Gangliosides: Differentiation Markers for Murine T Helper Lymphocyte Subpopulations T_H1 and T_H2

Frank Ebel,[‡] Edgar Schmitt,[§] Jasna Peter-Katalinić,[∥] Bernhard Kniep,[‡] and Peter F. Mühlradt*,[‡]

GBF-Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, FRG, Institut für Immunologie, Universität Mainz, Mainz, FRG, and Physiologisch-Chemisches Institut, Universität Bonn, Bonn, FRG

Received May 26, 1992; Revised Manuscript Received July 22, 1992

ABSTRACT: On the basis of the pattern of lymphokines they secrete, murine T helper clones can be divided into two subsets, T_H1 and T_H2. This concept of two different T helper effector cells helps to explain the diversity of immune reactions occurring in different parts of the body. The in vivo localization of T helper subtypes is of great interest, but up to now no biochemical or surface markers were available to distinguish between them. We analyzed the glycolipids from altogether 12 murine T_H1 and T_H2 cell lines or clones. A comparison of the gangliosides by thin-layer chromatography showed differences between the T_H1 and T_H2 cells. Binding studies with specific antibodies to asialo backbone structures after degradation by neuraminidases showed that the main gangliosides from these lymphocytes shared a common $GgOse_4$ backbone and thus differed only in their degree or position of sialylation. Two disialogangliosides appeared to be characteristic. They were isolated from the D10.G4.1 T_H2 cell clone and identified by fast atom bombardment mass spectrometry as IVNeuAc,IINeuAc-$GgOse_4Cer$ (G_{D1a}) and IVNeuAc,IIINeuAc-$GgOse_4Cer$ ($G_{D1\alpha}$), respectively. G_{D1a} was characteristically only detected in T_H2 cells, whereas $G_{D1\alpha}$ was preferably, but not exclusively, expressed by T_H1 lymphocytes. Although G_{D1a} was also found in the lung, heart, kidney, and spleen, its expression within the murine immune cells under investigation was unique to T_H2 lymphocytes. Scarcely any G_{D1a} was found in thymocytes, B cells, or CD8 positive (cytolytic) T cells, but significant expression was seen in CD4 positive (helper) T cells which include the T_H2 subpopulation. According to these data, G_{D1a} can be considered as a useful marker for the T_H2 helper subtype in the mouse.

A complex pattern of glycosphingolipids (GSLs)[1] is expressed on immune and most other cells of the body. This pattern may change during differentiation and development, and some selected GSLs can be regarded as differentiation antigens in humans or in mice (Mühlradt et al., 1989; Feizi, 1985). Our earlier analyses of the gangliosides from murine T blasts and cloned T cells with helper or cytolytic functions suggested that these T cell subpopulations differ in their gangliosides (Müthing et al., 1989).

Whereas cytolytic and helper T cells can be distinguished by their CD8 or CD4 antigens, respectively, no cell surface markers are known for the functionally distinct subpopulations of helper T cells. On the basis of the pattern of lymphokines they secrete, cloned murine T helper cells can be divided into two subsets, T_H1 and T_H2. T_H2 cells primarily provide help to B cells, whereas T_H1 cells are known to support inflammatory processes such as the delayed-type hypersensitivity reaction (Mosmann et al., 1989). Questions as to the in vivo localization of such T helper subsets, however, have remained unresolved because of the lack of an appropriate cell surface marker. Suggestive evidence for the in vivo existence of T_H1- and T_H2-like lymphocytes came from T helper cell lines, which were established after a relatively short cultivation time with IL-2 or IL-4 (Schmitt et al., 1990), and from Taguchi et al. (1990), who succeeded in counting T_H1- and T_H2-like lymphocytes from murine gut-associated tissue in an elegant ELISPOT assay.

In this study we have analyzed the gangliosides from several murine T_H1 and T_H2 cell lines or clones. We present evidence that the gangliosides from T_H1 and T_H2 cells differ. One prominent ganglioside in particular was characteristic for T_H2 cells. Its structure was elucidated as IVNeuAc,IINeuAc-$GgOse_4Cer$ (G_{D1a}). G_{D1a} was found only in traces in T_H1 cells. The presence of this ganglioside was investigated in different mouse organs and purified lymphocyte subpopulations. According to our data, G_{D1a} can be considered as a useful marker for the T_H2 helper subtype.

MATERIALS AND METHODS

Animals and Reagents. All mice were purchased from the Zentralinstitut für Versuchstierzucht (Hannover, FRG) with the exception of C3H/HeJ, which were obtained from Charles River (Sulzfeld, FRG). Mice, all female, were used at the age of 7–10 weeks. Concanavalin A (ConA) was obtained from Pharmacia (Freiburg, FRG), human recombinant IL-2 from BHK cells was a generous gift from Dr. Hauser (GBF, Braunschweig), and purified protein derivative (PPD, tuberculin "GT") was purchased from Behring (Marburg, FRG). Bovine brain gangliosides (BBG) were obtained from Supelco (Bellefonte, PA). Rat monoclonal antibodies against Thy-1.2 and Lyt-2 were obtained from Becton-Dickinson (Heidelberg, FRG), phycoerythrin-labeled antiserum against rat

* Address correspondence to this author at GBF, Mascheroder Weg 1, D-3300 Braunschweig, FRG.
‡ GBF.
§ Universität Mainz.
∥ Universität Bonn.

[1] Abbreviations: BBG, bovine brain gangliosides; ConA, concanavalin A; DIG, digoxigenin–succinyl-ε-aminocaproic acid hydrazide; GSL(s), glycosphingolipid(s); IL-2, interleukin-2; IL-4, interleukin-4; PBS, phosphate-buffered saline without calcium and magnesium. The designation of the following glycosphingolipids follows the IUPAC–IUB recommendations (1977): $GgOse_3Cer$ or gangliotriaosylceramide or asialo-G_{M2}, GalNAcβ1–4Galβ1–4GlcCer; $GgOse_4Cer$ or gangliotetraosylceramide or asialo-G_{M1}, Galβ1–3GalNAcβ1–4Galβ1–4GlcCer; $GgOse_5$ or gangliopentaosylceramide, GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4GlcCer; II^3NeuAc-$GgOse_4Cer$, G_{M1}; IV^3NeuAc,II^3NeuAc-$GgOse_4Cer$, G_{D1a}; $II^3(NeuAc)_2$-$GgOse_4Cer$, G_{D1b}; IV^3NeuAc,III^6NeuAc-$GgOse_4Cer$, $G_{D1\alpha}$; $IV^3(NeuAc)_2$-$GgOse_4Cer$, G_{D1c}; IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4Cer$, G_{T1b}.

0006-2960/92/0431-12190$03.00/0

immunoglobulins was from Medac (Hamburg, FRG), fluorescein-labeled anti mouse immunoglobulin antiserum was from Daco (Hamburg, FRG), and rat monoclonal antibody against L3T4 was a kind gift of Dr. F. W. Fitch, University of Chicago.

Culture Media. All cell lines and clones except the D10.G4.1 cells were cultured in Iscove's modified Dulbecco's medium (Gibco, Eggenstein, FRG), supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol (ME), 10 IU penicillin, 100 μg/mL streptomycin, and 5% heat-inactivated fetal calf serum (FCS). D10.G4.1 and sorted T cells were propagated in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine, 50 μM ME, and 7.5% heat-inactivated FCS.

Cell Lines. The LNC (LNC = lymph node cells) cell lines were established as described previously (Schmitt et al., 1990). Briefly, PPD-specific T cell lines were obtained from BALB/c mice (I-A^d) immunized with complete Freund's adjuvant. These lines were propagated by serial restimulations of 10^6 T cells with 5×10^6 γ-irradiated (2000 rad) syngenic spleen cells and 200 μg of PPD/mL. LNC.2 and LNC.2 A.4 were cultivated with human recombinant IL-2 (10 units/mL) and LNC.4 and LNC.4 A.4 with mouse recombinant IL-4 (40 units/mL).

The T_H2 cell clone ST2/K.9, specific for pig and bovine insulin associated with I-$A^{b\times k}$ (Spaeth & Rüde, 1985), and the PPD-specific T_H2 cell clone LNC.4/2K1, a subclone of the LNC.4 cell line, were grown with 40 units/mL mouse recombinant IL-4. The autoreactive T_H1 cell clone F7.15A (I-$A^{b\times k}$) (Plachov et al., 1988), the T_H1 cell clone M4, specific for bovine insulin associated with I-$A^{b\times k}$ (Fischer et al., 1988), the T_H1 cell clone F2.1, specific for pig insulin in association with I-$A^{b\times k}$ (Plachov et al., 1988), the T_H1 line B10 BI, specific for bovine insulin in association with I-A^b (Hampl et al., 1991), and the T_H2 cell clone D10.G4.1, specific for conalbumin associated with I-A^k (Kaye et al., 1983), were grown with 10 units/mL human recombinant IL-2. All lines and clones were stimulated bimonthly with irradiated syngenic spleen cells and the appropriate antigens.

Isolation and Mitogen Stimulation of Murine Lymph Node T Lymphocytes. Single-cell suspensions from inguinal and mesenteric lymph nodes of BALB/c mice were prepared by gently crushing the nodes between the frosted ends of two glass slides. Cells were washed twice with PBS and incubated with the first (anti L3T4, anti Lyt-2) and the appropriate second fluorescein- or phycoerythrin-labeled antibody for 1 h on ice. Cell sorting was performed on an EPICS-753 (Coulter Electronics, Krefeld, FRG), and surface markers were analyzed with a FACScan II (Becton-Dickinson). For mitogen stimulation, 5×10^5 sorted lymph node T cells/mL were cultured with ConA (2 μg/mL) and propagated for 9 days with human recombinant IL-2 (30 units/mL).

Preparation of Thymocytes. Thymuses were isolated from 4-week-old BALB/c mice. Organs were washed twice with PBS, and a single-cell suspension of thymocytes was prepared by passing the tissue through a 47 μm mesh nylon sieve.

Preparation of T Cell-Depleted Spleen Cells. Single-cell suspensions of BALB/c spleen cells were obtained by passing the minced tissue through a 47 μm mesh nylon sieve. Erythrocytes were lysed with 0.15 M NH_4Cl/Tris, pH 7.2. The remaining leukocytes were centrifuged through FCS, washed twice with PBS, and incubated with monoclonal anti Thy-1.2 IgM antibody (a generous gift of Dr. Opitz, Bayer AG, Wuppertal, FRG) for 1 h at 4 °C. Thy-1.2 positive T cells were lysed by incubation with 20% rabbit complement (Behring) in PBS/1% bovine serum albumin (BSA) for 1 h at 37 °C. Dead cells were agglutinated with high phosphate buffered saline (Mishell & Shiigi, 1980). Briefly, cells were suspended in high phosphate buffered saline (2 g of KH_2PO_4, 8.57 g of Na_2HPO_4, and 4.5 g of NaCl in 1 L of water, pH 7.4) containing 5% FCS and sedimented by centifugation. The pellet was resuspended in this buffer by vigorous mixing with a Pasteur pipet, and aggregates were removed by centrifugation for 10 s at 50*g*. Living cells were obtained from the supernatant.

Metabolic Labeling of D10.G4.1 Cells. Radioactive labeling was performed in the normal medium for 16 h with 37 kBq/mL [1-^{14}C]-D-glucosamine HCl (Amersham Buchler, Braunschweig, FRG) and 37 kBq/mL [1-^{14}C]-D-galactose (Amersham Buchler) at a cell density of 4×10^6 cells/mL. To deplete the labeled cells of radioactive monosaccharides, they were washed twice with 0.85% NaCl, 1 mM galactose, and 1 mM glucosamine before lipid extraction.

Isolation of Gangliosides. Before lipid extraction, cells were routinely washed twice with PBS, suspended in chloroform/methanol (2/1 v/v), and stored at −20 °C. Gangliosides were isolated as previously described (Müthing et al., 1989). Briefly, cells or minced organs were extracted successively with 2/1, 1/1, and 1/2 (v/v) chloroform/methanol. The combined evaporated extracts were dissolved in methanol and desalted by use of RP18 SepPak columns (Millipore, Milford, MA) (Williams & McCluer, 1980). The freeze-dried lipids from D10.G4.1 cells and organs were subjected to alkaline hydrolysis to destroy phospholipids. Chloroform/0.6 M methanolic NaOH (1/1 v/v) was added to the dry material, and incubation was performed for 1 h at 37 °C. The products were dialyzed (Visking 36, Loewe Biochemica, München, FRG) three times against 5 L of H_2O at 4 °C, freeze-dried, dissolved in chloroform/methanol/water (30/60/8 v/v), and applied to a DEAE-Sepharose CL-6B (Pharmacia) column. The column was washed with 10 volumes of the same solvent, and gangliosides were eluted either stepwise with different concentrations of sodium acetate in methanol or in one batch with chloroform/methanol/0.8 M sodium acetate (30/60/8 v/v). Fractions from the DEAE-Sepharose column were evaporated and desalted with an RP18 SepPak column. All other cell extracts were applied to a DEAE-Sepharose CL-6B column, eluted with chloroform/methanol/0.8 M sodium acetate, and finally desalted with an RP18 SepPak column.

Analytical and Preparative Thin-Layer Chromatography. High-performance thin-layer chromatography plates (HPTLC plates, 10 cm × 10 cm × 0.1 mm, E. Merck, Darmstadt, FRG) were used for analytical and preparative purposes. Two solvent systems were used: I, chloroform/methanol/0.5% $CaCl_2$ in water (50/40/10 v/v); II, chloroform/methanol/2.5 M NH_3 in aqueous 0.25% KCl (42/34/8 v/v). Radioactive GSLs were detected by autoradiography with Hyperfilm-βmax (Amersham Buchler). For preparative use, the HPTLC plates were prerun in methanol/diethyl ether (4/1 v/v) and chloroform/methanol/pyridine/water (2/4/1/1 v/v) to remove contaminants. After chromatography of GSLs and localization by autoradiography, bands were scraped off and GSLs were extracted with chloroform/methanol/pyridine/water (2/4/1/1 v/v) and chloroform/methanol/water (10/10/3 v/v). Finally, the GSL material was run over an RP18 SepPak column to remove solubilized silica gel.

Immunochemical Detection of GSLs on HPTLC Chromatograms by the DIG Stain Technique. After separation of the GSLs, plates were fixed by chromatography with polyisobutylmethacrylate (Plexigum P 28, Roehm & Haas,

Darmstadt, FRG) in *n*-hexane (HPLC grade, Rathburne Chemicals, Walkerburn, Peeblesshire, UK). The GSLs were stained by a carbohydrate-specific immunochemical method based on periodate oxidation and subsequent coupling with a hapten that is detected by enzyme-linked antibodies (DIG stain technique; Kniep & Mühlradt, 1990).

Neuraminidase Treatment of Gangliosides on HPTLC Chromatograms. Gangliosides were first separated by HPTLC with solvent system I, and then the plates were fixed as described above and treated at 37 °C with either 10 munits/mL *Vibrio cholerae* neuraminidase (EC 3.2.1.18) (Behring) in 0.05 M sodium acetate and 9 mM $CaCl_2$, pH 5.5, for 2 h or with 10 munits/mL *Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18) (Boehringer, Mannheim) in 0.1 M sodium acetate buffer, pH 4.8, including 0.05% sodium taurodesoxycholate (Sigma, Deisenhofen, FRG) for 10 h.

Immunostaining of GSLs on HPTLC Chromatograms. GSLs were chromatographed in the appropriate solvent system. Plates were fixed as described above and blocked with PBS/1% BSA at 37 °C for 30 min. A monoclonal antibody against $GgOse_3Cer$ (asialo-G_{M2}; Young et al., 1979) was produced with the hybridoma 2D4, obtained from the American Type Culture Collection. Rabbit antiserum specific for $GgOse_4$-Cer (asialo-G_{M1}) (Bethke et al., 1986) was produced according to the method of Kasai et al. (1980). Goat anti mouse IgM antiserum and goat anti rabbit Ig antiserum (Dianova, Hamburg, FRG), both labeled with alkaline phosphatase, were used as second antibodies at a dilution of 1/1000. Incubation with the first antibody was performed overnight at 4 °C, whereas incubation with the appropriate second antibody was performed over 4 h at 25 °C. The plate was washed three times with PBS/0.05% Tween 21 and once with 0.1 M glycine, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, pH 10, before the bound antibodies were visualized by development with 0.05% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Biomol, Hamburg, FRG) in this glycin buffer.

Cholera Toxin Subunit B Binding Assay. Gangliosides were separated on HPTLC plates, and the plates were fixed and blocked as described above. Incubation with cholera toxin B subunit (0.5 mg/mL) (Sigma) was performed for 1 h at 22 °C at a dilution of 1/1000 in PBS/1% BSA. After washing three times with PBS/0.5% Tween 21, plates were incubated at 6 °C overnight with anti cholera toxin B subunit antiserum (Calbiochem, Bad Soden, FRG). Bound antibodies were detected with alkaline phosphatase-labeled rabbit anti goat antiserum (Dianova) at a dilution of 1/1000 in PBS/1% BSA as described above.

Fast Atom Bombardment Mass Spectroscopy (FAB-MS). FAB-MS was carried out using a ZAB-HF mass spectrometer (VG Analytical, Manchester, England) fitted with an Ion Tech FAB gun as described (Egge & Katalinić, 1987). Native gangliosides were analyzed by negative ion FAB-MS in triethanolamine as a matrix. Permethylation of gangliosides was performed as described (Ciucanu & Kerek, 1984). After cleanup by column chromatography on Sephadex LH-20 (elution by chloroform/methanol, 1/1 v/v) and repeated Iatrobeads chromatography (Peter-Katalinić & Egge, 1990), the permethylated gangliosides were analyzed by positive ion FAB-MS in thioglycerol. Spectra were run in upscan mode at a speed of 3 decade/s and acquired on a AMD DP10 data system fitted with SAM-II (KWS) hardware and SUSY software (KWS) (AMD Intectra, Beckeln, FRG).

Methylation Analysis. The permethylated compound Y was hydrolyzed, reduced, and peracetylated according to Weisshaar et al. (1991). Analyses of the carbohydrate derivatives were performed on a Kratos MS 50 fast scan mass spectrometer connected to a Carlo Erba Mega Series gas chromatograph equipped with a 30-m DB1 column.

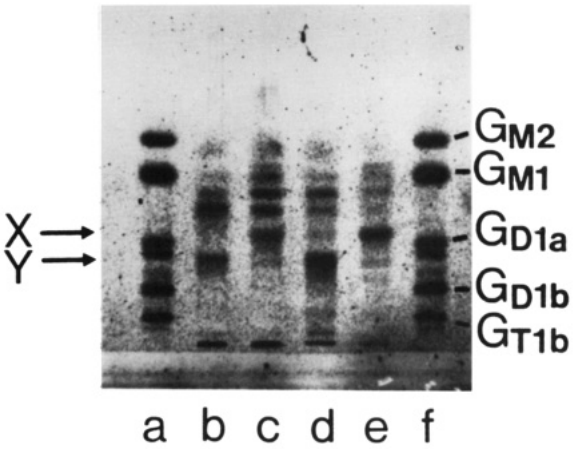

FIGURE 1: Immunochemical staining of gangliosides from LNC T helper cell lines with the DIG stain technique. Gangliosides were chromatographed in chloroform/methanol/0.5% $CaCl_2$ in water (50/40/10 v/v). Material from 8×10^6 cells was applied to each lane. Lanes a and f: bovine brain gangliosides (5 μg) plus G_{M2} (0.6 μg). Lane b: gangliosides from LNC.2 cells (T_H1). Lane c: gangliosides from LNC.4 cells (T_H2). Lane d: gangliosides from LNC.2 A.4 cells (T_H1). Lane e: gangliosides from LNC.4 A.4 cells (T_H2). Positions of hypothetical marker gangliosides are marked X and Y, respectively.

RESULTS

T Helper Cell Lines of the T_H1- and T_H2-Types Show Different Ganglioside Patterns. Gangliosides from two T_H1 and two T_H2 cell lines were isolated, separated by HPTLC, and detected by the DIG stain technique. Although the ganglioside patterns of each cell line differed somewhat, there was a striking similarity within the same helper subtypes. Two compounds in the disialoganglioside region appeared to be especially characteristic of the two T helper subtypes, and they were designated X and Y (Figure 1). X was present only in T_H2 cells, whereas Y was preferably expressed by T_H1 cells. To obtain preliminary information about the structures of X and Y, gangliosides were subjected to treatment with neuraminidases from *V. cholerae* or *A. ureafaciens* on the HPTLC plate and then stained with antibodies against asialogangliosides. Neuraminidase from *V. cholerae* cleaves off sialic acids at positions III and IV of the $GgOse_4$ backbone, whereas *A. ureafaciens* neuraminidase, in the presence of detergent, additionally cleaves *N*-acetylneuraminic acid (NANA), but not *N*-glycolylneuraminic acid (NGNA), at the internal position II (Hirabayashi et al., 1986; Müthing et al., 1988) (for ganglioside structures, see Table I). Of X and Y only the latter was degraded to $GgOse_4Cer$ by *V. cholerae* neuraminidase treatment and was then detectable by a specific antibody against $GgOse_4$ (Figure 2A). Y could thus be either IVNeuAc,IIINeuAc-$GgOse_4Cer$ ($G_{D1\alpha}$), a ganglioside found in ConA-stimulated murine T cells (Müthing et al., 1989), or IV$(NeuAc)_2$-$GgOse_4Cer$ (G_{D1c}), previously detected in murine thymoma and thymocytes (Bartoszewicz et al., 1986; Nakamura et al., 1991). In contrast, X was only susceptible to treatment with *A. ureafaciens* neuraminidase plus detergent, yielding the same asialoganglioside as Y that could be stained with anti $GgOse_4$ antibody (Figure 2B). This finding and the migratory properties of X suggested that it could be identical to IVNeuAc,IINeuAc-$GgOse_4Cer$ (G_{D1a}).

No staining occurred when the *A. ureafaciens* neuraminidase treated gangliosides X and Y were reacted with the

Table I: Structures of Selected Gangliosides

G_{M1}	Galβ(1→3)GalNAcβ(1→4)Galβ(1→4)Glcβ(1→1')Cer 3 ↑ NeuAcα2
G_{D1a}	Galβ(1→3)GalNAcβ(1→4)Galβ(1→4)Glcβ(1→1')Cer 3 3 ↑ ↑ NeuAcα2 NeuAcα2
G_{D1a}	Galβ(1→3)GalNAcβ(1→4)Galβ(1→4)Glcβ(1→1')Cer 3 6 ↑ ↑ NeuAcα2 NeuAcα2
G_{D1c}	Galβ(1→3)GalNAcβ(1→4)Galβ(1→4)Glcβ(1→1')Cer 3 ↑ NeuAcα2 8 ↑ NeuAcα2

monoclonal antibody 2D4 (data not shown). The 2D4 antibody is specific for the terminal GalNAcβ1–4Gal structure of both $GgOse_3$ and $GgOse_5$ (Müthing et al., 1987). None of the antibodies against asialo structures showed immunostaining of gangliosides prior to neuraminidase treatment.

Gangliosides X and Y in T_H1 and T_H2 Cell Clones. Since the helper subtypes were originally defined with cloned T cells, we extended our investigations to T helper clones. As expected from the data obtained with T_H2 lines, ganglioside X was again exclusively expressed by T_H2 clones (Figure 3B). These data were confirmed with an additional T_H1 line (B10 BI) and a clone isolated from the T_H2 line LNC.4 (data not shown). In agreement with our findings with the LNC cell lines, Y was prominent in the gangliosides from T_H1 clones but was also present in somewhat lower amounts in T_H2 cells (Figure 3A).

Isolation and Characterization of the Gangliosides X and Y. The gangliosides X and Y were isolated from the conalbumin-specific T_H2 clone D10.G4.1. To facilitate isolation, 2×10^8 D10.G4.1 cells were metabolically labeled with [^{14}C]galactose and [^{14}C]glucosamine HCl and were mixed with 1×10^{10} unlabeled D10.G4.1 cells. The gangliosides were fractionated by DEAE-sepharose chromatography into mono- and disialogangliosides. As expected, gangliosides X and Y eluted like disialogangliosides (data not shown). Each band from the disialo fraction was isolated by preparative thin-layer chromatography and appeared to be homogeneous in two different solvent systems (Figure 4A,B). Whereas 23% of the radioactivity in the ganglioside fraction was found in ganglioside X, 6% was found in ganglioside Y. X and Y from the clones behaved in the same way as the corresponding compounds from the helper cell lines in all respects: both yielded $GgOse_4Cer$ after enzymatic desialylation as the two upper bands including ganglioside X were sensitive only to *A. ureafaciens* neuraminidase (Figure 4C,D), whereas the two lower bands including Y were sensitive to *V. cholerae* neuraminidase (Figure 4C,D).

Ganglioside X, IVNeuAc,IINeuAc-$GgOse_4Cer$ (G_{D1a}), should also be detectable by a cholera toxin B subunit binding assay after degradation to G_{M1} by *V. cholerae* neuraminidase treatment (Spiegel et al., 1986). Furthermore, this assay should detect G_{D1a} with a *N*-glycolylneuraminic acid at position II, a G_{D1a} species which would elude detection by *A. ureafaciens* neuraminidase plus immunostaining (see above). We therefore analyzed all T helper cell lines and clones under these conditions. As expected, band X was strongly stained. One additional, slower moving band with G_{D1a} properties was also detected. This ganglioside, corresponding to the band in lane c of Figure 4D, was present in T_H2 cells and only in traces in T_H1 cells. Its structure was identified as G_{D1a} (with the C_{16} and C_{18} fatty acid moieties) by FAB-MS (data not shown).

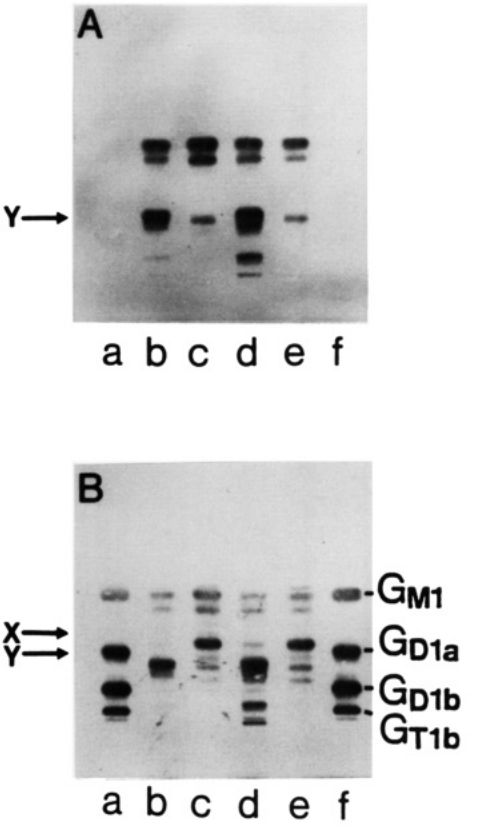

FIGURE 2: Detection of $GgOse_4$ backbone in gangliosides from LNC T helper cell lines. Gangliosides were chromatographed in chloroform/methanol/0.5% $CaCl_2$ in water (50/40/10 v/v). Plates were fixed, and gangliosides were treated with neuraminidase and then stained with anti-$GgOse_4Cer$ antibody. (A) Staining pattern after *V. cholerae* neuraminidase treatment. (B) Staining pattern after *A. ureafaciens* neuraminidase treatment. Lanes a and f: BBG (1 μg) plus G_{M2} (0.12 μg). Lane b: gangliosides from LNC.2 cells (T_H1). Lane c: gangliosides from LNC.4 cells (T_H2). Lane d: gangliosides from LNC.2 A.4 cells (T_H1). Lane e: gangliosides from LNC.4 A.4 cells (T_H2). Material from 1.2×10^6 cells was applied to each lane.

FAB-MS of Native and Permethylated Disialogangliosides. Sufficient amounts of gangliosides X and Y were obtained for subsequent FAB-MS analysis to confirm the proposed structures.

In the negative ion FAB-MS of the native X (Figure 5A), the G_{D1a}-specific sequence was represented by the fragment ions at m/z = 648/646 (Cer^- 24:0/18 and 24:1/18), 810 ($HexCer^-$), 1263 ($NeuAcHex_2Cer^-$), 1466 ($NeuAcHex_2HexNAcCer^-$), and 1628 ($NeuAcHex_3HexNAcCer^-$). They were accompanied by a series of 28 amu lower mass ions, representing another G_{D1a} homologue with Cer 22:0/18 (Figure 5A). The molecular ions appeared as $[M - H]^-$ and $[M + Na^+ - 2H]^-$ at m/z = 1919 and 1941, respectively, for the G_{D1a} 24:0/18 and 24:1/18 homologues and at m/z = 1891 and 1913 for the 22:0/18 homologue. A minor component, possibly belonging to the G_{D1b} type, was present in the sample as documented by the ions at m/z = 972 (Hex_2Cer^-), m/z = 1175 ($Hex_2HexNAc\ Cer^-$), and m/z = 599 ($NeuAc_2^-$) (Figure 5A).

Due to the presence of impurities, the negative ion FAB-MS of ganglioside Y was only of poor quality (not shown). After permethylation and cleanup by LH-20 and Iatrobeads chromatography, this sample was analyzed by positive ion FAB-MS (Figure 5B). $NeuAc^+$ was observed at m/z = 376–

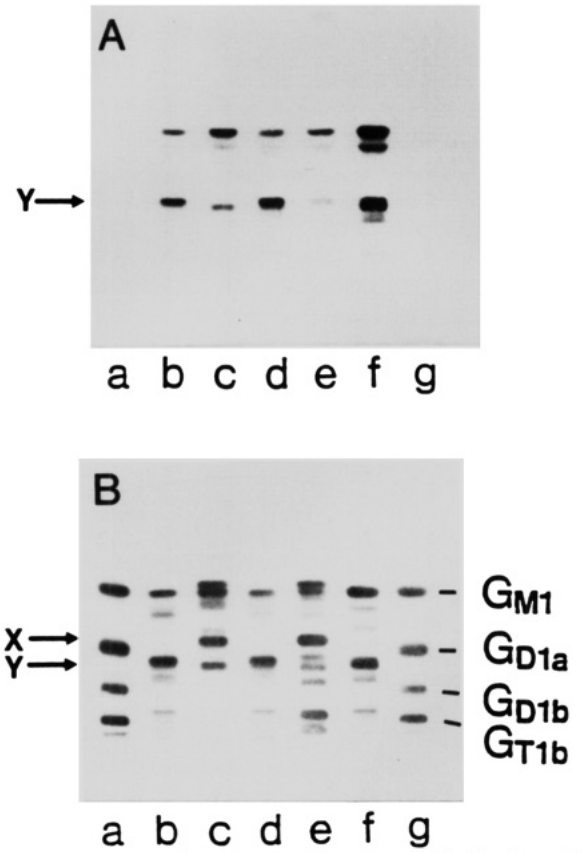

FIGURE 3: Detection of $GgOse_4$ backbone in gangliosides from T_H1 and T_H2 clones. Immunostaining with anti-$GgOse_4Cer$ antibody was performed after in situ neuraminidase treatment as described in Figure 2. (A) Staining pattern after *V. cholerae* neuraminidase treatment. (B) Staining pattern after *A. ureafaciens* neuraminidase treatment. Lanes a and g: BBG (1 μg) plus G_{M2} (0.12 μg). Lane b: gangliosides from F7.15A cells (T_H1). Lane c: gangliosides from D10.G4.1 cells (T_H2). Lane d: gangliosides from F2.1 cells (T_H1). Lane e: gangliosides from ST2/K.9 cells (T_H2). Lane f: gangliosides from M4 cells (T_H1). Material from 1.2×10^6 cells was applied to each lane.

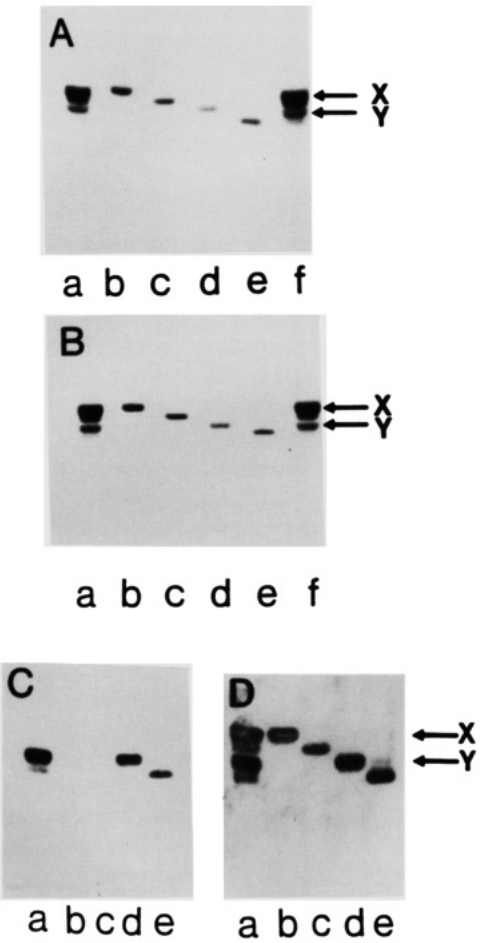

FIGURE 4: HPTLC-purified ^{14}C-labeled disialogangliosides from D10.G4.1 cells (T_H2). (A) Chromatography in chloroform/methanol/0.5% $CaCl_2$ (50/40/10 v/v), autoradiography of purified disialogangliosides. (B) Chromatography in chloroform/methanol/0.5 M NH_3 in aqueous 0.25% KCl (42/34/8), autoradiography of purified disialogangliosides. (C) and (D) Immunostaining with anti-$GgOse_4Cer$ antibody after in situ neuraminidase treatment as described in Figure 2. (C) Staining pattern after *V. cholerae* neuraminidase treatment. (D) Staining pattern after *A. ureafaciens* neuraminidase treatment. Autoradiography: purified gangliosides (lanes b–e), 400 cpm; disialoganglioside fraction (lanes a and f), 2000 cpm. Immunostaining was done with one-fifth of the material required for autoradiography.

344, $NeuAc_2HexHexNAc^+$ at m/z = 1186, Cer^+ at m/z = 660 (major species 24:0/18), and molecular ion MH^+ at m/z = 2271 (Figure 5B), as expected for $G_{D1\alpha}$ (24:0/18). This structure was confirmed by methylation analysis, yielding 2,3,6-tri-*O*-methyl-Gal, 2,3,6-tri-*O*-methyl-Glc, 2,4,6-tri-*O*-methyl-Gal, and 4-*O*-methyl-GalNAc (data not shown). A contaminating minor component, possibly derived from a disialylated gangliopentaosylceramide, was also present. It was characterized by ions at m/z = 548 (Cer^+) and 1070 ($NeuAcHexHexNAc_2^+$).

IVNeuAc, IINeuAc-GgOse$_4$Cer (G_{D1a}) in Different Murine Lymphocyte Subsets. Gangliosides were analyzed from B cells, thymocytes, and CD4 positive and CD8 positive T cells from BALB/c mice. B cells were prepared from spleen cells after elimination of Thy-1.2 positive T cells by complement lysis. L3T4 (CD4) positive and Lyt-2 (CD8) positive lymph node T cells were isolated by fluorescence-activated cell sorting (FACS). In order to obtain sufficient material, the sorted T cells were expanded by ConA stimulation and culturing for 9 days. The purity of the respective lymphocyte populations was ascertained by staining with appropriate antibodies (see Table II). Using neuraminidase treatment and immunostaining, X (G_{D1a}) was found only in traces in B cells, thymocytes, and CD8 positive T cells, whereas it was present in CD4 positive T cells, although in lower quantities than in the T_H2 clone D10.G4.1 (Figure 6B). Compound Y, in all probability IVNeuAc,IIINeuAc-$GgOse_4Cer$ ($G_{D1\alpha}$), was present in significant amounts in thymocytes and both CD4 positive and CD8 positive T cells, but was absent from B cells (Figure 6A).

IVNeuAc,IINeuAc-GgOse$_4$Cer (G_{D1a}) in Different Organs of the Mouse. We next analyzed the gangliosides from the liver, spleen, heart, kidney, and lung from BALB/c mice. Y ($G_{D1\alpha}$) was found in the spleen and in minor amounts in the lung (Figure 7A). X (G_{D1a}) was found in the spleen, heart, kidney, and lung, but not in the liver (Figure 7B). Using the cholera toxin subunit B binding assay after in situ *V. cholerae* neuraminidase treatment, a band with the migration properties of G_{D1a} was detected in gangliosides from liver, suggesting the presence of N-glycolylated G_{D1a} in that organ (data not shown).

DISCUSSION

Up to this point there were no surface marker molecules known to distinguish T_H1 from T_H2 T helper cells. Gangliosides, being part of the outer leaflet of the cytoplasmic membrane, represent possible candidates for such markers. It should be emphasized, however, that expression of gan-

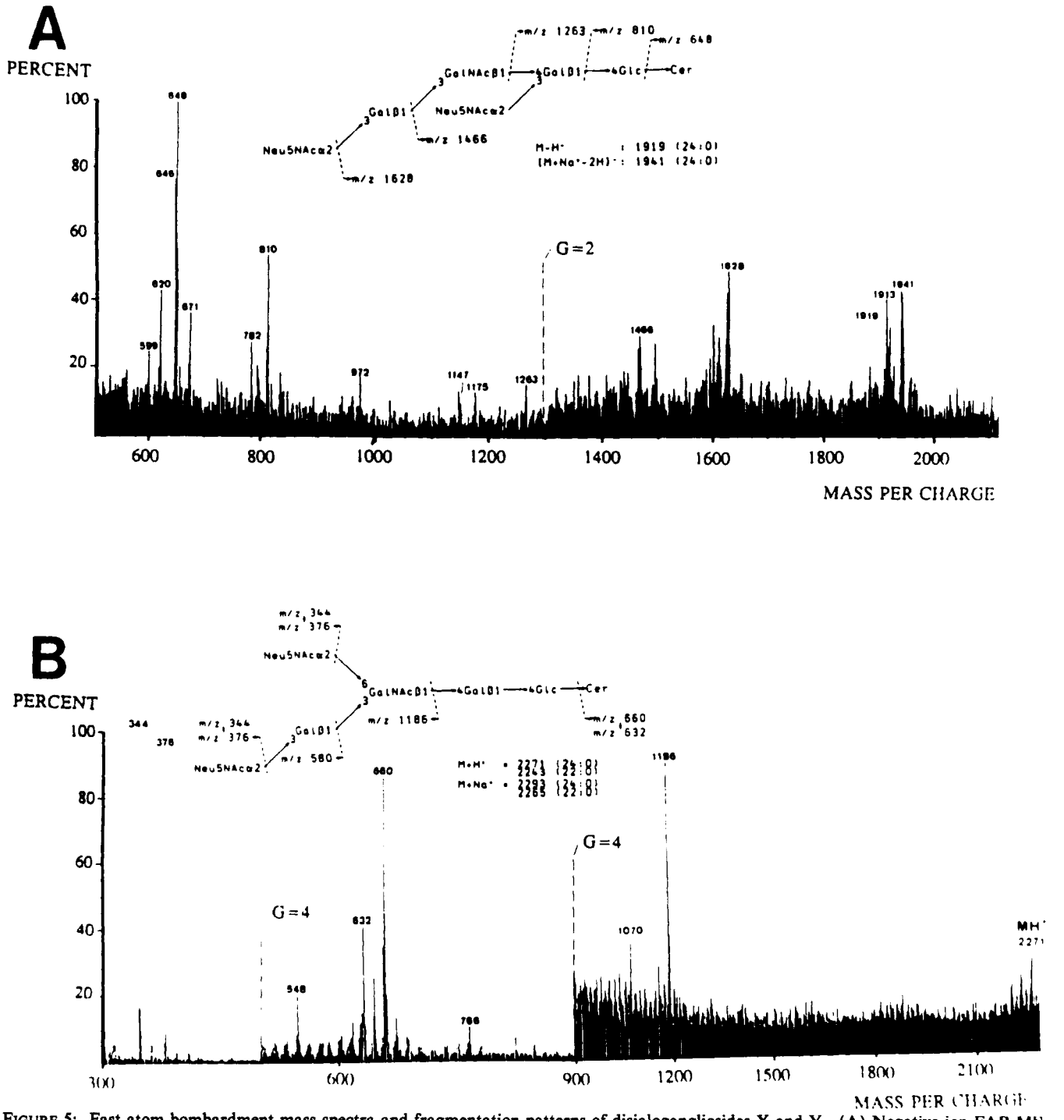

FIGURE 5: Fast atom bombardment mass spectra and fragmentation patterns of disialogangliosides X and Y. (A) Negative ion FAB-MS of native ganglioside X. (B) Positive ion FAB-MS of permethylated ganglioside Y.

Table II: Surface Markers on Purified Lymphocyte Subpopulations[a]

	sorted lymph node cells (%)			
serological markers	CD4- (L3T4)+	CD8- (Lyt-2)+	T cell-depleted spleen cells (%)	thymocytes (%)
Thy-1.2	99	98	<2	92
Lyt-2	5	97	<2	83[b]
L3T4	93	<2	<2	85[b]
mouse Ig	<2	<2	88	<2

[a] % positive. [b] 75% L3T4 and Lyt-2 double positve.

glioside markers can be species-specific. In analyzing the gangliosides of seven murine T_H1 and five T_H2 cell lines or clones, respectively (see Table III), we found a subtype-specific ganglioside pattern within the two T helper subsets with characteristic bands of X and Y. Using HPTLC and immunostaining with an anti $GgOse_4Cer$ antibody after *A. ureafaciens* or *V. cholerae* neuraminidase treatment, and confirming these data by FAB-MS of the isolated gangliosides, X was identified as IVNeuAc,IINeuAc-$GgOse_4Cer$ (G_{D1a}) (24:0/18 and 24:1/18), and ganglioside Y was identified as IVNeuAc,IIINeuAc-$GgOse_4Cer$ (G_{D1a}) (24:0/18).

$G_{D1\alpha}$ was first described in a murine T lymphoma cell line (Murayama et al., 1986) and was previously considered by us to be a general T cell marker (Müthing et al., 1989). Indeed, gangliosides with $G_{D1\alpha}$ properties were detected in all T cell clones and lines, in sorted CD4 positive and CD8 positive lymph node cells, and in thymocytes, but not in B cells. Thus $G_{D1\alpha}$, although more prominent in the gangliosides from T_H1

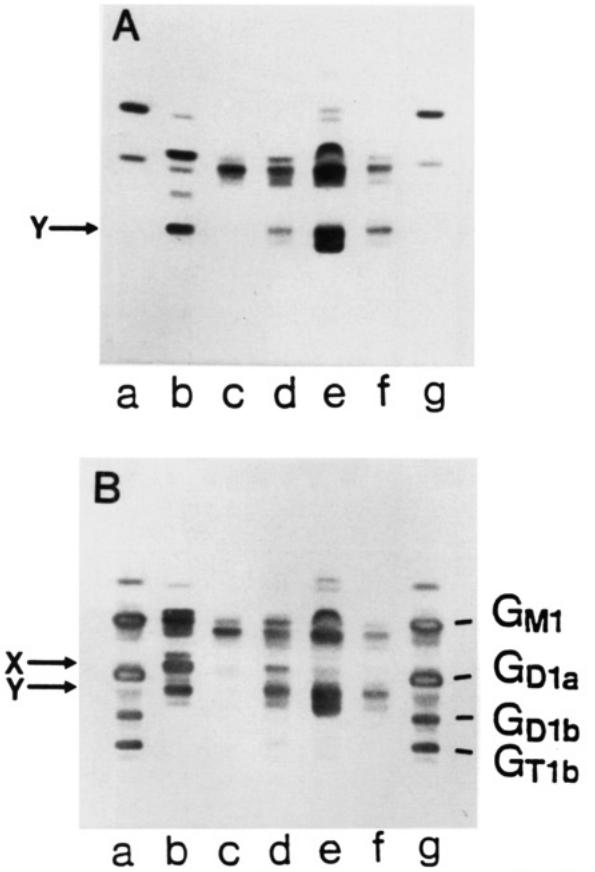

FIGURE 6: Detection of $GgOse_4$ backbone in gangliosides from purified murine lymphocyte subpopulations. Immunostaining was performed with anti-$GgOse_4Cer$ antibody after in situ neuraminidase treatment as described in Figure 2. (A) Staining pattern after *V. cholerae* neuraminidase treatment. (B) Staining pattern after *A. ureafaciens* neuraminidase treatment. Lanes a and g: BBG (1 μg) plus G_{M2} (0.12 μg). Lane b: gangliosides from 1.2×10^6 D10.G4.1 cells. Lane c: gangliosides from 1.4×10^7 T cell-depleted spleen cells. Lane d: gangliosides from 5×10^6 ConA-stimulated CD4 (L3T4) positive T cells. Lane e: gangliosides from 5×10^6 ConA-stimulated CD8 (Lyt-2) positive T cells. Lane f: gangliosides from 2×10^7 thymocytes.

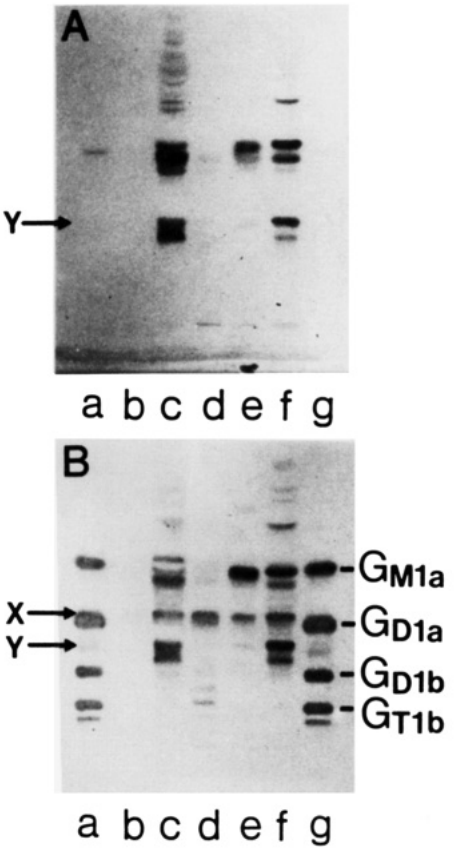

FIGURE 7: Detection of the $GgOse_4$ backbone in gangliosides from different organs of BALB/c mouse. Immunostaining was performed with anti-$GgOse_4Cer$ antibody after in situ neuraminidase treatment as described in Figure 2. (A) Staining pattern after *V. cholerae* neuraminidase treatment. (B) Staining pattern after *A. ureafaciens* neuraminidase treatment. Lanes a and g: BBG (1 μg) plus G_{M2} (0.12 μg). Lane b: gangliosides from 12.5 mg of wet weight liver tissue. Lane c: gangliosides from 12.5 mg of wet weight spleen tissue. Lane d: gangliosides from 22.5 mg of wet weight heart tissue. Lane e: gangliosides from 22.5 mg of wet weight kidney tissue. Lane f: gangliosides from 22.5 mg of wet weight lung tissue.

cells (Figure 1), is not a specific marker for T helper subtypes. In the organs investigated, $G_{D1\alpha}$ was found only in spleen and lung. G_{D1a}, on the other hand, was found in many murine organs, but within the investigated murine immune cells the expression of G_{D1a} seems to be a unique feature of T_H2 lymphocytes. Since G_{D1a} is one of the major gangliosides of T_H2 cells, it should be a useful marker for this helper subtype. Scarcely any G_{D1a} was found in thymocytes, resting B cells, or CD8 positive (cytolytic) T cells, but significant expression was seen in the CD4 positive (helper) T cells which include the T_H2 subpopulation. With the exception of B cells (Figure 6) and whole organs (Figure 7), activated growing cells were analyzed for their gangliosides. It is possible that expression of some gangliosides may be less pronounced in resting T cells. An antibody specifically detecting G_{D1a} would be very useful to clarify this question and, moreover, for staining lymphoid tissue and sorting T_H2 cells. To our knowledge no such antibody is presently available.

The pattern of ganglioside expression of a given T cell population appears to be conserved in mice, since the cell lines and clones under study were derived from genetically unrelated mouse strains. The conserved G_{D1a} expression in T_H2 cells may imply a T_H2-specific function for this ganglioside.

Carbohydrate structures and complementary lectins are known to be involved in cell–cell interactions and homing processes (Springer, 1990; Feizi, 1991). Recently a sialoadhesin from murine stromal tissue macrophages was described which showed a high-affinity binding to G_{D1a} in an HPTLC overlay (Crocker et al., 1991). Since there is evidence for a specific in vivo localization of T_H2 cells (Taguchi et al., 1990), it is tempting to speculate that G_{D1a} may be involved in T_H2-specific localization or T helper cell–macrophage interaction during antigen presentation.

A second known function of cell surface gangliosides is the regulation of the activity of functional membrane proteins.

Table III: Investigated T Helper Lines and Clones[a]

designation	cloned	mouse strain	helper subtype	expression G_{D1a} (X)	expression $G_{D1\alpha}$ (Y)
LNC.2	no	BALB/c	T_H1		+++
LNC.2 A.4	no	BALB/c	T_H1	(+)	+++
B10 BI	no	B10	T_H1		+++
F7.15A	yes	B10×B10.Br	T_H1		+++
F2.1	yes	B10×B10.Br	T_H1		+++
M4	yes	B10×B10.Br	T_H1		+++
LNC.4	no	BALB/c	T_H2	++	+
LNC.4 A.4	no	BALB/c	T_H2	++	+
ST2/K.9	yes	B10×B10.Br	T_H2	++	(+)
D10.G4.1	yes	AKR	T_H2	++	+
LNC.4/2K1	yes	BALB/c	T_H2	++	+

[a] With the exception of the clone LNC.4/2K1, which was derived from the LNC.4 line, all other clones and lines were random samples and not related.

Such an interaction with different growth factor receptors has been described (Bremer et al., 1986; Hanai et al., 1988), and recently the modulation of IL-2-stimulated T lymphocyte proliferation by G_{M1} has been reported (Sharom et al., 1991). Whether G_{D1a} plays any functional role in the cellular activity of the T_H2 cells, or in the cell surface interaction with other cells, remains to be elucidated.

ACKNOWLEDGMENT

We thank Mrs. Maria Höxter for preparative cell sorting, Dr. M. Nimtz for GC–MS analysis of the permethylated ganglioside Y, and Dr. D. A. Monner for his critical reading of the manuscript.

REFERENCES

Bartoszewicz, Z., Koscielak, J., & Pacuszky, T. (1986) *Carbohydr. Res. 151*, 77–88.

Bethke, U., Müthing, J., Schauder, B., Conradt, P., & Mühlradt, P. F. (1986) *J. Immunol. Methods 89*, 111–116.

Bremer, E. G., Schlessinger, J., & Hakomori, S. (1986) *J. Biol. Chem. 261*, 2434–2440.

Ciucanu, I., & Kerek, F. (1984) *Carbohydr. Res. 131*, 209–218.

Crocker, P. R., Kelm, S., Dubois, C., Martin, B., McWilliam, A. S., Shotton, D. M., Paulson, J. C., & Gordon, S. (1991) *EMBO J. 10* (7), 1661–1669.

Egge, H., & Peter-Katalinić, J. (1987) *Mass Spectrom. Rev. 6*, 331–393.

Feizi, T. (1985) *Nature 314*, 53–57.

Feizi, T. (1991) *Trends Biochem. Sci. 16*, 84–86.

Fischer, H. G., Opel, B., Reske, K., & Reske-Kunz, A. B. (1988) *Eur. J. Immunol. 18*, 1151–1158.

Hampl, J., Gradehandt, G., Plachov, D., Gattner, H. G., Kalbacher, H., Voelter, W., Meyer-Delius, M., & Rüde, E. (1991) *Mol. Immunol. 28*, 479–487.

Hanai, N., Dohi, T., Nores, G. A., & Hakomori, S. (1988) *J. Biol. Chem. 263*, 6296–6301.

Hirabayashi, Y., Koketsu, K., Higashi, H., Suzuki, Y., Matsumoto, M., Sugimoto, M., & Ogawa, T. (1986) *Biochim. Biophys. Acta 876*, 178–182.

IUPAC–IUB Commission on Biochemical Nomenclature, (1977) *Eur. J. Biochem. 79*, 11–21.

Kasai, M., Iwamori, M., Nagai, Y., Okumuru, K., & Tada, T. (1980) *Eur. J. Immunol. 10*, 175–180.

Kaye, J., Porcelli, S., Tite, J., Jones, B., & Janeway, C. A. (1983) *J. Exp. Med. 158*, 836–856.

Kniep, B., & Mühlradt, P. F. (1990) *Anal. Biochem. 188*, 5–8.

Mishell, B. B., & Shiigi, S. M. (1980) *Selected Methods in Cellular Immunology*, pp 25–26, W. H. Freeman, San Francisco.

Mosmann, T. R., & Coffman, R. L. (1989) *Annu. Rev. Immunol. 7*, 145–174.

Mühlradt, P. F., Müthing, J., & v. Kleist, R. (1989) in *Gangliosides and Cancer* (Oettgen, H. F., Ed.) pp 185–195, Verlag Chemie, Weinheim, FRG.

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-Katalinić, J., Egge, H., & Mühlradt, P. F. (1989) *Biochemistry 28*, 2923–2929.

Murayama, K., Levery, S. B., Schirrmacher, V., & Hakomori, S. (1986) *Cancer Res. 46*, 1395–1402.

Nakamura, K., Suzuki, M., Taya, C., Inagaki, F., Yamakawa, T., & Suzuki, A. (1991) *J. Biochem. 110*, 832–841.

Peter-Katalinić, J., & Egge, H. (1990) *Methods Enzymol. 193*, 713–733.

Plachov, D., Fischer, H. G., Reske-Kunz, A. B., & Rüde, E. (1988) *Mol. Immunol. 25*, 611–620.

Schmitt, E., Van Brandwijk, R., Fischer, H. G., & Rüde, E. (1990) *Eur. J. Immunol. 20*, 1709–1715.

Sharom, F. J., Chiu, A. L. H., & Chu, J. W. K. (1991) *Biochim. Biophys. Acta 1094*, 35–42.

Spaeth, E., & Rüde, E. (1985) *Eur. J. Immunol. 15*, 1177–1183.

Spiegel, S., Yamada, K. M., Hom, B. E., Moss, J., & Fishman, P. M. (1986) *J. Cell Biol. 102*, 1898–1906.

Springer, T. A. (1990) *Nature 346*, 425–434.

Taguchi, T., McGhee, J. R., Coffman, R. L., Beagley, K. W., Eldridge, J. H., Takatsu, K., & Kiyono, H. (1990) *J. Immunol. 145*, 68–77.

Weisshaar, G.; Hiyama, J., Renwick, A. G. C., & Nimtz, M. (1991) *Eur. J. Biochem. 195*, 257–268.

Williams, M. A., & McCluer, R. H. (1980) *J. Neurochem. 35*, 266–269.

Young, W. W., MacDonald, E. M. S., Nowinski, R. C., & Hakomori, S. (1979) *J. Exp. Med. 150*, 1008–1019.