied. The purity was consistently around 85%, containing about 10% B cells and a few percent non-T, non-B cells. The B-cell-enriched fraction, obtained from nylon-wool-adherent cells followed by immunomagnetic T cell depletion, consisted of B cells (~60%), T cells (~5%), and non-T, non-B cells (>30%), with a recovery of about 10%. Morphologic evaluation of the non-T, non-B fraction revealed the presence of only several percent monocytes and even fewer large granular lymphocytes, indicating that monocytes and granular lymphocytes are minor constituents of this fraction. The remaining non-T, non-B cells were not identified. Gangliosides and neutral glycolipids were prepared from T cells, the B-cell-enriched fraction, and thymocytes and identified by several techniques.

Gangliosides of Spleen T Cells and Thymocytes. Thymocytes contained gangliosides belonging to the mono-, di-, tri-, and tetrasialoganglioside families, wherease in spleen T cells, only mono- and disialogangliosides were detected (Figure 1). The content of gangliosides per cell was found to be about 10-fold higher in T cells than in thymocytes as shown in Figure 1, where the gangliosides from 8×10^8 thymocytes and those from 8×10^7 T cells are compared. Identical results were obtained in three preparations from thymocytes and two preparations from T cells. GM3 was the major monosialoganglioside in thymocytes, whereas it was only a minor constituent of the monosialoganglioside fraction from spleen T cells. In agreement with our previous studies, the predominant ganglioside of thymocytes was GD1c-(NeuGc,NeuGc) (Nohara et al., 1991). The present study revealed that T cells also contain GD1c(NeuGc,NeuGc) as the overwhelmingly abundant ganglioside. This was further confirmed by immunostaining with anti-GA1 antibody after in situ hydrolysis with *V. cholera* sialidase (Figure 2). In addition to GD1c, three prominent gangliosides, two mono-

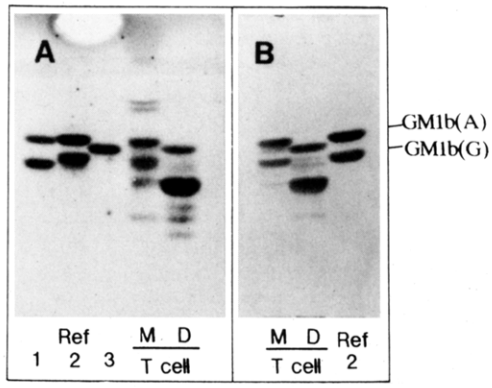

FIGURE 2: Detection of GA1 after in situ sialidase treatment. Monosialogangliosides (M) and disialogangliosides (D) from T cells (8×10^7 cells) were chromatographed on a Si·HPF TLC plate using 1-propanol/28% NH_4OH/water (75:5:25) and visualized with resorcinol reagent (A) or immunostained using anti-GA1 antibody after in situ sialidase treatment (B). Ref (reference glycolipids): 1, GM1(NeuAc) and GM1(NeuGc); 2, GM1b(NeuAc) and GM1b-(NeuGc) (300 ng of each as sialic acid); 3, GD1α(NeuAc,NeuAc).

sialogangliosides, and one disialoganglioside were detected in T cells with anti-GA1 antibody after sialidase hydrolysis (Figure 2B). The coincidence of the R_f values with those of standards identified the monosialogangliosides as GM1b-(NeuAc) and GM1b(NeuGc) and the disialoganglioside as GD1α(NeuAc,NeuAc). However, as shown in the chromatograms in Figure 1, GD1α(NeuAc,NeuAc) was not found in thymocytes, though gangliosides corresponding to GM1b-(NeuAc) and GM1b(NeuGc) could be detected.

Previously, we have shown that CTB induces proliferation of rat thymocytes by binding to monosialoganglioside GM1 (Spiegel et al., 1985; Dixon et al., 1987). Others have further substantiated a role for monosialogangliosides in T and B cell proliferation (Dugas et al., 1991; Woogen et al., 1987) suggesting an immunomodulatory role for these monosialogangliosides (Campbell & Munson, 1987; Francis et al., 1992). Thus, it was of interest to examine, by the sensitive overlay technique, the existence of monosialogangliosides that bind to CTB. There was about 10-fold higher CTB binding in T cells than in thymocytes as indicated in Figure 3, where gangliosides from 8×10^7 thymocytes and 8×10^6 T cells are compared. Three monosialogangliosides in thymocytes and four in T cells were detected by immunostaining with CTB (Figure 3A). In agreement with a previous study (Spiegel et al., 1985), two were identified as GM1(NeuAc) and GM1-

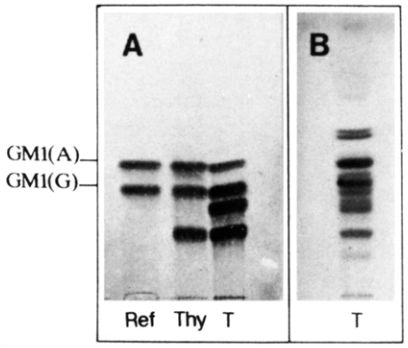

FIGURE 3: Detection of monosialogangliosides that bind to CTB. (A) Monosialogangliosides from thymocytes (Thy, 8×10^7 cells) and T cells (T, 8×10^6 cells) were chromatographed on a Si·HPF TLC plate with 1-propanol/28% NH_4OH/water (75:5:25) and immunostained with CTB as described in Materials and Methods. (B) Monosialogangliosides from T cells were visualized with resorcinol reagent. Ref (reference glycolipids): GM1(NeuAc) and GM1-(NeuGc) (12 ng of each as sialic acid).

(NeuGc) by coincidence with reference standards. From Figure 2A,B, the predominant band in the monosialogangliosides of T cells consisted of GM1b(NeuAc) and GM1b-(NeuGc) and the amounts of GM1(NeuAc) and GM1-(NeuGc) were significantly less than that of GD1c.

Gangliosides of the B-Cell-Enriched Fraction. In contrast to the T lineage cells, the most abundant ganglioside of the B-cell-enriched fraction was GM3(NeuAc) (Figure 4). Gangliosides corresponding to GM3(NeuGc) and GM1(NeuGc) were also found to be major monosialogangliosides. In addition, minor amounts of αGal-LacNAc-GM1 and αGal-$(LacNAc)_2$-GM1 were also detected, while LacNAc-GM1 was not evident. One of the major disialogangliosides coincided with sialyl-LacNAc-GM1. In addition to those gangliosides which are synthesized through GM3 and GM1, GD1c was the predominant disialoganglioside found in this B cell fraction.

Neutral Glycolipids of Thymocytes, T Cells, and the B-Cell-Enriched Fraction. In T cells, GA1 was nearly the exclusive neutral glycolipid present (Figure 5A). As shown in Figure 5B, using anti-GA1 antibody, both bands of the doublets were confirmed to be GA1, most probably arising due to acyl-chain heterogeneity. On the other hand, thymocytes contained only minor amounts of GA1, and the major glycolipids were identified as galactosylceramide (Figure 5A) and globoside (Figure 5C). A glycolipid corresponding to αGal,Fuc-GA1 was also recognized in thymocytes (Figure 5A). It should be noted that the amount of neutral glycolipids in thymocytes was much lower than that in T or B cells as shown in Figure 5A, where glycolipids from 1×10^9 thymocytes and those from 1×10^8 T cells or the B-cell-enriched fraction are compared. In the B-cell-enriched fraction, the glycolipids corresponding to GA1, αGalFuc-GA1, and globoside were the major neutral glycolipids (Figure 5A). These neutral glycolipids were also identified by thin-layer chromatography, based on mobilities in a different solvent system composed of chloroform/methanol/NH_4OH (60:35:8) (data not shown).

DISCUSSION

In this study, characteristic features of glycosphingolipids in rat T lineage cells were revealed by comparing the gangliosides and neutral glycolipids of spleen T- and B-cell-enriched fractions and thymocytes. Although previously we found that GD1c(NeuGc,NeuGc) was only a minor component of gangliosides of rat spleen lymphocytes (Nohara et al., 1992), it was clearly demonstrated in the present study that GD1c-(NeuGc,NeuGc) was the predominant ganglioside in splenic T cells. This ganglioside is also the predominant ganglioside of rat thymocytes (Nohara et al., 1991). In addition to GD1c-(NeuGc,NeuGc), three other GA1-derived gangliosides, GM1b(NeuAc), GM1b(NeuGc), and GD1α(NeuAc,NeuAc), were found in rat T cells in this study. Interestingly, GM1b-(NeuGc) is likely to be the precursor of GD1c(NeuGc,NeuGc) and GM1b(NeuAc) to be the precursor of GD1α(NeuAc,-NeuAc), since gangliosides GD1c(NeuGc,NeuAc) and GD1α-(NeuAc,NeuGc) were not detectable. Previously, GD1α has been detected in various T cell lines, in sorted CD4-positive and CD8-positive lymph nodes, but not in B cells (Müthing et al., 1989; Eble et al., 1992). However, only trace amounts of GD1α(NeuAc,NeuAc) were detected in murine thymocytes and found mainly in mature peripheral T blasts, suggesting that the synthesis of GD1α correlates with maturation or stimulation of T cells (Müthing et al., 1989). In agreement, in the present study, we found that GD1α(NeuAc,NeuAc) was not discernible in rat thymocytes, although gangliosides corresponding to GM1b(NeuAc) and GM1b(NeuGc) were readily detected. Thus, in rats, this ganglioside most probably is only expressed on mature T cells and perhaps only on certain populations of mature T cells.

Previously, we have demonstrated that CTB, which is known to interact specifically with the oligosaccharide of GM1 exposed on the cell surface, can induce proliferation of rat thymocytes which was accompanied by rapid influx of calcium (Spiegel et al., 1985; Dixon et al., 1987). In the present study, it was demonstrated that there are three monosialogangliosides which can bind to CTB in thymocytes and four in T cells. Although two of these gangliosides were identified as GM1-(NeuAc) and GM1(NeuGc), we can only speculate about the identity of the others. Previously, a ganglioside with CTB-binding activity was isolated from mouse spleen and shown to be Galβ1-3GalNAcβ1-4GM1b(NeuGc) (Nakamura et al., 1987). This ganglioside, which contains a terminal tetrasaccharide structure identical with that of GM1, is also a derivative of GM1b. The unknown gangliosides which bind CTB in T cells and thymocytes could be Galβ1-3GalNAcβ1-4GM1b containing NeuAc and NeuGc. It is not yet possible to determine which of these gangliosides is responsible for the effects of CTB on lymphocytes. The future availability of specific mAbs against these individual gangliosides may make it possible to sort out the importance of these minor monosialogangliosides.

In contrast to T lineage cells, the gangliosides synthesized through GM3 and GM1 were found to be predominantly expressed in the B-cell-enriched fraction. Previously, we isolated four novel gangliosides containing LacNAc-GM1 structures (Nohara et al., 1990a, 1992). In the present study, αGal-LacNAc-GM1, αGal-$(LacNAc)_2$-GM1, and sialyl-LacNAc-GM1 were detected in the B-cell-enriched fraction, whereas LacNAc-GM1 was barely detectable. In addition, significant amounts of GD1c were also detected in the B-cell-enriched fraction. However, as this fraction contained about 5% T cells and 30% non-T, non-B cells, it is not clear whether GD1c in this fraction was due to contamination with T cells and/or other types of cells or whether it is present in both T and B cells.

GA1 is known to be a specific antigen of thymocytes and natural killer cells in mouse (Habu et al., 1980; Kasai et al., 1980; Young et al., 1980), whereas in rat, it can be found mainly on peripheral T cells and macrophages and to a lesser degree on thymocytes (Arndt et al., 1981). Furthermore, 5% of thymus and 60–80% of lymph node T cells were immuno-

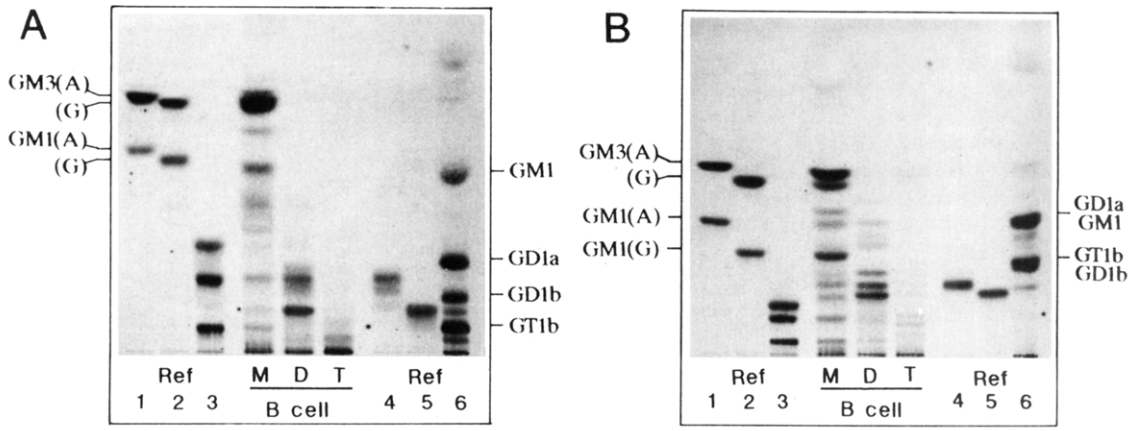

FIGURE 4: TLC profiles of gangliosides from the B-cell-enriched fraction. The gangliosides from the B-cell-enriched fraction were separated on a DEAE-Sephadex column into mono- (M), di- (D), and tri- and tetrasialogangliosides (T). Each fraction from 1.6×10^8 cells was applied on HPTLC silica gel 60 plates, developed using chloroform/methanol/0.2% $CaCl_2$ (60:40:9) (A) or 1-propanol/28% NH_4OH/water (75:5:25) (B), and visualized with resorcinol reagent. Ref (reference glycolipids): 1, GM3(NeuAc) and GM1(NeuAc); 2, GM3(NeuGc) and GM1(NeuGc); 3, LacNAc-GM1, αGal-LacNAc-GM1, and αGal-$(LacNAc)_2$-GM1; 4, sialyl-LacNAc-GM1; 5, GD1c; 6, total gangliosides from rat brain.

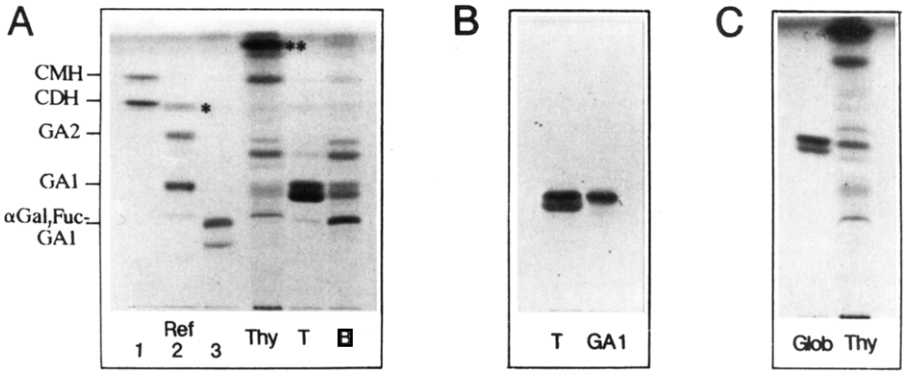

FIGURE 5: Neutral glycolipids of thymocytes, T cells, and the B-cell-enriched fraction. Neutral glycolipids from thymocytes (Thy, 1×10^9 cells), T cells (T, 1×10^8 cells), and the B-cell-enriched fraction (B, 1×10^8 cells) were separated on Si-HPF TLC plates, developed with chloroform/methanol/0.2% $CaCl_2$ (60:35:8), and visualized with 20% sulfuric acid (A and C) or immunostained with anti-GA1 antibody (B). (A) Ref (reference glycolipids): 1, CMH and CDH; 2, GA2 and GA1; 3, αGal,Fuc-GA1 (contaminated with αGal,Fuc-GM1). * = sodium cholate, and ** = orcinol negative, nonglycolipid component. (C) Glob, globoside.

stained by anti-GA1 antibody (Momoi et al., 1982). The present study further indicates that GA1 is almost the exclusive neutral glycolipid of T cells, whereas much lower amounts are present in thymocytes. GA1 was also detected in the B-cell-enriched fraction. However, its presence may be due to contamination with T cells and/or other cell types.

Previously, globoside was reported to be specific for alloantigen-activated T cells in mouse (Gruner et al., 1981). In addition, αGal,Fuc-GA1 was also found in rat spleen and thymus (Taki et al., 1985). However, in the present study, the glycosphingolipids corresponding to αGal,Fuc-GA1 and globoside were the major neutral glycolipids in the B-cell-enriched fraction rather than in T cells. αGal,Fuc-GA1 was found not only in rat bone marrow cells, spleen, and thymus but also in rat ascites hepatoma cells AH7974F (Taki et al., 1985). It has been proposed that αGal,Fuc-GA1 may be commonly present in immunocytes and tumor cells and may be involved in the escape mechanism of tumor cells from host immunosurveillance systems. In this work, the glycosphingolipid corresponding to αGal,Fuc-GM1 was the major component of neutral glycolipids of the B-cell-enriched fraction and of thymocytes. Furthermore, GM1b and GD1α, which were also detected in rat ascites hepatoma cells (Taki et al., 1986), were also present in T cells.

In summary, the predominance of GA1 and GD1c and the presence of characteristic gangliosides derived from GA1 were demonstrated in rat T cells. The involvement of these unique glycosphingolipids in signal transduction pathways remains to be eluciated.

ACKNOWLEDGMENT

We thank Prof. M. Ohashi (Ochanomizu University) for the generous gift of GD1α and valuable discussions and Dr. H. Fujimaki (NIES) for helpful advice on separation of spleen lymphocytes. We also thank Drs. M. Isoda (RIKEN) for cytometric analyses, T. Kobayashi (NIES) for helpful discussions, and A. Furuyama (NIES) for advice on morphologic evaluation, and Ms. Y. Kataoka for technical assistance.

REFERENCES

Arndt, R., Thiele, H.-G., Hamann, A., Graning, G., Raedler, A., Momoi, T., & Wiegandt, H. (1981) *Eur. J. Immunol. 11*, 21–26.

Campbell, J. S., & Munson, A. E. (1987) *J. Pharmacol. Exp. Ther. 242*, 895–904.

Chu, J. W., & Sharom, F. J. (1991) *Cell Immunol. 132*, 319–338.

Gruner, K. R., van Eijk, R. V. W., & Muhlradt, P. F. (1981) *Biochemistry 20*, 4518–4522.

Dixon, S. J., Stewart, D., Grinstein, S., & Spiegel, S. (1987) *J. Cell. Biol. 105*, 1153–1161.

Dugas, B., Eugene, N. P., Genot, E., Huerta, J. M., Braquet, P., & Kolb, J. P. (1991) *Eur. J. Immunol. 21*, 495–500.

Ebel, F., Schmitt, E., Peterkatalinic, J., Kniep, B., & Mühlradt, P. F. (1992) *Biochemistry 31*, 12190–12197.
Francis, M. L., Ryan, J., Jobling, M. G., Holmes, R. K., Moss, J., & Mond, J. J. (1992) *J. Immunol. 148*, 1999–2005.
Grassi, F., Lopalco, L., Lanza, P., Ciccomascolo, F., Cazzola, F., DiMartino, A., Kirschner, G., Callegaro, L., Chieco-Bianchi, L., & Siccardi, A. G. (1990) *Eur. J. Immunol. 20*, 145–150.
Habu, S., Kasai, M., Nagai, Y., Tamaoki, N., Tada, T., Herzenberg, L. A., & Okumura, K. (1980) *J. Immunol. 125*, 2284–2288.
Hakomori, S. (1990) *J. Biol. Chem. 265*, 18713–18716.
Hawkes, R., Niday, E., & Gordon, J. (1982) *Anal. Biochem. 119*, 142–147.
Hersey, P., Schibeci, S. D., & Cheresh, D. A. (1989) *Cell Immunol. 119*, 263–278.
Hirabayashi, Y., Taki, T., & Matsumoto, M. (1979) *FEBS Lett. 100*, 253–257.
Jackson, K. M., Yates, A. J., Orosz, C. G., & Whitacre, C. C. (1987) *Cell. Immunol. 104*, 169.
Julius, M. H., Simpson, E., & Herzenberg, L. A. (1973) *Eur. J. Immunol. 3*, 645–649.
Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., & Tada, T. (1980) *Eur. J. Immunol. 10*, 175–180.
Ladisch, S., Becker, H., & Ulsh, L. (1992) *Biochim. Biophys. Acta 1125*, 180–188.
Marcus, D. M. (1984) *Mol. Immunol. 21*, 1083–1091.
Matsumoto, M., Taki, T., Samuelsson, B., Pascher, I., Hirabayashi, Y., Li, S.-T., & Li, Y. T. (1981) *J. Biol. Chem. 256*, 9737–9741.
Miller, H. C., & Esselman, W. J. (1975) *J. Immunol. 115*, 839–843.
Momoi, T., Nakajima, K., Sakakibara, K., & Nagai, Y. (1982) *J. Biochem. 91*, 301–310.
Morrison, W. J., Young, K., Offner, H., & Vandenbark, A. A. (1993) *Cell. Mol. Biol. Res. 39*, 159–1655.
Müthing, J., & Mühlradt, P. F. (1988) *Anal. Biochem. 173*, 10–17.
Müthing, J., Egge, H., Kniep, B., & Mühlradt, P. F. (1987) *Eur. J. Biochem. 163*, 407–416.
Müthing, J., Schwinzer, B., Peter-Katalinic, J., Egge, H., & Mühlradt, P. F. (1989) *Biochemistry 28*, 2923–2929.
Nakamura, K., Hashimoto, Y., Suzuki, M., Suzuki, A., & Yamakawa, T. (1984) *J. Biochem. 96*, 949–957.
Nakamura, K., Suzuki, M., Inagaki, F., Yamakawa, T., & Suzuki, A. (1987) *J. Biochem. 101*, 825–835.
Nakamura, K., Suzuki, M., Taya, C., Inagaki, F., Yamakawa, T., & Suzuki, A. (1991) *J. Biochem. 110*, 832–841.
Nohara, K., Suzuki, M., Inagaki, F., Ito, H., & Kaya, K. (1990a) *J. Biol. Chem. 265*, 14335–14339.
Nohara, K., Suzuki, M., Inagaki, F., Ito, H., & Kaya, K. (1990b) *J. Biochem. 108*, 684–688.
Nohara, K., Suzuki, M., Inagaki, F., & Kaya, K. (1991) *J. Biochem. 110*, 274–278.
Nohara, K., Suzuki, M., Inagaki, F., Sano, T., & Kaya, K. (1992) *J. Biol. Chem. 267*, 14982–14986.
Nohara, K., Sano, T., & Shiraishi, F. (1993) *J. Biol. Chem. 268*, 24997–25000.
Offner, H., Thieme, T., & Vandenbark, A. A. (1987) *J. Immunol. 139*, 3295–3305.
Ohashi, M., (1981) in *Glycoconjugates* (Yamakawa, T., Osawa, T., & Handa, S., Eds.) pp 33–34, Japan Scientific Societies Press, Tokyo.
Olivera, A., & Spiegel, S. (1992) *Glycoconjugate J. 9*, 109–117.
Pessina, A., Mineo, E., Masserini, M., Neri, M. G., & Cocuzza, C. E. (1989) *Biochim. Biophys. Acta 1013*, 206–211.
Repke, H., Barber, E., Ulbricht, S., Buchner, K., Hucho, F., Kopp, R., Scholz, H., Rudd, C. E. & Haseltine, W. A. (1992) *J. Immunol. 149*, 2585–2591.
Reynolds, C. W., Timonen, T., & Herberman, R. B. (1981) *J. Immunol. 127*, 282–287.
Saggioro, D., Sorio, C., Calderazzo, F., Callegaro, L., Panozzo, M., Berton, G., & Chieco-Bianchi, L. (1993) *J. Biol. Chem. 268*, 1368–1375.
Spiegel, S. (1989) in *Gangliosides in Cancer* (Oettgen, H., Ed.) pp 17–29, VCH Publishers, New York.
Spiegel, S., Fishman, P. H., & Weber, J. W. (1985) *Science 230*, 1285–1287.
Sugano, K., Saito, M., & Nagai, Y. (1978) *FEBS Lett. 89*, 321–325.
Svennerholm, L. (1963) *J. Neurochem. 10*, 613–623.
Svennerholm, L., Mansson, J.-E., & Li, Y.-T. (1973) *J. Biol. Chem. 248*, 740–742.
Taki, T., Kimura, H., Gasa, S., Nakamura, M., & Matsumoto, M. (1985) *J. Biol. Chem. 260*, 6219–6225.
Taki, T., Hirabayashi, Y., Ishikawa, H., Ando, S., Kon, K., Tanaka, Y., & Matsumoto, M. (1986) *J. Biol. Chem. 261*, 3075–3078.
Woogen, S. D., Ealding, W., & Elson, C. O. (1987) *J. Immunol. 139*, 3764–3770.
Young, W. W., Hakomori, S., Durdik, J. M., & Henney, C. S. (1980) *J. Immunol. 124*, 199–201.