

Structural Characterization of Gangliosides from Resting and Endotoxin-Stimulated Murine B Lymphocytes†

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ABSTRACT: B lymphocytes from CBA/J mice were stimulated in splenocyte cultures for 72 h with various endotoxins. Bisphosphoryl lipid A from *Escherichia coli* had the highest stimulatory effect followed by LPS of *Citrobacter freundii* and *Salmonella minnesota* as measured by [³H]thymidine uptake. Gangliosides of stimulated B cells (metabolically labeled with D-[1-¹⁴C]galactose and D-[1-¹⁴C]glucosamine) and unlabeled gangliosides from resting B cells (prepared from spleens without stimulus) were analyzed by high-performance TLC, DEAE anion-exchange HPLC, and immunostaining procedures. Contents of ganglioside-derived sialic acids, quantified by HPLC as their fluorescent derivatives, decreased from stimulated to resident B lymphocytes in the following order: LPS *S. minnesota* > LPS *C. freundii* > bisphosphoryl lipid A *E. coli* > resting B cells. Gangliosides of resting B cells contained more *N*-glycolyl- than *N*-acetylneuraminic acid, whereas inverse ratios were found in activated cells, indicating a shift from *N*-glycolyl- to *N*-acetylneuraminic acid due to stimulation. Furthermore, a higher disialoganglioside content was characteristic for activated B cells. Fast atom bombardment mass spectrometry was performed with permethylated mono- and disialoganglioside fractions of LPS *S. minnesota* and LPS *C. freundii* stimulated B cells. Major gangliosides were G_{M1a} and G_{D1a} beside minute amounts of G_{D1b}. The structural heterogeneity in the gangliosides was caused by (a) *N*-substitution of the sialic acids with either acetyl or glycolyl groups, (b) variation in the long-chain base (sphingosine, sphinganine), and (c) substitution of the ceramide moiety by fatty acids of different chain length and degree of unsaturation (C_{16:0}, C_{24:0,24:1}). In summary, these findings indicate the predominance of the G_{M1a} pathway in murine B lymphocytes whereas G_{M1b}-type gangliosides are preferentially expressed in T lymphocytes as well as macrophages.

Gangliosides are a diverse group of glycosphingolipids (GSLs)¹ composed of long-chain base, fatty acid, and carbohydrate moieties (Hakomori, 1981). Structures and functions have been widely reviewed (Stults et al., 1989; Thompson & Tillack, 1985; Zeller & Marchase, 1992). Gangliosides are characterized by the presence of one or more sialic acid units in the oligosaccharide chain. The parent compounds are *N*-acetylneuraminic acid (Neu5NAc) and *N*-glycolylneuraminic acid (Neu5NGc), which play crucial roles in various biological functions (Schauer, 1985, 1988).

Because gangliosides are primarily localized in the outer leaflet of plasma membranes, they are ideally positioned to interact with neighboring cells and components of the extracellular milieu.

In the past, GSLs of murine immune cells (Stein et al., 1978) have been analyzed with the aim of detecting GSLs which might be specifically expressed by, e.g., thymocytes (Schwartz & Gajewski, 1983), macrophages (Mercurio et al., 1984; Yohe et al., 1985; Yohe & Ryan, 1986) and B lymphocytes (Yohe et al., 1990; Chaby et al., 1986). Gangliosides of the G_{M1b} pathway (i.e., G_{M1b}, GalNAc-G_{M1b}, and G_{D1a}) were found to be expressed by Con A stimulated murine T lymphocytes (Müthing et al., 1987, 1989). Since G_{M1b}-type gangliosides were also detected in GSL extracts of mouse spleen (Nakamura et al., 1984, 1987) and in lymphoid tumor cell lines (Murayama et al., 1986; Bartoszewicz et al., 1986; Rokukawa et al., 1988; Laferté et al., 1987; Müthing et al., 1991), these structures were suggested to be T cell specific. Recent reports of Yohe et al. (1991, 1992) indicated the presence of G_{M1b} in stimulated murine macrophages as well as the expression of G_{M1b}-type gangliosides in a murine myelomonocytic leukemic cell line, respectively. However, this ganglioside pathway appears to be dominant in murine immune cells. Reports of several groups have indicated involvement of gangliosides in immunological functions (Marcus, 1982), e.g., as receptors for immunoregulatory agents (Liu et al., 1982; Parker et al., 1984) and as compounds with immunoregulatory potency (Ladisch et al., 1983; Bergelson et al., 1989). Furthermore, GSLs may play a role in immune cell circulation and/or localization in lymphoid organs. Since no detailed structural data of B lymphocyte gangliosides have

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¹ Abbreviations: AcP LipA, bisphosphoryl lipid A; FAB-MS, fast atom bombardment mass spectrometry; GSL(s), glycosphingolipid(s); HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Neu5NAc, *N*-acetylneuraminic acid; Neu5NGc, *N*-glycolylneuraminic acid; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SBA, soybean agglutinin. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations (1977) and the ganglioside nomenclature system of Svennerholm (1963): Gangliotetraosylceramide or GgOse₄Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; G_{M1a}, II³Neu5NAc-GgOse₄Cer; G_{M1b}, IV³Neu5NAc-GgOse₄Cer; G_{D1a}, IV³Neu5NAc-II³Neu5NAc-GgOse₄Cer; G_{D1b}, II³(Neu5NAc)₂-GgOse₄Cer; G_{D1a}, IV³Neu5NAc,II⁶Neu5NAc-GgOse₄Cer; G_{T1b}, IV³Neu5NAc,II³(Neu5NAc)₂-GgOse₄Cer; G_{Q1b}, IV³(Neu5NAc)₂,II³(Neu5NAc)₂-GgOse₄Cer.

been available yet, we spent our efforts in extending the previous work in further structure elucidation of gangliosides from murine B cells. For this purpose, B lymphocytes were stimulated with various endotoxins in splenocyte cultures, and qualitative as well as quantitative differences in ganglioside expression of stimulated and resident B cells were investigated. Preliminary results have been reported (Pörtner et al., 1992).

MATERIALS AND METHODS

Animals. Female CBA/J inbred mice 6–8 weeks of age were purchased from Gl. Bomholtgard Ltd. (Ry, Denmark) and from the Zentralinstitut für Versuchstierzucht (Hannover, Germany).

Spleen Cell Cultures. Mice splenocytes were prepared as described previously (Müthing et al., 1987), and leukocytes were counted in Türk's solution with a hemocytometer. B lymphocytes were specifically stimulated in splenocyte cultures with various mitogenic endotoxins (see below) in DMEM containing penicillin G (65 µg/mL), streptomycin (100 µg/mL), and 44 mM NaHCO₃, supplemented with 5% (v/v) heat-inactivated FCS (Boehringer, Mannheim, Germany). The cells were cultured in a humidified atmosphere of 10% CO₂ in air at 37 °C.

Mitogens. LPS from *Salmonella minnesota* was purchased from Sigma (Deisenhofen, Germany). LPS from *Citrobacter freundii* was extracted with phenol/water and purified by reextraction with phenol/chloroform/petroleum ether, ultracentrifugation, electro dialysis, and precipitation with divalent cations as described in detail elsewhere (Galanos et al., 1979). Bisphosphoryl lipid A (AcP Lip A) was prepared from LPS of the Re-mutant strain F515 of *Escherichia coli* by hydrolysis in acetate buffer as described elsewhere (Brade & Brade, 1985).

Determination of DNA Synthesis. The mitogenic response of endotoxins was obtained in microtiter plates (NUNC, Wiesbaden, Germany). Mitogens were added to 1 × 10⁶ splenocytes to give a final total volume of 0.2 mL. Splenocytes were incubated with various concentrations of endotoxins and cultivated from 24 h up to 98 h to determine the optimal mitogenic response of the respective endotoxins. Twelve hours before harvest, [³H]thymidine (2 Ci/mmol or 74 GBq/mmol; Amersham Buchler, Braunschweig, Germany) at a final concentration of 2 µCi/mL was added to the cultures. Cells were collected in a 12-well cell harvester (Skatron, Tranby, Norway) by pouring them onto glass fiber filters (Skatron, 1-µm pore diameter), washed successively with PBS, 10% trichloroacetic acid, and 95% ethanol, and dried. The filters were placed into scintillation vials, and 3 mL of Aqualuma plus (Baker, Gross-Gerau, Germany) was added to each vial. The samples were counted 1 h later in a Tri-Carb 1900 CA liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). The data were presented as counts per minute (cpm) per 10⁶ living leukocytes originally placed in culture.

B Cell Preparation for Ganglioside Isolation. Splenocytes were stimulated for 72 h with LPS from *S. minnesota* (50 µg/mL), LPS from *C. freundii* (3.2 µg/mL), and AcP Lip A from *E. coli* (1.6 µg/mL) in DMEM as described above. The cells were cultured in 90-mL volumes in tissue culture flasks (NUNC) at a concentration of 5 × 10⁶ cells/mL. Stimulated as well as resting B cells (splenocyte preparations without stimulus) were isolated by agglutination with soybean agglutinin (SBA, Medac, Hamburg, Germany) according to Reisner et al. (1976) with minor modifications. About 3.2 × 10⁹ splenocytes were washed with 20 mL of PBS containing 1 mg/mL glucose and incubated with 8 mL of SBA in PBS

(1 mg/mL, 4 × 10⁸ cells/mL) for 10 min at room temperature. Two-milliliter aliquots were then gently layered on top of 4 mL of FCS/PBS (1/1, v/v). After 15 min at room temperature, agglutinated B lymphocytes (bottom fraction) were suspended in 4 mL of 0.2 M D-galactose in PBS, incubated for 10 min at room temperature, and washed twice with PBS. Viability was determined by trypan blue exclusion with a hemocytometer. Finally, the cells were suspended in chloroform/methanol (2/1, v/v) and stored at -20 °C.

Metabolic Labeling of B Cell Gangliosides. Stimulated B lymphocytes were labeled for 72 h in splenic cultures (see above) with 0.5 µCi/mL (18.5 kBq/mL) D-[1-¹⁴C]glucosamine hydrochloride (54 mCi/mmol or 2 GBq/mmol) and 0.5 µCi/mL (18.5 kBq/mL) D-[1-¹⁴C]galactose (57 mCi/mmol or 2.11 GBq/mmol; Amersham Buchler). B lymphocytes were separated by SBA agglutination and gravity sedimentation as described above.

Isolation of Gangliosides. B cell gangliosides were isolated as described for mouse T lymphocytes by Müthing et al. (1987) and were further purified after the anion-exchange chromatography step on a small Iatrobeads column (6RS-8060; Macherey-Nagel, Düren, Germany) as described by Ueno et al. (1978).

High-Performance Liquid Chromatography. Whole gangliosides were fractionated by anion-exchange HPLC on a column of Fractogel DEAE 650-S (1.0 cm × 15 cm, 25–40-µm average particle size; E. Merck, Darmstadt, Germany). The HPLC system (Gilson Abimed, Langenfeld, Germany) consisted of three M 303 pumps, a high-pressure M 811 mixer, and a computer-control module. The column was rinsed with 3 column volumes of chloroform/methanol/water (30/60/8 by volume). The whole ganglioside fraction was applied, and after being rinsed with 3 column volumes of the same solvent and methanol, gangliosides were eluted with a programmed gradient up to 0.1 M methanolic ammonium acetate. The flow rate was 0.5 mL/min, and fractions were collected every 4 min (2-mL fractions). Aliquots of each fraction were transferred to scintillation vials. After evaporation of the organic solvent, 3 mL of Lipoluma (Baker) was added to each vial, and the radioactivity was determined as described above.

Determination of Sialic Acids. Neu5NAc and Neu5NGc of gangliosides were identified and quantified as their fluorescent derivatives by HPLC essentially as described by Hara et al. (1987). Gangliosides were dissolved in 0.2 mL of 25 mM H₂SO₄ and heated for 1.5 h at 80 °C. Released sialic acids were converted with 1,2-diamino-4,5-methylenedioxybenzene (DMB, Sigma) into fluorescent derivatives. DMB solution (7.0 mM) was prepared by dissolving DMB dihydrochloride in water containing 1 M β-mercaptoethanol and 18 mM sodium hydrosulfite (Hara et al., 1987); 200 µL of the DMB solution was added to the cooled hydrolysate, and the mixture was heated at 60 °C for 2.5 h in the dark. The reaction was stopped in ice/water, and aliquots were used for HPLC analysis. Reference Neu5NAc was purchased from Biomol (Hamburg, Germany) and Neu5NGc from Sigma. HPLC was carried out with a Kontron D 450 system consisting of two HPLC type 420 pumps, a type 460 autosampler, an SFM 25 fluorometer, and a MT 450 data system (Büntemeyer et al., 1991). Isocratic HPLC was performed using an RP18 column (250 mm × 4.6 mm, ODS 5 µm; Beckman, München, Germany) as the stationary phase and acetonitrile/methanol/water (8/8/84 by volume) as the mobile phase. The flow rate was 1.2 mL/min. It was operated at an excitation wavelength of 373 nm and an emission wavelength >448 nm.

Reference Gangliosides. Human brain gangliosides were purchased from Supelco (Bellefonte, PA). Reference gangliosides from Con A stimulated murine T lymphocytes were isolated as described by Müthing et al. (1987) and are listed in Table II.

Thin-Layer Chromatography. Silica gel precoated high-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.24 mm; E. Merck) were used. Gangliosides were separated in chloroform/methanol/water (120/85/20 by volume) containing 2 mM CaCl₂ and were visualized by resorcinol (Svennerholm, 1957). ¹⁴C-Labeled gangliosides were located by autoradiography on Hyperfilm-³H (Amersham Buchler).

HPTLC Immunostaining Assay with Cholera Toxin B Subunit (Choleraegenoid). The HPTLC binding method using cholera toxin B subunit for specific detection of G_{M1a} has been developed by Magnani et al. (1980). We modified the described method as follows. Gangliosides were chromatographed on HPTLC plates, and the silica gel was fixed with poly(isobutyl methacrylate). The plate was incubated with choleraegenoid (Sigma, no. C-7771, 250 ng/mL) in PBS/1% (w/v) BSA (solution A) for 2 h at room temperature. Goat anti-choleraegenoid antiserum (Calbiochem, no. 227040, Frankfurt, Germany; 1:1000 in solution A) and alkaline phosphatase conjugated rabbit anti-goat IgG (Dianova, Hamburg, Germany; 1:1000 in solution A) were used for the immunostaining procedure (1 h at room temperature). Alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolyl phosphate as described (Müthing & Mühlrad, 1988).

To reveal the presence of gangliosides with a G_{M1a} core, i.e., G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b}, neuraminidase is employed to convert these gangliosides to G_{M1a} prior to treatment with choleraegenoid. Silica gel fixed plates were incubated with 50 milliunits/mL *V. cholerae* neuraminidase (Behring, Marburg, Germany) for 18 h at 37 °C in 0.05 M sodium acetate/9 mM CaCl₂, pH 5.5. The subsequent immunostaining assay with choleraegenoid was performed as described above. The technique has been originally developed by Wu and Ledeen (1988).

Detection of Gangliosides of the G_{M1b} Type on HPTLC Plates. Terminally sialylated gangliosides with a GgOse₄-Cer backbone (G_{M1b}, G_{D1a}) were detected as described (Müthing & Mühlrad, 1988). Briefly, gangliosides were chromatographed on HPTLC plates. After silica gel fixation, the plates were incubated with 5 milliunits/mL *V. cholerae* neuraminidase for 2 h at room temperature in the buffer described above. Polyclonal anti-GgOse₄Cer antiserum (1:1000) and alkaline phosphatase conjugated goat anti-rabbit IgG antiserum (1:1000) were used for the immunostaining procedure. Bound antibodies were visualized with 5-bromo-4-chloro-3-indolyl phosphate (Müthing et al., 1991; Müthing & Mühlrad, 1988).

The overlay assay for detection of GalNAc-G_{M1b} with a highly specific antiserum has been recently described by Müthing et al. (1991).

Mass Spectrometric Analysis of Gangliosides. The mono- and disialoganglioside fractions, obtained from resting and from stimulated murine B lymphocytes, were structurally characterized by positive (permethylated fractions) ion mode FAB-MS as previously reported (Peter-Katalinić & Egge, 1990). The permethylation was carried out as described by Ciucanu and Kerek (1984). Permethylated gangliosides were separated from reagents by exhaustive extraction with chloroform and chromatographed on a 1 cm × 23 cm Sephadex

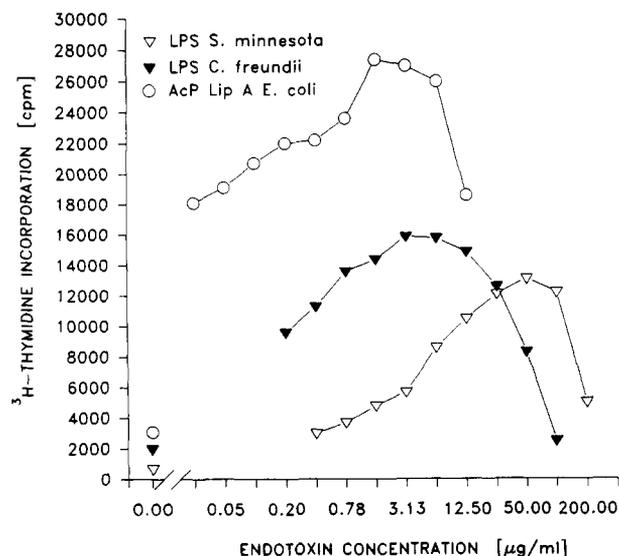


FIGURE 1: [³H]Thymidine incorporation into endotoxin-stimulated spleen lymphocytes. 10⁶ leukocytes/0.2 mL were stimulated for 72 h with various concentrations of endotoxins. Twelve hours before harvesting, the cultures were pulsed with 2 µCi/mL [³H]thymidine. Each point represents the mean of duplicate cultures.

LH-20 column (Pharmacia Fine Chemicals, Freiburg, Germany) with chloroform/methanol (1/1, by volume) as eluent. Final purification was performed on a small Iatrobeads column (0.5 cm × 5 cm, Iatrobeads 6RS-8060) by elution with chloroform, chloroform/methanol (98/2, v/v, 95/5, v/v, and 95/10, v/v), and chloroform/methanol/water (75/24/4, v/v/v).

Fast atom bombardment mass spectrometry (FAB-MS) was performed on a ZAB HF mass spectrometer (VG Analytical, Manchester, Great Britain). The mass spectra were acquired as single scans in the upscan mode on an AMD DP10 data system fitted with SAM II (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany).

RESULTS

Mitogenic Responses of B Lymphocytes to Endotoxins. Certain mitogens such as PHA and Con A stimulate T cells whereas LPSs stimulate B lymphocytes (Andersson et al., 1972; DeFranco, 1987). LPSs as well as lipid A, the endotoxic center of bacterial LPS, elicit a variety of physiological responses in mice (Rietschel et al., 1991). Mitogenic responses of B lymphocytes are generally measured by the uptake of [³H]thymidine. In order to determine the responses to endotoxins from different bacterial sources, murine B lymphocytes were stimulated in splenocyte cultures with LPSs from *S. minnesota* and *C. freundii* and AcP Lip A from *E. coli*. Spleen cells were incubated with various concentrations of endotoxins; 72 h was found to induce optimal B cell mitogenesis as measured by [³H]thymidine uptake (see Materials and Methods). A comparison of the mitogenic responses of B lymphocytes to LPSs from *S. minnesota* and *C. freundii* and to AcP Lip A from *E. coli* is shown in Figure 1. The endotoxins produced B cell activation over a wide range of concentrations. AcP Lip A had the highest stimulatory effect at a concentration of 1.6 µg/mL compared to optimal LPS doses of *C. freundii* (3.2 µg/mL) and *S. minnesota* (50 µg/mL). To analyze the ganglioside expression of resident and endotoxin-stimulated B lymphocytes, these optimal LPS doses were used for B cell production followed by ganglioside isolation (see below).

Table I: B Cell Yields and Parameters of B Cell Preparations from Endotoxin-Stimulated and Resident Splenocytes

endotoxin	stimulus concn ($\mu\text{g}/\text{mL}$)	total leukocytes ^a	B cell yield ^b (cells/% of leukocytes)	[³ H]thymidine uptake ^c (cpm)	viability ^d (%)
LPS <i>S. minnesota</i>	50	6.1×10^9	$1.2 \times 10^9/20\%$	13100	82
LPS <i>C. freundii</i>	3.2	6.9×10^9	$1.3 \times 10^9/19\%$	15900	73
AcP Lip A <i>E. coli</i>	1.6	7.2×10^9	$3.1 \times 10^9/42\%$	27400	89
<i>e</i>		5.3×10^9	$2.2 \times 10^9/41\%$		82

^a 104 mice spleens were prepared, respectively. ^b Obtained by SBA agglutination. ^c 72-h stimulation of 10^6 leukocytes. ^d Determined by trypan blue exclusion. ^e Without stimulus.

Table II: Structures of Gangliosides from Con A Stimulated Mouse Splenic T Lymphocytes^a

ganglioside fraction	fatty acid component	structure	symbol
I	C _{24:1}	IV ³ Neu5NAc-GgOse ₄ Cer	G _{M1b} (Neu5NAc)
II	C _{16:0}	IV ³ Neu5NAc-GgOse ₄ Cer	G _{M1b} (Neu5NAc)
	C _{24:0} , C _{24:1}	IVNeu5NGc-GgOse ₄ Cer	G _{M1b} (Neu5NGc)
III	C _{24:0} , C _{24:1}	IVNeu5NAc-GgOse ₅ Cer	GalNAc-G _{M1b} (Neu5NAc)
	C _{16:0}	IVNeu5NGc-GgOse ₄ Cer	G _{M1b} (Neu5NGc)
	C _{16:0}	IVNeu5NAc-GgOse ₅ Cer	GalNAc-G _{M1b} (Neu5NAc)
	C _{24:0}	IVNeu5NGc-GgOse ₅ Cer	GalNAc-G _{M1b} (Neu5NGc)
IV	C _{16:0}	IVNeu5NGc-GgOse ₅ Cer	GalNAc-G _{M1b} (Neu5NGc)
	C _{24:0} , C _{24:1}	IVNeu5NAc,IIIIVNeu5NAc-GgOse ₄ Cer	G _{D1a} (Neu5NAc, Neu5NAc)
V	C _{16:0}	IVNeu5NAc,IIIIVNeu5NAc-GgOse ₄ Cer	G _{D1a} (Neu5NAc, Neu5NAc)
VI	C _{16:0}	IVNeu5NAc,IIIIVNeu5NAc-GgOse ₄ Cer	G _{D1a} (Neu5NAc, Neu5NAc)

^a Data drawn from Müthing et al. (1987, 1989).

B Cell Production. The approach for the separation of B lymphocytes from splenocyte cultures is based on differential agglutinability of leukocytes with lectins. Endotoxin-stimulated as well as resident B lymphocytes were agglutinated by soybean agglutinin (SBA) from *Glycine max* and separated by gravity sedimentation according to Reisner et al. (1976). B cell yields and parameters of the B cell preparation are summarized in Table I. [³H]Thymidine uptake correlated with B cell yields obtained by the different endotoxins used in this study. Stimulation by LPS of *S. minnesota* (50 $\mu\text{g}/\text{mL}$) gave 20%, LPS of *C. freundii* (3.2 $\mu\text{g}/\text{mL}$) 19%, and AcP Lip A (1.6 $\mu\text{g}/\text{mL}$) 42% agglutinated B lymphocytes from total leukocytes compared to 41% resting B cells prepared from mouse spleen without stimulus (see Table I).

Metabolically Labeled Gangliosides from Endotoxin-Stimulated B Lymphocytes. Significant differences between the ganglioside patterns of Con A stimulated T cells and LPS-stimulated B cells have been reported by Rosenfelder et al. (1979). Structural characterizations have shown the considerable heterogeneity of gangliosides from Con A stimulated T lymphocytes (Müthing et al., 1987, 1989). The main components found in T cells were IVNeu5NAc/Neu5NGc-GgOse₄Cer (G_{M1b}), IVNeu5NAc/Neu5NGc-GgOse₅Cer (GalNAc-G_{M1b}), and IVNeu5NAc,IIIIVNeu5NAc-GgOse₄Cer (G_{D1a}). The reference gangliosides from T cells are listed in Table II.

Metabolically labeled gangliosides from stimulated B and T cells are given in Figure 2. Whereas the autoradiography of gangliosides from T cells shows a continuum of bands on the HPTLC plate (Figure 2, lane a, fractions I–VI), the ganglioside patterns of endotoxin-stimulated B cells are characterized by two clusters, e.g., *S. minnesota* (Figure 2, lane b). Gangliosides of the upper cluster chromatographed analogously to G_{M1a}, and gangliosides of the lower cluster showed chromatographic behavior like G_{D1a} of reference gangliosides from human brain. Similar ganglioside patterns of B cells were obtained by the three different endotoxins used in our experiments. Slight differences due to some faint additional bands superior to the “G_{M1a} cluster” were observed in the ganglioside fractions of the LPS-stimulated B cells compared to AcP Lip A stimulated B cells (see below). Since

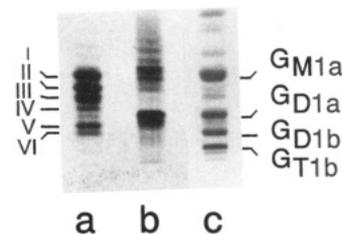


FIGURE 2: Autoradiography of ¹⁴C-labeled gangliosides from Con A stimulated T lymphocytes (lane a) and LPS *S. minnesota* (lane b) stimulated B lymphocytes. 1000 cpm each were applied to the HPTLC plate and chromatographed. The exposure time was 360 h. Ten micrograms of human brain gangliosides (lane c) was stained with resorcinol. T cell gangliosides of bands I–VI are listed in Table II.

chromatographic data are not structurally definitive, we further characterized the gangliosides by immunological methods (overlay technique), anion-exchange HPLC, and fast atom bombardment mass spectrometry as demonstrated in the following sections.

Detection of Gangliosides with a G_{M1a} Core. G_{M1a} can be made visible on the HPTLC plate with cholera toxin using the well-working overlay technique. The gangliosides G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b} can be detected by conversion with *V. cholerae* neuraminidase to G_{M1a} prior to treatment with cholera toxin according to Wu and Ledeen (1988). The suitability of this highly specific overlay assay is demonstrated in Figure 3A using a reference ganglioside mixture from human brain containing the mentioned G_{M1a}-type gangliosides. G_{M1a} was immunostained by cholera toxin without neuraminidase treatment (Figure 3A, lane a) and the higher sialylated gangliosides with G_{M1a} backbone with preceding neuraminidase treatment of the plate (Figure 3A, lane b). Gangliosides G_{M1a}, G_{D1a}, and G_{T1b} were identified in all B cell ganglioside fractions by “in situ” staining with cholera toxin. As shown in Figure 3B, 1×10^6 resting B cells produced three well-detectable G_{M1a} and two G_{D1a} immunostained bands, representing the main B cell gangliosides. One weak G_{D1b} and G_{T1b} band each was detected by usage of this sensitive assay. Resting as well as stimulated B cells showed similar overlay patterns. Gangliosides from 5×10^5 AcP Lip A *E. coli*

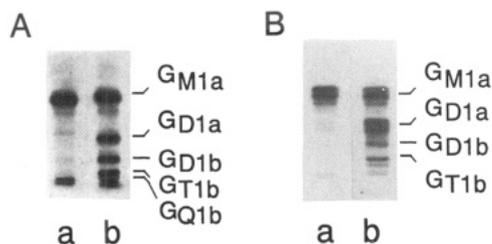


FIGURE 3: Detection of G_{M1a} -type gangliosides in resting murine B lymphocytes. Fifty nanograms of reference gangliosides from human brain (A) and ganglioside aliquots corresponding to 10^6 resting B lymphocytes (B) were chromatographed and immunostained with cholera toxin without (lanes a) and after *V. cholerae* neuraminidase treatment (lanes b). The positions of human brain gangliosides are marked at the margins.

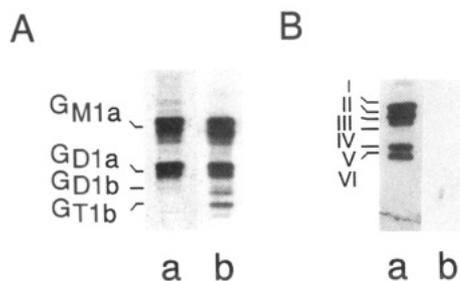


FIGURE 4: HPTLC immunostaining of gangliosides from murine B lymphocytes stimulated with AcP Lip A from *E. coli*. (A) 1000 cpm of ^{14}C -labeled gangliosides from 5×10^5 stimulated B lymphocytes were chromatographed. Lane a, autoradiography, exposure time 312 h; lane b, immunostaining with cholera toxin after *V. cholerae* neuraminidase treatment of the same chromatogram. The positions of human brain gangliosides are marked at the margin. (B) Gangliosides of 2×10^6 Con A stimulated T cells (lane a, positive control) and 2×10^6 AcP Lip A stimulated B cells (lane b) were chromatographed, and the plates were overlaid with *V. cholerae* neuraminidase. GgOse₄Cer bands were detected with specific antibodies. T cell gangliosides of bands I–VI are marked at the margin and are listed in Table II.

stimulated B cells gave strong G_{M1a} and G_{D1a} bands and sparse G_{D1b} and G_{T1b} bands (Figure 4A, lane b). The corresponding autoradiography indicated congruency of metabolically labeled and immunostained gangliosides (Figure 4A, lane a). Higher sensitivity of the immunostaining procedure traced out weak G_{D1b} and G_{T1b} bands, which became visible in the autoradiography by prolonged film exposure (not shown).

B cell gangliosides were checked for the presence of G_{M1b} -type gangliosides by immunostaining with a specific anti-GgOse₄Cer antibody on HPTLC plates after *V. cholerae* neuraminidase treatment as described by Müthing and Mühlradt (1988). G_{M1b} and G_{D1a} , the characteristic gangliosides of Con A stimulated T cells (Figure 4B, lane a; see Table II), were not detected in the ganglioside fractions of resident and stimulated B cells. As an example, the negative stain of gangliosides from LPS *S. minnesota* stimulated B cells is shown in Figure 4B (lane b). Analysis of gangliosides of 2×10^6 T and B cells each showed predominance of the G_{M1b} pathway in T cells. However, when the plate was overloaded with B cell gangliosides ($>1 \times 10^7$ cells), trace amounts of G_{M1b} and G_{D1a} were detected in all B cell preparations. This fact will be a point of discussion (see below). GalNAc- G_{M1b} , highly expressed on Con A stimulated T cells, was not expressed by resident and stimulated B lymphocytes. All the immunochemical data were confirmed by fast atom bombardment mass spectrometry (see below).

Mono- and Disialogangliosides from B Lymphocytes. B cell gangliosides were separated into mono- and disialogangliosides by anion-exchange HPLC on DEAE-Fractogel with

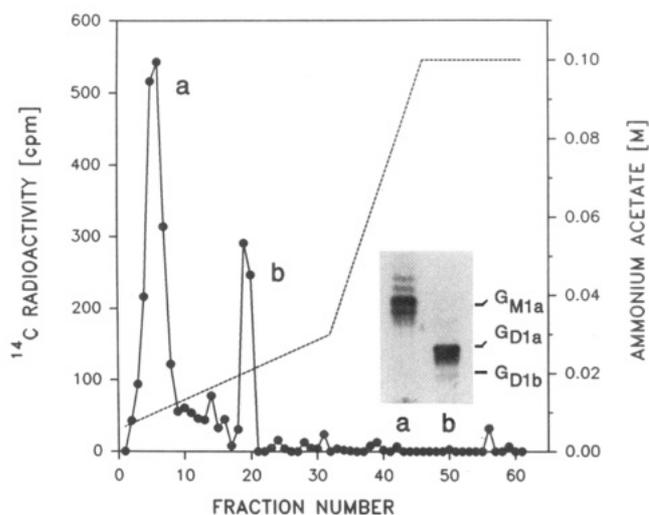


FIGURE 5: Anion-exchange HPLC elution profile of ^{14}C -labeled gangliosides from LPS *C. freundii* stimulated B lymphocytes. 34 000 cpm of labeled gangliosides were applied onto a Fractogel DEAE column and eluted with a gradient of ammonium acetate in methanol. Two-milliliter fractions were collected every 4 min, and the radioactivity of 10% aliquots was determined in a liquid scintillation counter. The monosialogangliosides (fractions 2–10, pool a) and the disialogangliosides (fractions 15–22, pool b) were further characterized by HPTLC (see insert). 1000 cpm each were chromatographed; exposure time 264 h. The positions of human brain gangliosides are marked at the margin.

gradient elution of ammonium acetate in methanol. As an example, the elution profile of ^{14}C -labeled gangliosides from LPS *C. freundii* is shown in Figure 5. Separated mono- and disialogangliosides, fractions 2–10 (pool a) and fractions 15–22 (pool b), respectively, were further analyzed by HPTLC (see insert of Figure 5). The monosialoganglioside cluster (pool a) and the disialoganglioside cluster (pool b) showed chromatographic behavior like human brain G_{M1a} and G_{D1a} , respectively. Ganglioside separation of AcP Lip A *E. coli* and LPS *S. minnesota* stimulated as well as resident B lymphocytes gave similar elution profiles (not shown); elution of unlabeled gangliosides from resident B cells was monitored by determining the sialic acid content of each fraction. The total content of ^{14}C -labeled gangliosides corresponding to 10^6 stimulated B cells and mono-/disialoganglioside ratios are depicted in Figure 6. The highest amounts of ^{14}C -labeled gangliosides were raised by stimulation with LPS *S. minnesota* (A) followed by LPS *C. freundii* (B) and AcP Lip A *E. coli* (C) containing 58%, 66%, and 57% monosialogangliosides, respectively.

Sialic Acid Profiles. Sialic acids of whole ganglioside fractions from stimulated T and B lymphocytes and resting B cells were identified and quantified by HPLC as their fluorescent DMB derivatives. Stimulated T cells expressed the highest amounts of GSL-bound sialic acids compared to B cells (see Figure 7). Sialic acid content decreased from stimulated to resident B lymphocytes in the following order: LPS *S. minnesota* > LPS *C. freundii* > AcP Lip A *E. coli* > resting B cells.

Distinct sialic acid profiles were obtained from mono- and disialogangliosides of resting and stimulated B cells. Both ganglioside fractions of resident B cells are characterized by higher Neu5NGc content (Figure 8B) whereas inverse ratios of sialic acids were detected in all fractions from stimulated B cells, e.g., AcP Lip A *E. coli* stimulus (Figure 8A). Furthermore, in all cases, sialic acid contents (Neu5NAc plus Neu5NGc) were higher in mono- than in disialoganglioside fractions (see Table III). From data of sialic acid concen-

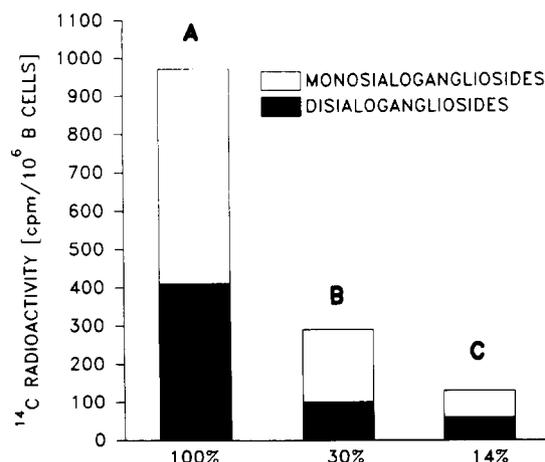


FIGURE 6: Distribution of ¹⁴C-labeled mono- and disialogangliosides within whole ganglioside fractions of 10⁶ B lymphocytes stimulated with LPS *S. minnesota* (A), LPS *C. freundii* (B), and AcP Lip A *E. coli* (C). Total amounts of ¹⁴C-labeled gangliosides are given at the bottom of the columns. ¹⁴C label raised by LPS *S. minnesota* was set to 100%.

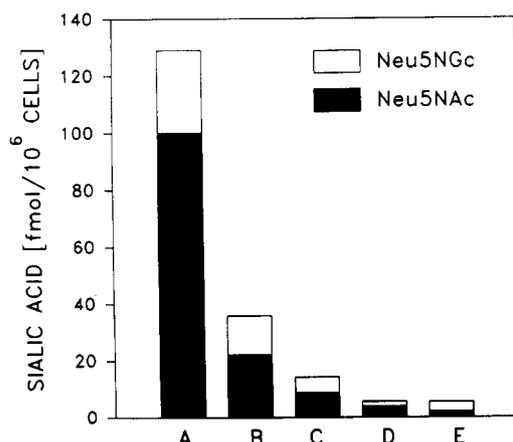


FIGURE 7: Content of Neu5NAc and Neu5NGc in whole ganglioside fractions of 10⁶ cells each Con A stimulated T lymphocytes (A), B lymphocytes stimulated with LPS *S. minnesota* (B), LPS *C. freundii* (C), AcP Lip A *E. coli* (D), and resting B cells (E). Neu5NAc and Neu5NGc, released by acid hydrolysis of gangliosides, were identified and quantified as their fluorescent DMB derivatives by isocratic HPLC using an RP₁₈ column as the stationary phase and acetonitrile/methanol/water (8/8/84 by volume) as the mobile phase.

trations, mono- and disialoganglioside contents were evaluated. The quotients of mono-/disialoganglioside concentrations (see Table III) indicated an increase of amounts of disialogangliosides in activated B cells (mono-/disialogangliosides \approx 2.3) compared to resting B cells (mono-/disialogangliosides \approx 4.8). The same shift in ganglioside sialylation has been described in resident and Con A stimulated T lymphocytes (Müthing et al., 1989).

FAB Mass Spectrometry. Due to limited quantities of material, further structural characterization of B cell gangliosides by FAB mass spectrometry was performed with the entire mono- and disialoganglioside fractions obtained by anion-exchange HPLC (see above). Due to the minute amounts of gangliosides obtained from resting and AcP Lip A *E. coli* stimulated B lymphocytes, structural characterization by FAB-MS gave no reproducible results. However, the compounds of the mono- and disialoganglioside fractions from LPS *S. minnesota* and LPS *C. freundii* stimulated B cells could be clearly deduced from their molecular and specific fragmentation ions. Structural features of Neu5NAc vs Neu5NGc,

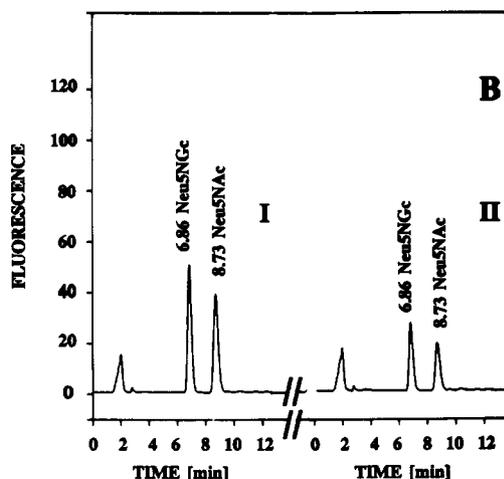
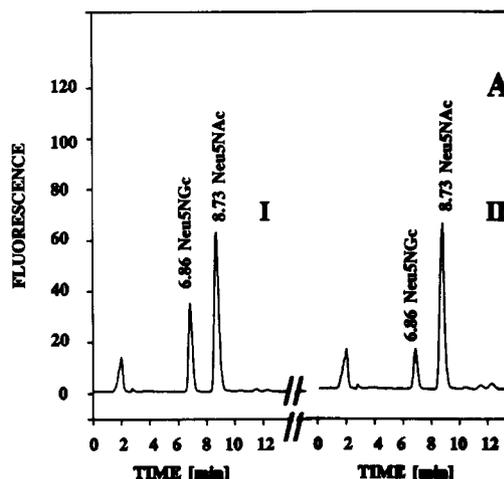


FIGURE 8: HPLC elution profiles of fluorescent DMB derivatives of sialic acids released from mono- (I) and disialogangliosides (II) of AcP Lip A *E. coli* stimulated (A) and resting B lymphocytes (B). Aliquots corresponding to 1.2×10^7 cells, respectively, were applied to the column. Neu5NAc and Neu5NGc were separated by isocratic elution with acetonitrile/methanol/water (8/8/84 by volume) on an RP₁₈ column. The flow rate was 1.2 mL/min.

Table III: Sialic Acid Contents and Ratios of Mono- and Disialoganglioside Fractions from Endotoxin-Stimulated and Resting B Lymphocytes

endotoxin	Neu5NAc ^a M/D ^c	Neu5NGc ^a M/D ^c	ganglio- sides ^{a,b} M/D ^c	ganglio- side quotient M/D ^c
LPS <i>S. minnesota</i>	14.10/10.54	5.93/7.24	20.03/8.89	2.25
LPS <i>C. freundii</i>	2.46/2.78	1.94/1.29	4.40/2.04	2.21
AcP Lip A <i>E. coli</i>	1.42/1.49	0.80/0.40	2.22/0.95	2.34
<i>d</i>	0.84/0.40	1.17/0.44	2.01/0.42	4.79

^a Given in femtomoles per 10⁶ cells. ^b Evaluated from sialic acid contents. ^c M = monosialogangliosides, D = disialogangliosides. ^d Resting B cells without stimulus.

the site of substitution, and the nature of sugar backbones and ceramide moieties were investigated as their permethylated derivatives in the positive ion mode. Their structural parameters determined from FAB-MS experiments are given in Table IV.

FAB-MS of Monosialogangliosides. In Figure 9A, major molecular structures of the G_{M1a} type present in the permethylated monosialoganglioside sample, containing Neu5NAc and Neu5NGc sialic acids, and C_{16:0} and C_{24:0} fatty acids obtained after LPS *S. minnesota* stimulation of B cells are shown. The molecular ions [M + H]⁺ of G_{M1a} (Neu5NAc,

Table IV: Structural Compositions of the Major Gangliosides from LPS *S. minnesota* and LPS *C. freundii* Stimulated B Lymphocytes Obtained by Positive FAB-MS of Their Permethyated Derivatives

[M + H] ⁺ of permethylated ganglioside	fatty acid	long-chain base	symbol	structure
1798/1800	C _{16:0}	sphingosine/sphinganine	G _{M1a}	II ₁ Neu5NAc-GgOse ₄ Cer
1910/1908	C _{24:0,24:1}	sphingosine	G _{M1a}	II ₁ Neu5NAc-GgOse ₄ Cer
1828/1830	C _{16:0}	sphingosine/sphinganine	G _{M1a}	II ₁ Neu5NGc-GgOse ₄ Cer
1940/1938	C _{24:0,24:1}	sphingosine	G _{M1a}	II ₁ Neu5NGc-GgOse ₄ Cer
2159/2161	C _{16:0}	sphingosine/sphinganine	G _{D1a}	IV ₁ Neu5NAc,II ₁ Neu5NAc-GgOse ₄ Cer
2269/2271	C _{24:0,24:1}	sphingosine	G _{D1a}	IV ₁ Neu5NAc,II ₁ Neu5NAc-GgOse ₄ Cer
2189/2191 ^a	C _{16:0}	sphingosine/sphinganine	G _{D1a}	IV ₁ Neu5NGc,II ₁ Neu5NAc-GgOse ₄ Cer
2299/2301	C _{24:0,24:1}	sphingosine	G _{D1a}	IV ₁ Neu5NGc,II ₁ Neu5NAc-GgOse ₄ Cer

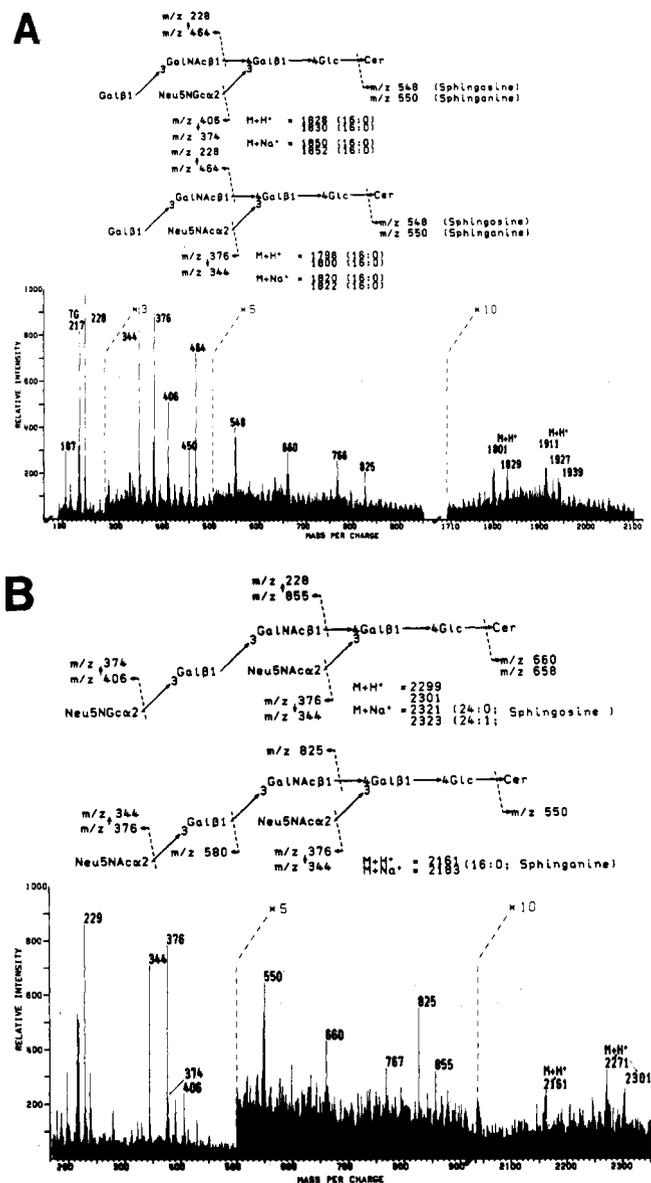
^a Traces only.

FIGURE 9: Positive-ion FAB-MS and fragmentation schemes of the monosialoganglioside fraction of LPS *S. minnesota* (A) and the disialoganglioside fraction of LPS *C. freundii* (B) stimulated B lymphocytes. In (A), the fragmentation schemes only of C_{16:0} fatty acid containing species are presented. The gangliosides are listed in Table IV. TG = thioglycerol.

C_{16:0}) and G_{M1a} (Neu5NGc, C_{16:0}) appeared at $m/z = 1798$ and 1828, respectively, and $[M + H]^+$ of G_{M1a} (Neu5NAc, C_{24:0}) and G_{M1a} (Neu5NGc, C_{24:0}) at $m/z = 1910$ and 1940, respectively. The subsets of sphinganine-derived G_{M1a} were present mostly in the species containing C_{16:0} fatty acid in the

ceramide portion, whereas in the C_{24:0}-containing species also unsaturated components ($[M + H]^+$ at $m/z = 1800$ and 1830, and $m/z = 1908$ and 1938, respectively) were detected. The existence of G_{M1a}-type gangliosides was deduced from the presence of the ion at $m/z = 464 \rightarrow 228$, $[\text{Hex1-3HexNAc}]^+$. The ion at $m/z = 825$, $[\text{Neu5NAc-Hex1-3HexNAc}]^+$, characteristic for the G_{M1b} type was present at very low intensity, indicating the predominance of the G_{M1a} type over the G_{M1b} type (ca. 9:1), which is in agreement with the HPTLC overlay analysis (see above).

FAB-MS of Disialogangliosides. Major molecular structures in the disialoganglioside fractions belong to the G_{D1a} series carrying Neu5NAc on the internal galactose and Neu5NAc or Neu5NGc units on the terminal galactose as shown for LPS *C. freundii* stimulated B cells in Figure 9B. Further heterogeneity arises from the variation in the long-chain base and fatty acid in the ceramide portion like in the monosialoganglioside fraction (see above). The molecular ions $[M + H]^+$ of G_{D1a} [Neu5NAc₂ (C_{24:0,24:1}) and Neu5NGc, Neu5NAc, (C_{24:0,24:1})] appeared at $m/z = 2269/2271$ and $m/z = 2299/2301$, respectively. The C₂₄ fatty acid portion with and without the double bond is linked to sphingosine, whereas the C_{16:0} species of G_{D1a} (Neu5NAc₂, C_{16:0}) is linked to sphingosine and sphinganine long-chain base ($[M + H]^+$ at $m/z = 2159/2161$). The predominance of G_{D1a}-type over G_{D1b}-type gangliosides is documented by the fragment ions $m/z = 825$ $[\text{Neu5NAc-Hex1-3HexNAc}]^+$ and $m/z = 855$ $[\text{Neu5NGc-Hex1-3HexNAc}]^+$. Neu5NGc was present only at the terminal galactose, since no molecular MH⁺/MNa⁺ ions for Neu5NGc₂-GgOse₄Cer have been detected. The unusual mixed $[\text{Neu5NAc-Neu5NGc}]^+$ fragment (Nakamura et al., 1991) at $m/z = 767$ characteristic for G_{D1b}-type ganglioside was of only low intensity, indicating the predominance of the G_{D1a} type as shown by HPTLC overlay analysis as well (see above). These results reveal the expression of the sphinganine-type long-chain base only in the ceramide species containing C_{16:0} fatty acid chain in both mono- and disialoganglioside fractions. The ratio of sphingosine to sphinganine was approximately 5/4 in the monosialoganglioside and 4/5 in the disialoganglioside fraction (see Figure 9). No sphinganine was present in the ceramide portions carrying C_{24:0,24:1} fatty acids. Gangliosides containing saturated long-chain base were the major components in LPS *C. freundii* whereas in LPS *S. minnesota* activated B cells only low amounts of sphinganine could be detected. Due to the lack of material of isolated gangliosides from resting and AcP Lip A *E. coli* stimulated B cells, their structures could not be confirmed by FAB-MS. However, thin-layer chromatographic and immunological data (see above) suggest the expression of gangliosides identical to those found in LPS *S. minnesota* and LPS *C. freundii* stimulated B lymphocytes. In this context, it should be stressed that the overall expression of gangliosides

in AcP Lip A *E. coli* stimulated B lymphocytes was particularly low whereas the cell growth rate of this subpopulation, as monitored by [³H]thymidine incorporation, and B cell yield were particularly high (see Table I).

DISCUSSION

Expression of G_{M1a}-Type Gangliosides in Murine B Lymphocytes. Changes in both the quantity and the structural complexity of B cell gangliosides upon stimulation by different endotoxins compared to resident cells were studied. Major gangliosides detected in all B cell ganglioside fractions were G_{M1a} and G_{D1a} besides minute amounts of G_{D1b}. The structural heterogeneity of gangliosides was caused by four factors: (a) N-substitution of the sialic acids with either acetyl or glycolyl groups; (b) the site of glycosidic linkage by Neu5NAc and/or Neu5NGc in disialogangliosides; (c) variation in the long-chain base (sphingosine, sphinganine) and (d) fatty acid (C₂₄ and C₁₆) in the ceramide portion.

Absence of G_{M1b}-Type Gangliosides in Murine B Lymphocytes. Rosenfelder et al. (1979) first described differing ganglioside patterns of metabolically labeled murine T and B lymphoblasts. Studies on murine thymus gangliosides by Schwarting and Gajewski (1983) indicated the presence of G_{M1b}-type gangliosides. In the murine immune system, G_{M1b} and GalNAc-G_{M1b} were found in spleen (Nakamura et al., 1984, 1987), splenic T lymphoblasts (Müthing et al., 1987), and thymocytes (Müthing et al., 1989). Increased amounts of GalNAc-G_{M1b} were detected in Con A stimulated splenic T cells compared to unstimulated thymocytes by Müthing et al. (1989). These data have been recently confirmed by Horikawa et al. (1991). The uncommon ganglioside G_{D1a}, a III⁶GalNAc sialylation product of G_{M1b}, was found to be characteristic for stimulated T cells (Müthing et al., 1989) and has also been detected in murine T helper subpopulations (Ebel et al., 1992). Due to the expression of G_{M1b}-type structures on lymphoid tumor cell lines (Murayama et al., 1986; Bartoszewicz et al., 1986; Laferté et al., 1987; Müthing et al., 1991), these gangliosides were suggested to be T cell specific at this stage of research. The first evidence of G_{M1b} expression in murine macrophage came from Saito et al. (1980). The presence of G_{M1b} as a major component of murine macrophages was clearly demonstrated in the recent work of Yohe et al. (1991). In addition to G_{M1b}, GalNAc-G_{M1b} and Gal-GalNAc-G_{M1b} were detected in a murine myelomonocytic leukemic cell line with macrophage like properties by the same group (Yohe et al., 1992). Therefore, gangliosides of the G_{M1b} pathway seem to be dominant and characteristic of murine T lymphocytes, macrophages, and related cell lines, but the functional importance of these compounds in the immune system is not yet known. As shown in this study, GalNAc-G_{M1b} was not detected, but small amounts (about 5%) of G_{M1b} were present in B cell preparations, separated by use of soybean agglutinin. Since the agglutinated fraction may contain about 5% T lymphocytes (Reisner et al., 1976), low amounts of G_{M1b} may be derived from T cells present in separated B cell preparations.

Ganglioside Content of Resting and Stimulated B Lymphocytes. Absolute amounts of gangliosides determined by incorporated radioactivity differed depending on the B cell stimulus used. Stimulation with LPS *S. minnesota* resulted in the highest ganglioside label (set to 100%), followed by LPS *C. freundii* and AcP Lip A *E. coli* expressing only 30% and 14% of the *S. minnesota* label, respectively. Interestingly, metabolical ganglioside and [³H]thymidine label were inversely correlated; i.e., AcP Lip A *E. coli* raised the highest

[³H]thymidine but lowest ganglioside label whereas LPS *S. minnesota* stimulus led to the highest ganglioside expression but induced the lowest DNA synthesis. No significant alteration in the mono- to disialoganglioside ratio due to stimulation was observed, showing an average amount of about 60% monosialogangliosides determined by ¹⁴C-labeling. Quantitatively, GSL-derived sialic acids correlated with ganglioside metabolic radiolabeling. Comparison of GSL-bound sialic acids of stimulated T cells and stimulated and resting B cells indicated further profound differences in both lymphocyte subpopulations. The highest sialic acid content of stimulated B cells, represented by LPS *S. minnesota* with 38 fmol/10⁶ cells, corresponds to 130 fmol/10⁶ T cells. Differences within the B cell subpopulations due to stimulation were obvious when ratios of Neu5NAc vs Neu5NGc were compared. While resting B cells express more Neu5NGc- than Neu5NAc-substituted gangliosides, inverse ratios were found in the ganglioside fractions of stimulated B cells. When mono-/disialoganglioside ratios of resting and stimulated B lymphocytes were compared, increased amounts of disialogangliosides were expressed in activated B cells. The same shift has been observed in resting and Con A stimulated T lymphocytes (Müthing et al., 1989). Therefore, the changes in sialic acid expression may be a common principle in leukocyte stimulation and/or differentiation.

SUMMARY

G_{M1a}-type gangliosides, i.e., G_{M1a} and G_{D1a}, were found to be the main gangliosides in resting and stimulated mouse B lymphocytes. Our data show that there are profound differences in the expression of gangliosides in murine leukocytes. Whereas T lymphocytes (Müthing et al., 1987) as well as macrophages (Yohe et al., 1991) express gangliosides of the G_{M1b} pathway, the G_{M1a} pathway is predominant in B cells. It is likely that gangliosides and GSLs in general may play a role either in immune cell circulation or in their localization in lymphoid organs.

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