Glycosphingolipid Antigens in Cultured Microvascular Bovine Brain Endothelial Cells: Sulfoglucuronosyl Paragloboside as a Target of Monoclonal IgM in Demyelinative Neuropathy

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Abstract. Since a number of anti-glycosphingolipid (GSL) antibody activities have been demonstrated in patients with various neurological disorders, the presence of common antigens between brain microvascular endothelial cells (BMECs) and the nervous tissues presents a potential mechanism for the penetration of macromolecules from the circulation to the nervous system parenchyma. We first investigated GSL composition of cultured bovine BMECs. Bovine BMECs express GM3(NeuAc) and GM3(NeuGc) as the major gangliosides, and GM1, GD1a, GD1b, GT1b, as well as sialyl paragloboside and sialyl lactosaminylparagloboside as the minor species. Sulfoglucuronosyl paragloboside was also found to be a component of the BMEC acidic GSL fraction, but its concentration was lower in older cultures. On the other hand, the amounts of neutral GSLs were extremely low, consisting primarily of glucosylceramide. In addition, we analyzed the effect of anti-SGPG IgM antibody obtained from a patient of demyelinative polyneuropathy with macroglobulinemia against cultured BMECs. Permeability studies utilizing cocultured BMEC monolayers and rat astrocytes revealed that the antibody facilitated the leakage of [carboxy-^{14}C]-inulin and ^{125}I-labeled human IgM through BMEC monolayers. A direct cytotoxicity of this antibody against BMECs was also shown by a leakage study using [^{51}Cr]-incorporated BMECs. This cytotoxicity depended on the concentration of the IgM antibody, and was almost completely blocked by preincubation with the pure antigen, sulfoglucuronosyl paragloboside. Our present study strongly supports the concept that immunological insults against BMECs induce the destruction or malfunction of the blood–nerve barrier, resulting in the penetration of the immunoglobulin molecule to attach peripheral nerve parenchyma.

THE vascular endothelial cells (ECs)[1] are highly versatile cells and are structurally and metabolically involved in various barrier functions. The characteristic features of ECs include the presence of a nonthrombogenic luminal surface, expression of von Willebrand factor, prostacycline, and endothelin, and formation of a highly selective barrier to the passage of plasma constituents into the tissue parenchyma. ECs are functionally as well as morphologically heterogeneous in various tissues, and this property is very important for their specialized function in individual tissue and organ function. Brain microvascular endothelial cells (BMECs) are highly specialized cells in this respect and are considered to make up the structural basis of the blood–brain barrier (BBB) and blood–nerve barrier (BNB). Since BMECs are the only cell groups in the nervous system which are continuously exposed to blood constituents, the information conveyed via the surface receptors on BMECs are considered to be very important for the regulation of BBB/BNB function and, subsequently, for the homeostasis of various cations, nutrients, and growth factors in the central and peripheral nervous systems.

Gangliosides are sialic acid-containing glycosphingolipids (GSLs) and are located almost exclusively on the outer leaflet of the plasma membrane. They have been implicated in many cell surface phenomena (Hakomori, 1981), including cellular recognition and adhesion, cellular communication, and modulation of immune responses; hence, knowl-

1. *Abbreviations used in this paper*: BBB, blood–brain barrier; bFGF, basic fibroblast growth factor; BMEC, brain microvascular endothelial cell; BNB, blood–nerve barrier; DM, dissecting media; EC, endothelial cell; GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; MAG, myelin-associated glycoprotein; SGGL, sulfoglucuronosyl glycolipid; SGPG, sulfoglucuronosyl paragloboside, IV³HSO₃-Glc-UAn-Lc₄Cer; SGLPG, sulfoglucuronosyl lactosaminyl paragloboside, VI³HSO₃-GlcUAnLc₆Cer; SPG, sialyl paragloboside, IV³NeuAcnLc₄Cer; SLPG, sialyl lactosaminyl paragloboside, VI³NeuAcnLc₆Cer; sialyl Lewisˣ, IV³Neu-AcIII³FucnLc₄Cer.

edge about GSLs including gangliosides as surface antigens and receptors in BMEC is of crucial importance to the understanding of the pathogenesis of nervous system disorders affecting BBB/BNB. Because a number of anti-GSL antibody activities have been demonstrated in patients with various neurological disorders (Yu et al., 1993), the presence of common antigens between BMECs and the nervous tissues presents a potential mechanism for the penetration of macromolecules from the circulation to the nervous system parenchyma. However, information on the GSL composition in ECs is limited to human umbilical cord vein EC so far (Gillard et al., 1987), and no work has been performed on BMECs.

We recently developed a method to yield sufficient quantities of highly purified bovine BMECs for biochemical analyses. One of the aims of the present study is to determine the GSL composition of cultured bovine BMECs. In addition, we demonstrate the immunological attack of anti-sulfoglucuronosyl glycolipid (SGGL) monoclonal IgM obtained from a patient with demyelinative neuropathy against BMECs, using our in vitro model of BBB/BNB and [^{51}Cr]-incorporated BMEC monolayer.

Materials and Methods

Culture Media for BMECs

Dissecting medium (DM): Medium 199 (GIBCO BRL, Grand Island, NY) supplemented with 5% equine plasma-derived serum (EPDS) (Sigma Chemical Co., St. Louis, MO), 20 mM sodium bicarbonate, 50 μg/ml heparin (Sigma Chemical Co.), 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B (GIBCO BRL), and 20 mM N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid](Hepes; pH 7.2). Bovine BMEC growth medium: DME (GIBCO BRL) supplemented with 15% EPDS and 4% fetal bovine serum (BioWhittaker, Inc., Walkersville, MD), 1 ng/ml human recombinant basic fibroblast growth factor (bFGF) (Collaborative Biomedical Product, Bedford, MA), and 50 μg/ml heparin. Percoll gradient: 43 ml of percoll (Pharmacia Fine Chemicals, Piscataway, NJ), 5 ml of 10× Medium 199, 1.5 ml of 1 M Hepes, 0.5 ml of 100× antibiotic antimycotic solution, 50 ml of DM with 10% of EPDS. The solution was mixed well, aliquoted to 50-ml Oak Ridge centrifuge tubes, and centrifuged at 25,100 g in a fixed angle rotor for 70 min (Beckman JA-17; Beckman Instrs., Carlsbad, CA).

Isolation of BMECs

Isolation of bovine BMECs was achieved using a modified method of Gordon et al. (1991), which was originally designed for rat capillary endothelial cells. Bovine brains were removed from freshly killed animals at a local slaughterhouse and delivered to the laboratory at 4°C. They were rinsed twice with DM, and the pia mater and surface vessels were carefully removed using fine forceps. The cerebral cortex was then minced to 2- to 3-mm cubes, rinsed several times in DM, and homogenized using a Wheaton-Dounce Teflon homogenizer. The homogenate was further dissociated with 0.005% (wt/vol) dispase (grade 1, Boehringer Mannheim Corp., Indianapolis, IN) in DM at 37°C for 2 h in a shaking water bath. After centrifugation (800 g, 5 min), the pellet was suspended with a dextran solution (mol wt 70,000; 15% wt/vol in DM; Sigma Chemical Co.), and the whole suspension was centrifuged (4°C, 4,500 g for 10 min, using a Beckman JS 13.1 swinging bucket rotor). The pellet was resuspended with the dextran solution, and centrifuged again. After two centrifugations, microvessels and some single cells including red blood cells were obtained in the pellet. The undesirable fractions including myelin and brain parenchyma were floated. The pellet was recovered with DM and filtered through a 130-μm nylon mesh (Nitex; Tetko Inc., Elmsford, NY) to remove large vessels. The filtrate was further digested using collagenase/dispase [0.035% (wt/vol) in 10 ml DM; Boehringer Mannheim Corp.] at 37°C, for 12 h in a shaking water bath. The collagenase dispase-treated microvessels were centrifuged and suspended in 1.0 ml DM, layered across the top of a Percoll

gradient and centrifuged at 4°C, 1,650 g, for 10 min. EC clusters were present in approximately the middle third of the gradient (just below the thick band consisting of cell debris and single cells), and were collected and washed twice in DM to remove Percoll. They were filtered through double layers of 15-μm mesh to remove single cells, which were the largest sources of non ECs, and the unfiltered cell clusters were placed into type I collagen (Collaborative Biomedical Products, Bedford, MA) coated dishes. Cells were maintained at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. The medium was changed three times a week.

After 18 h, the first migrated ECs were observed. When the EC colonies grew sufficiently large for cloning (usually more than 100 EC cells), the medium was replaced by 0.25% pancreatin solution (GIBCO BRL). The colonies for cloning (free of contamination of non-ECs including pericytes) were marked prior to the enzymatic treatment. The colonies were detached as clumps of ECs, and the marked colonies were picked up using a Pasteur pipete and were briefly dissociated in 0.1% trypsin in Ca^{2+}, Mg^{2+}-free Hanks' solution for 3–4 min. Cells were inoculated again on 35-mm Petri dishes coated with type I rat tail collagen. After 5–12 d, confluent EC cells, free of contaminating non-EC cells, were subcultured at a split ratio of 1:10.

Identification of BMECs

BMECs were identified by the following four criteria: a cobblestone-like appearance, immunoreactivity to anti-von Willebrand factor antigen antibody (data not shown), uptake of DiI-Ac-LDL (1, 1'-dioctacecyl-3,3,3'3, tetramethyl indocarbocyanine perchlorate acetylated low density lipoprotein (Biomedical Technologies Inc., Stoughton, MA) (Voyta et al., 1984), and the ability to adhere to leukocytes or myeloid cells after stimulation with inflammatory cytokines such as interleukin 1β. To label with DiI-Ac-LDL, cells were incubated with 10 μg/ml of DiI-Ac-LDL at 37°C in normal media for 4 h. The cells were then washed once with probe-free media for 10 min, fixed with 4% paraformaldehyde for 30 min, and viewed under a fluorescent microscope. BMECs showed cobblestone appearance and incorporated the bright DiI-Ac-LDL particles into their cytoplasm (Fig. 1).
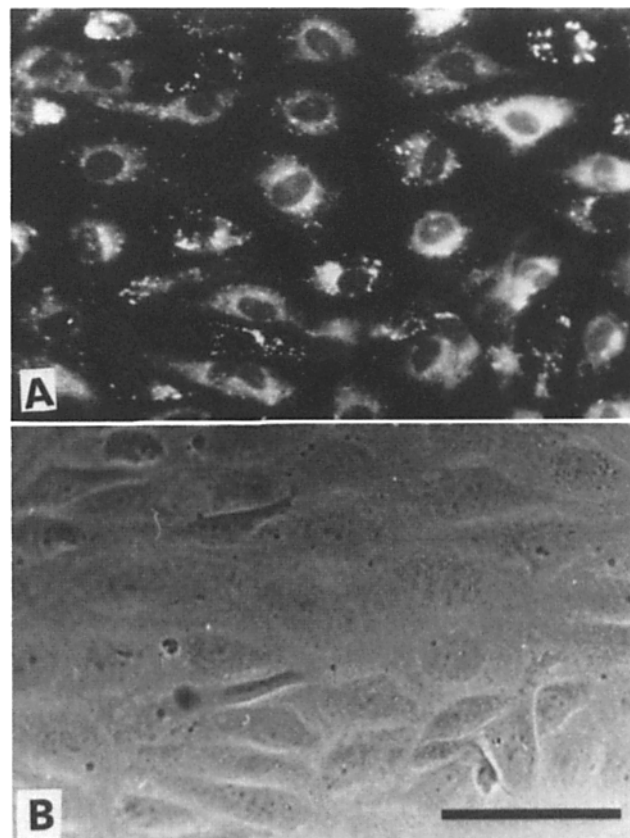

Figure 1. Staining of BMECs with DiI-Ac-LDL. (*A*) Fluorescent micrograph; (*B*) phase contrast micrograph of the same field. Bar, 100 μm.

Only the lots of BMECs with more than 99% of DiI-Ac-LDL positive cells were used for further analyses.

Adhesion of HL-60 cells, known to possess sialyl Lewis[x] (IV[3] NeuAcIII₃FucnLc₄Cer) antigen which has been shown to be a specific ligand for E-selectin (Phillips et al., 1990) on activated BMECs, was assayed. The BMECs monolayer, confluently grown in a collagen-coated 24-well plate, was stimulated with 5 ng/ml of human recombinant interleukin 1β (Beckton-Dickinson, Franklin Lakes, NJ). Four hours later, HL-60 cells ($1.0 \times 10^6/0.5$ ml/well) were added and incubated for 20 min with rotation (100 rpm) at room temperature. The number of attached cells were counted directly under light microscopy. Significantly larger number of HL-60 cells attached to stimulated BMECs ($12310 \pm 1050/mm^2$; Fig. 2 A) than to nonstimulated BMECs ($410 \pm 60/mm^2$; Fig. 2 B).

Adhesion of human leukocytes to activated BMEC monolayer was also investigated. Leukocytes were isolated by Histopague-1077 (Sigma Chemical Co.) density gradient centrifugation of EDTA-treated blood from healthy volunteers. Four hours after stimulation with interleukin 1β, leukocytes ($0.25 \times 10^6/0.5$ ml/well) were added and incubated for 20 min with rotation (100 rpm) at room temperature. Then the cells were fixed with 2.5% glutaraldehyde overnight and the number of cells attached to the surface of BMEC monolayer was counted. Significantly larger numbers of leukocytes was attached to the interleukin β-stimulated BMEC ($P < 0.05$, $1250 \pm 280/mm^2$ vs. $200 \pm 20/mm^2$; Fig. 2 C and 2 D).

Isolation of Glycosphingolipids

Cultured BMECs, confluently grown and $\sim 1 \times 10^7$ cells in number, were harvested by scraping from the petri dishes, washed two times with PBS, pH 7.3, and homogenized in 0.5 ml of distilled water. Cells grown in vitro from days 14–35 were used for GSL analysis, except for the quantitation of SGGLs. The latter was performed utilizing cultures of 7 or 14 d in vitro because SGGLs became undetectable on TLC plate after long period of cultivation. Protein contents were determined by the method of Lowry et al. (1951). Lipids were extracted with 5 ml of chloroform/methanol (1:1 by volume), and 5 ml of chloroform/methanol (1:2 by volume), successively. After evaporating the organic solvents under a nitrogen stream, the lipids were

dissolved in 0.5 ml of chloroform/methanol/water (30:60:8 by volume; solvent A), and applied to a Sephadex LH-20 column (0.5 × 48 cm, 25-ml bed volume; Pharmacia Fine Chemicals), pre-equilibrated with solvent A. After the first 8 ml of the effluent was discarded, the next 8 ml of the effluent was collected as a GSL fraction. The GSL fraction was then applied to a DEAE-Sephadex A-25 column (2.0-ml bed volume; Pharmacia Fine Chemicals). The neutral GSL fraction was eluted with 10 ml of solvent A and 10 ml of methanol, and the acidic GSL fraction was eluted with 10 ml of 0.2 M sodium acetate in methanol and 0.4 M sodium acetate in methanol. The acidic lipid fraction was evaporated to dryness and the residue was dissolved in 0.5 ml of solvent A, and then desalted by Sephadex LH-20 column as described above. The recovered gangliosides and SGGLs were developed on a high-performance thin-layer chromatographic plate (HPTLC; Merck, Darmstadt, Germany) with the solvent system of chloroform/methanol/water containing 0.22% $CaCl_2·2H_2O$ (50:45:10 by volume, solvent system I) or chloroform methanol 5N ammonia 0.4% $CaCl_2·2H_2O$ (50:45:5:5 by volume, solvent system II), and were visualized by spraying the plate with the resorcinol-HCl or orcinol-sulfuric acid reagent (Ando et al., 1978; Sekine et al., 1984).

The neutral GSL fraction was evaporated to dryness. The residue was subjected to acetylation according to the method of Handa (1963) to remove the phospholipids. The acetylated GSLs were purified by Iatrobeads column chromatography (0.5 × 10 cm) by stepwise elution with 10 ml each of chloroform n-hexane (1:1 by volume), chloroform, chloroform methanol (4:1 by volume), and chloroform methanol (2:1 by volume). The acetylated GSLs were eluted with chloroform methanol (4:1 by volume). The eluant was evaporated to dryness and the residue was subjected to mild alkaline treatment (0.5 ml of 0.4N NaOH in methanol) at 40°C for 2 h. The reaction mixtures were applied to a Sephadex LH-20 column to remove salts as described above. Neutral GSL composition was examined by HPTLC. Prior to chromatography, the upper half of the plate was sprayed with 1% sodium borate and the entire plate was activated for 30 min at 120°C. Following application of the sample, the plate was developed with the solvent system of chloroform/methanol/water (65:35:5 by volume, solvent system III). The neutral GSLs were visualized by spraying with the orcinol-sulfuric acid reagent.
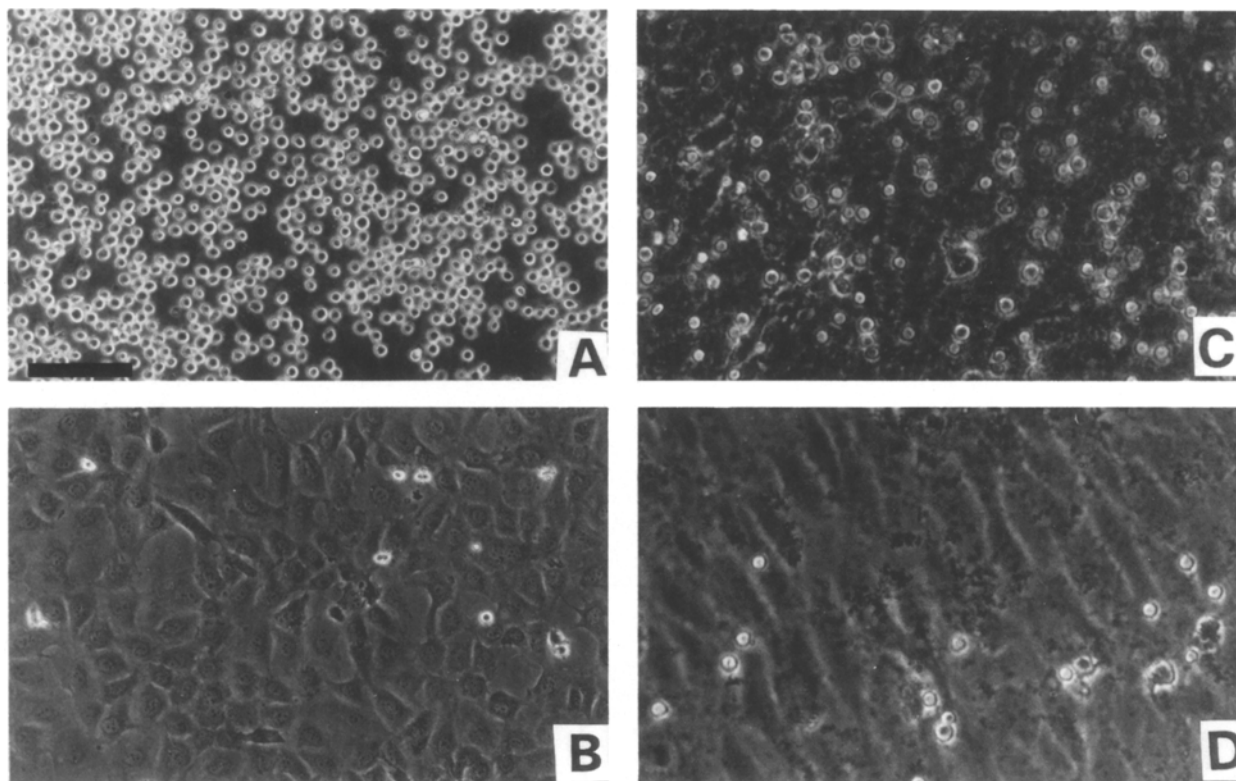

Figure 2. Attachment of HL-60 cells (A) and (B), and human leukocytes (C) and (D) to the surface of BMECs. (A) Activated and (B) nonactivated BMECs (HL-60 cells). (C) Activated and (D) nonactivated BMECs (human leukocytes). Pictures C and D were taken after overnight fixation with 2.5% glutaraldehyde. Bar, 100 μm.

HPTLC-immunostaining of GM3 Gangliosides

Immunostaining of gangliosides was performed on an HPTLC plate as described previously (Kasai et al., 1984). The acidic GSL fractions of the brain, BMEC and standard GM3 (GM3[NeuAc] and GM3 [NeuGc] from bovine adrenal medulla [Ariga et al., 1984]) were developed on an HPTLC plate in the solvent systems as described above. The plate was then dipped in a 0.2% polyisobutylmethacrylate (Aldrich Chemical Co., Milwaukee, WI) solution in hexane for 30 s. After drying the plate in air, the plate was overlaid with an avian polyclonal antibody against GM3(NeuGc) (courtesy of Dr. N. Kasai, Hokkaido University, Japan) at a dilution of 1:200 with PBS containing 0.3% gelatin (dilution buffer) at 4°C overnight. The plate was then incubated with peroxidase-conjugated goat anti–rabbit immunoglobulin antibody (Cappel, West Chester, PA) at a dilution of 1:200 for 1 h at room temperature. Gangliosides were visualized with a PBS solution containing 0.5% 4-chloro-1-naphtol and 0.01% H_2O_2.

Quantification of Gangliosides

The content of GM3 was determined by densitometric scanning of the chromatographic plate visualized with the resorcinol–HCl reagent as described previously (Ando et al., 1978). Because the amounts of gangliosides other than GM3 were below the detection limit of the resorcinol–HCl reagent, quantification of these gangliosides was performed using more sensitive ligands and specific antibodies against each GSL as described below. The content of gangliotetraose-series gangliosides including GM1 and GD1a and neolacto-series gangliosides were determined by the GSL-overlay technique with in situ treatment of gangliosides with sialidase and cholera toxin B-subunit (List Biological Labs. Inc., Campbell, CA) or an anti-paragloboside antibody (Myoga et al., 1988) (kindly provided by Dr. T. Taki) as described previously (Saito et al., 1985; Miyatani et al., 1990a). The chromatogram obtained was scanned by a Shimadzu CS-910 scanning densitometer equipped with a Shimadzu CR1A data processor (Shimadzu Scientific Co., Kyoto, Japan) at 580 nm in a reflectance mode. Each ganglioside was quantitated based on the standard curves obtained from the standard gangliosides developed on the same plate.

Immunocytochemistry of GM3(NeuAc)

Cultured BMECs on 35-mm dishes were fixed with 4% paraformaldehyde for 15 min and washed with PBS at least three times. After incubation in PBS with 3% nonimmunized rabbit serum, rat polyclonal anti-GM3(NeuAc) antibody, diluted 1:100 in PBS, was applied at 4°C for 72 h. The dishes were washed three times and peroxidase-conjugated rabbit anti–rat immunoglobulin Ab (Cappel, 1:200 dilution) was applied for 1 h at room temperature. Then the dish was incubated in diaminobenzidine tetrahydrochloride (Sigma Chemical Co.; 600 μg/ml in PBS with 0.03% H_2O_2) for 5 min to visualize the labeled sites.

Monoclonal IgM that Reacts with SGGLs

The serum from a patient with demyelinative neuropathy and IgM paraproteinemia (LT-serum) was provided by Dr. A. Pachner (Yale University School of Medicine, New Haven, CT). This monoclonal IgM recognizes a carbohydrate epitope that is shared by myelin-associated glycoprotein (MAG) (Braun et al., 1982), a group of low molecular weight glycoproteins in peripheral nerve (Inuzuka et al., 1984), glycoproteins of the N-CAM family (Kruse et al., 1984, 1985), human natural killer cells (Schuller-Petrovic et al., 1983), and at least three acidic GSLs, sulfoglucuronosyl paragloboside (SGPG), sulfoglucuronosyl lactosaminyl paragloboside (SGLPG), and band X (Chou et al., 1986; Ariga et al., 1987; Kusunoki et al., 1987). The anti-MAG and anti-SGGL activities of this serum were examined by an immunoblot technique and HPTLC-immunostaining method, respectively (Kohriyama et al., 1987). A purified IgM preparation was obtained from the ammonium sulfate precipitation of the LT serum (LT-IgM). The precipitate was dialyzed against water, and the IgM fraction was finally separated by Sephacryl G-300 (Pharmacia Fine Chemicals) and freeze-dried. Control human IgM was purchased from Sigma Chemical Co. It was also dialyzed against water and freeze-dried.

Quantification of SGPG

The total GSL fraction, including both acidic and neutral GSLs, was applied directly to an HPTLC plate (Yoshino et al., 1993). Quantitative analysis of SGGLs was achieved by the method described previously (Kohriyama et al., 1987) with slight modifications. After the GSL fraction and authentic

SGPG (from 2 to 120 ng in quantity) were chromatographed in the solvent system I, the plate was dipped in 0.2% polyisobutylmethacrylate solution in hexane for 30 s and air dried. The the plate was incubated with LT-serum at a dilution of 1:1,000 in dilution buffer, followed by peroxidase conjugated rabbit anti–human IgM (μ-chain specific, 1:1,000; Cappel). The plate was incubated with cyclic diacylhydrazide solution (Amersham Corp., Arlington Heights, IL) for 1 min and exposed on an x-ray film for 15 s. SGPG was quantitated based on the standard curves generated by densitometric scanning of known amounts of SGPG developed on the same plate.

Sulfate Incorporation into BMEC Monolayer

BMEC monolayer (in vitro 45 d, confluently grown on 60-mm culture dish) was incubated with 1 mCi of [^{35}S]-Na$_2$SO$_4$ (New England Nuclear, Boston, MA) for 24 h at 37°C and harvested. Total lipids were then extracted from the homogenized cells as described above and the GSL-fraction was cochromatographed with authentic SGPG and the acidic lipid fraction obtained from bovine cauda equina in two different solvent systems I and II. Then the plate was exposed to an x-ray film for 72 h and immunostained with LT-serum as described above.

Immunostaining of Living BMECs with LT-IgM

BMECs grown on collagen-coated 35-mm petri dishes (Beckton-Dickinson) was incubated with heat-inactivated EC media containing 0.1% LT-IgM for 1 h. After washing three times with Hanks' solution, cells were further incubated in heat-inactivated EC media containing FITC-conjugated rabbit anti–human IgM (μ-chain specific, 1:40 dilution; Dako, Carpinteria, CA) for 30 min. BMECs were washed three times with Hanks' solution and immediately fixed with 4% paraformaldehyde for 15 min.

^{125}I-labeling of Normal and Patient's IgM

A portion of the freeze-dried IgM from LT-serum and control was [^{125}I]-iodinized according to the method of Bolton and Hunter (1973), using the Bolton–Hunter reagent, N-succinimidyl 3-(4 hydroxy, 5-[^{125}I]iodophenyl) proprionate, purchased from Amersham Corp. The iodinated IgM fraction was desalted with a PD-10 column (Pharmacia Fine Chemicals).

Isolation and Culture of Rat Astrocytes

Primary cultures of astrocytes were prepared from newborn rat cerebral cortex using a modified method of McCarthy and de Vellis (1980). The cerebral hemispheres were aseptically removed from newborn (Sprague-Dawley, postnatal days 1–2) rats and placed into ice-cooled Ca^{2+}, Mg^{2+}-free Hanks' solution. The meninges were carefully removed and the hemispheres were minced into small fragments. After incubation in 0.005% dispase (Boehringer Mannheim Corp.) solution for 2 h at 37°C, the tissue was centrifuged at 150 g for 3 min, and the supernatant was replaced with DME supplemented with 10% FBS. The digested tissue was then dissociated by trituration 15 times with a Pasteur pipete, filtered through a 125-μm nylon mesh screen (Tetko, Inc.), and seeded into 75-cm^2 culture flasks (Falcon Plastics, Cockeysville, MD). Cells were maintained in an atmosphere of 5% CO$_2$ in a humidified incubator. The medium was changed three times a week. After 7–9 d in vitro, the flasks were filled with flat astrocytes and small, round oligodendrocyte precursors (OA-2 progenitors). The flasks were vigorously shaken (200 rpm) on an orbital shaker (Lab-line Instruments, Melrose Park, IL) for 48 h to detach loosely attached OA-2 progenitors and macrophages. The media containing detached cells were discarded and the cells still remaining at the bottom (almost 95% as astrocytes) were used for coculture with BMECs or temporarily stored in liquid nitrogen.

Coculture of Bovine BMECs and Rat Astrocytes

Fig. 3 shows the schematic drawing of the BMEC/astrocyte coculture system. ECs obtained from brain microvessels are known to gain high impedance and mimic the actual BMECs making up the BBB and BNB in vivo when cocultured with astrocytes (Meresse et al., 1989; Rubin et al., 1991). Both (luminal and abluminal) sides of the culture plate inserts (Millicell-CM, pore size 0.4 μm, 30 mm in diameter; Milipore, Bedford, MA) were coated with rat tail collagen type I (Collaborative Biomedical Products) and human fibronectin (Biomedical Technologies Inc.). Astrocytes were plated at a density of 1 × 10^6 cells/insert on the bottom side with the filter placed upside down. After 8 h, the filters were properly positioned in 6-well culture plates (Beckton-Dickinson) and were examined to ensure complete coverage by cultured astrocytes. 7 to 10 d after plating, bovine BMECs were
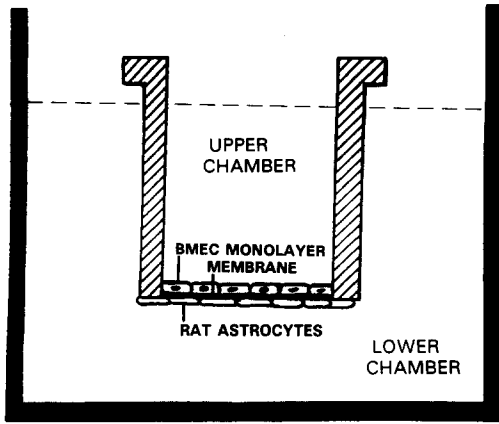
Figure 3. Schematic drawing of the experimental system for permeability study. Rat tail type I collagen- and human fibronectin-coated membrane was sandwitched between BMEC monolayer and rat astrocytes. Neither cells can traverse to the other side because of the small (0.4 μm) pore size of the membrane. This system mimics the actual brain microvascular system in vivo because the BMECs were closely cultured but not directly in touch with astrocytes and the challenged substances always came from the upper chamber, equivalent to the intraluminal space of the capillaries in vivo.

seeded on the upper side of the filter at a concentration of 5×10^5 cells/insert and the media was changed to EC media. These cocultures were used for [carboxy-^{14}C]-inulin or [^{125}I]-IgM flux determinations after 7 d. The electric resistance across BMECs cultures on Millicell-CM was measured with a WPI EVOM apparatus (World Precision Instruments, Sarasota, FL). The resistance of the filter with astrocytes was subtracted from the resistance of the cocultures to obtain the resistance due to the BMEC monolayers themselves. The resistance of these monolayers after 7 d of coculture averaged 439 ± 107 ohm·cm^2 (mean ± SD; $n = 22$) and only the BMEC monolayer which had a resistance of more than 350 ohm·cm^2 were utilized for flux measurements.

Flux Measurements and Data Analysis

The upper compartment was incubated with test media (0.1% LT-IgM or control IgM in heat-inactivated EC media), with or without 20% fresh human serum as a source of complement for 3 h. After washing two times with DME, 2.5 μCi of [carboxyl-^{14}C]-inulin (New England Nuclear) in 2.5 ml of EC media was added to the upper compartment. The appearance of [carboxyl-^{14}C]-inulin in the lower chamber was measured at various times (0, 10, 20, 30, 40, and 60 min) thereafter by scintillation counting of 100-μl
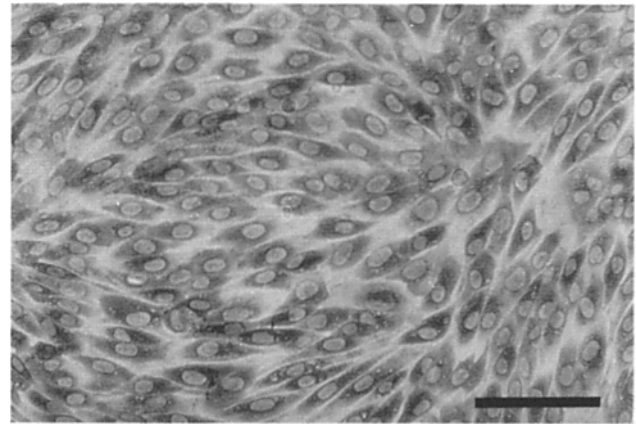

Figure 5. Immunocytochemistry with the anti-GM3(NeuAc) antibody. All cells grown in the monolayer were immunostained by this antibody. Bar, 100 μm.

aliquots of upper and lower chambers. For comparison, the flux across the EC-free filters were also measured. Care was taken to make sure that the fluid levels in the two chambers were equal (2.5 ml in the upper chamber and 3.0 ml in the lower). The chambers were kept at 37°C during the whole course of the experiment, and stirred (30 rpm, using an orbital shaker) to ensure uniform distribution of [carboxyl-^{14}C]-inulin traversed across the membrane.

The clearance of [carboxyl-^{14}C]-inulin was obtained as a measure of endothelial permeability. The clearance (in μl) of inulin (C_{inu}) was calculated as follows: $C_{inu} = V_L*CPM_L/CPM_U$, where V_L is the volume of the lower compartment in μl, CPM_L is radioactivity of [carboxy-^{14}C]-inulin in the lower chamber in CPM/μl, and CPM_U is the radioactivity of [carboxy-^{14}C]-inulin in the upper compartment in CPM/μl. The clearance rate (μl/min) was calculated by fitting the measured clearance for a single experiment to a straight line by least squares linear regression. The flux of [^{125}I]-IgM was also measured in the same manner. After incubation with the test media, ~50,000 cpm of [^{125}I]-IgM (LT or normal) was added to the upper compartment and the appearance of [^{125}I]-IgM in the lower chamber was estimated at various times by measuring 100-μl aliquots with a gamma counter.

^{51}Cr Release Assay

The confluent monolayers of BMECs in 24-well multiple plates were incubated for 18 h with the EC media supplemented with 5 μCi/ml of [^{51}Cr]-sodium chromate (1 mCi/ml in saline, 250-500 mCi/mg; 0.4 ml/well; New England Nuclear). Each well was washed two times with DME to remove
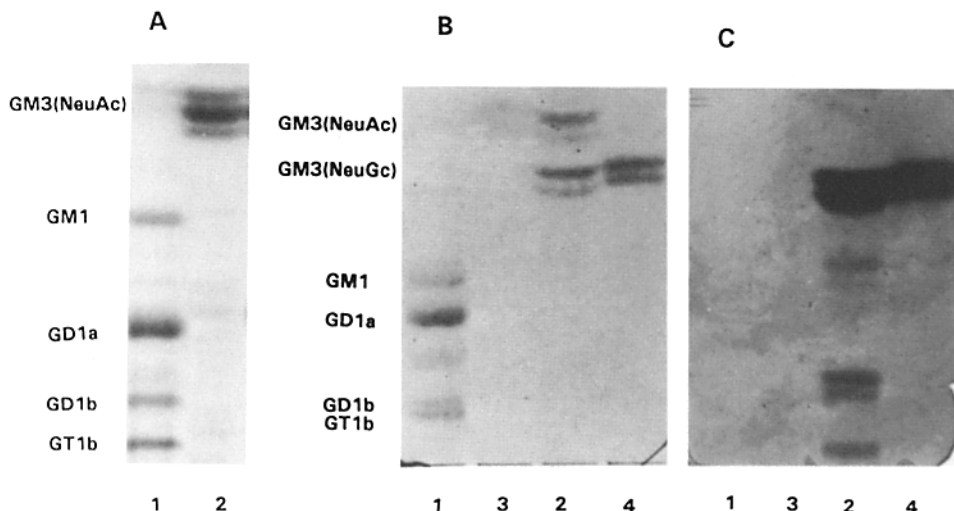

Figure 4. HPTLC-immunostaining with the anti-GM3 (NeuGc) antibody. Lane *1*, human brain ganglioside standards; lane *2*, BMEC gangliosides; lane *3*, standard GM3 (NeuAc); lane *4*, standard GM3(NeuGc). *A* was developed in the solvent system I. *B* and *C* were developed in the solvent system II. The bands of *A* and *B* were visualized with the resorcinol-HCl reagent. The bands of *C* were immunostained with anti-GM3 (NeuGc) antibody.
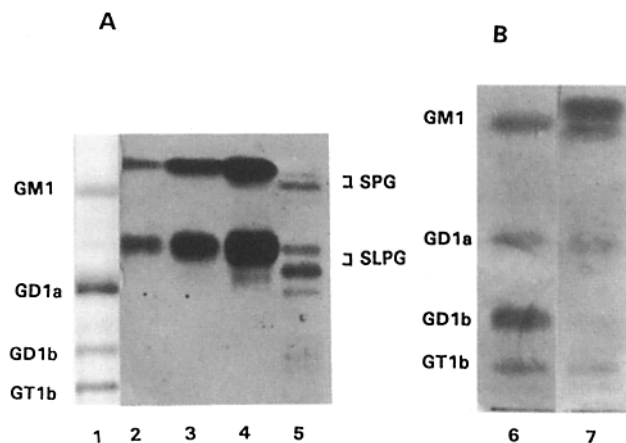
A

B

Figure 6. HPTLC-immunostaining of BMEC gangliosides. HPTLC-immunostaining of (A) SPG and SLPG and (B) ganglio-tetraose-series gangliosides in BMEC. Lane 1, human brain ganglioside standards were visualized with the resorcinol-HCl reagent; lanes 2-4, 0.1, 0.3, and 1.0 nmol of standard SPG (IV³NeuAcnLc₄Cer) and SLPG (VI³NeuAcnLc₆Cer), respectively; lane 5, BMEC gangliosides. The bands in lanes 2-5 were immunostained with anti-paragloboside antibody after sialidase treatment in situ. Lane 6, 1.25 pmol each of GM1 and GDla, and 2.5 pmol each of GDlb and GTlb; lane 7, BMEC gangliosides. Gangliotetraose-series gangliosides were detected by cholera toxin B-subunit after sialidase treatment in situ. The plates were developed in the solvent system I.

unbound radioactivity. The BMECs were than incubated with 0.4 ml per well of heat-inactivated EC media supplemented with: (a) 0.5% LT-IgM, (b) 0.5% LT-IgM plus 20% fresh human serum, (c) 0.1% LT-IgM, (d) 0.1% LT-IgM plus 20% fresh human serum, (e) 0.5% normal human IgM, (f) 0.5% normal human IgM plus 20% fresh human serum, (g) 0.1% normal human IgM, (n) 0.1% normal human IgM plus 20% fresh human serum, (i) 0.1% Triton X-100 for estimating maximum ^{51}Cr release, or (j) normal EC media for estimating spontaneous ^{51}Cr release. The 100-μl aliquot of incubation media was sampled from each well and the ^{51}Cr contents released in the media were measured with a gamma counter. Fresh human serum was used as a source of complements, and the four conditions (e-h) with normal human IgM served as controls. The results were expressed as percentage of specific release of ^{51}Cr as follows:

$$\frac{\text{Sample (cpm)} - \text{Spontaneous }^{51}\text{Cr release (cpm)}}{\text{Maximum }^{51}\text{Cr release (cpm)} - \text{Spontaneous }^{51}\text{Cr release (cpm)}} \times 100$$

Preincubation of LT-IgM with Sulfoglycosphingolipids

LT-IgM of various concentrations (from 0.0002% to 0.1%) was incubated for 3 h with a 10 times molar excess of genuine SGPG before application on [^{51}Cr]-incorporated BMECs. In addition, 0.05% LT-IgM solution was preincubated with a 10 times molar excess of SGPG or sulfatide to compare the effect of preincubation with these two sulfated glycosphingolipids.

Results

Glycosphingolipid Composition in BMEC

Except SGPG, the age of culture in vitro did not affect the GSL content significantly in the range of 14 to 35 d.

Ganglioside Composition in BMECs. The total lipid-bound sialic acid was 583 ng per mg protein for the BMECs. Fig. 4 shows the ganglioside pattern of the BMECs. Only three major gangliosides were observed in the general area of authentic GM3 using the neutral solvent system I (Fig. 4 A, lane 2). Other minor gangliosides included one slightly

above GM1, and another slightly above GDla. By using the solvent system II, the two major bands appeared as two doublets which had the same mobilities as authentic GM3-(NeuAc) and GM3(NeuGc) (Fig. 4 B, lane 2). The antibody against GM3(NeuGc) reacted with the lower doublet as well as standard GM3(NeuGc) but did not react with the upper doublet or authentic GM3(NeuAc) (Fig. 4 C). This suggests that the major gangliosides in BMECs consist of GM3(NeuAc) and GM3(NeuGc). The identity of GM3-(NeuAc) in BMECs was confirmed by immunocytochemical staining with a polyclonal antibody against GM3(NeuAc) (Fig. 5). Since bovine BMEC GM3 contains both N-acetyl and N-glycolyl neuraminic acid as shown above, these triple bands are presumably due to the presence of different sialic acid species as well as different fatty acids (Sekine et al., 1984).

The antibody against paragloboside recognized three bands in the area around SPG and SLPG after treatment of the parent GSLs with sialidase in situ (Fig. 6 A). After in situ sialidase treatment of the gangliosides on the HPTLC plate, cholera toxin B-subunit recognized four gangliosides, which corresponded to authentic GM1, GDla, GDlb, and GTlb. This suggests that BMECs express these four gangliotetra-ose-series gangliosides as minor components (Fig. 6 B). The amounts of these gangliosides, were smaller than those of the neolacto-series gangliosides such as sialyl paragloboside (SPG) or sialyl lactosaminyl paragloboside (SLPG) (Table I).

SGPG in BMECs. SGPG was found in the BMEC GSL fraction by the HPTLC-immunostaining method (Fig. 7 A). Neither SGLPG nor band X, previously found in human or bovine peripheral nerve (Kohriyama et al., 1987; Kusunoki et al., 1987), could be detected by this method. Fig. 7 B shows the quantitative change of the amount of SGPG at various in vitro days. The SGPG content of BMEC decreased with increasing age of culture. The concentration of SGPG was highest in 7 d samples, but not found in cells after 21 d in vitro, using the HTLC-immunostaining methods. However, after 24 h of incubation with 1 mCi of [^{35}S]-Na₂SO₄ in 38-d-old BMECs, a positive band was cochromatographed with authentic SGPG, suggesting the presence and synthesis of SGPG even in aged cultures of BMECs (Fig. 8).

Neutral Glycolipid Composition of BMECs

Neutral GSLs were isolated from confluent BMECs equiva-

Table I. Glycosphingolipid Composition of Cultured BMECs

Glycosphingolipid	ng/mg protein	n
GM3(NeuAc)	900 ± 152	5
GM3(NeuGc)	1258 ± 212	5
SPG	66 ± 25	4
SLPC	102 ± 45	4
GM1	27 ± 10	3
GDla	27 ± 10	3
GDlb	trace	
GTlb	trace	
SGPG (7d)	65 ± 25	5
SGPG (14d)	21 ± 4	5
GlcCer	379 ± 65	4

Values were expressed as ng ± SEM per mg protein. n, number of experiments.
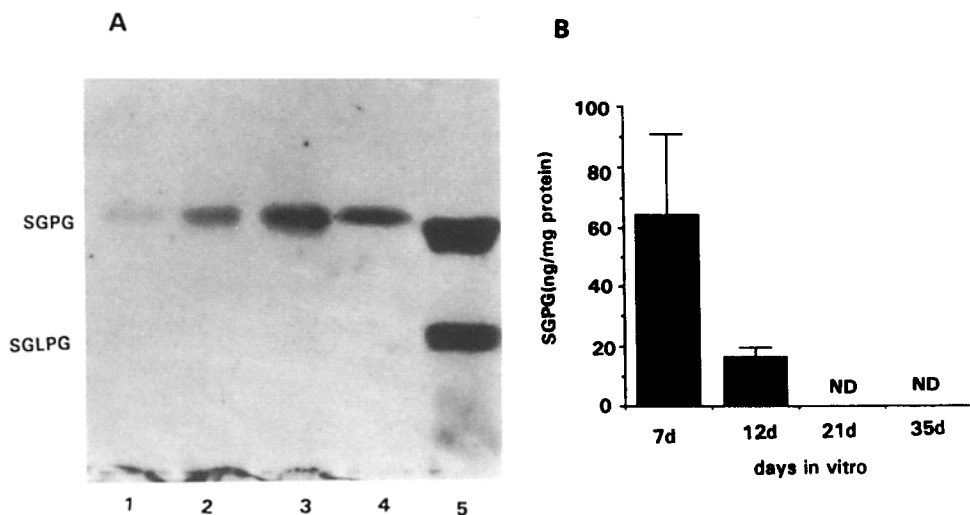
A **B**

SGPG

SGLPG

1 2 3 4 5

SGPG(ng/mg protein)

100

80

60

40

20

0

ND ND

7d 12d 21d 35d

days in vitro

lent to 1.5 mg of protein. Staining with the orcinol-sulfuric acid reagent revealed only two bands corresponding to GlcCer (Fig. 9). These double bands are presumably due to different fatty acid components (Rauvala, 1976; Ariga et al., 1982). Quantitation of these major neutral GSLs were carried out by gas–liquid chromatography of the trimethylsilyl derivatives of the individual sugar constituents (Vance and Sweeley, 1967). Glucose, but not galactose, was detected

A **B**

SGPG

SGLPG

SGPG

SGLPG

1 2 3 4 5 6

Figure 8. Thin-layer chromatograms of sulfated glycolipids. Sulfate incorporation into GSL fraction of BMECs (in vitro 8 d). Lanes *1, 2, 4,* and *5*: Fluorogram of [³⁵S]-Na₂SO₄ incorporation into BMEC glycolipids. Lanes *3* and *6*: Authentic samples of SGPG and SGLPG (1 ng each) immunostained with LT serum. *A* was developed in the solvent system II, and *B* was developed in the solvent system I. Radioactivities in the whole homogenate ranged from 3,200–4,700 cpm/μg protein and [³⁵S]-sulfate recovery into GSL fraction was 10–17% of total homogenate. Fluorogram showed at least six (*A*) and five (*B*) [³⁵S]-incorporated bands. The 1st band in *A* and the 2nd one in *B* were cochromatographed with authentic SGPG, indicating the production and presence of SGPG in BMEC cultures as old as 38 d in vitro. There was no band cochromatographed with SGLPG.

(data not shown). The amount of GlcCer was estimated to be ~380 ng/mg protein (Table I).

Effect of LT-IgM Against BMECs

The [carboxyl-¹⁴C]-inulin clearance through the empty and cocultured inserts challenged by 0.1% IgM was shown in Table II and Fig. 10, *a* and *b*. The [carboxyl-¹⁴C]-inulin clearance rate (μl/min) through cocultured inserts in control conditions (incubated with 0.1% normal IgM with or without complements) was almost 1/60 of that of the empty inserts, indicating that the BMEC monolayer cocultured with astrocytes provided an excellent barrier to [carboxyl-¹⁴C]-inulin fluxes ($p < 0.05$ at all times; Fig. 10 *a*). Incubation with LT-IgM produced a three-to fourfold increase of the [carboxyl-¹⁴C]-inulin clearance rate through membranes (Table II), but there was no significant difference between the condi-
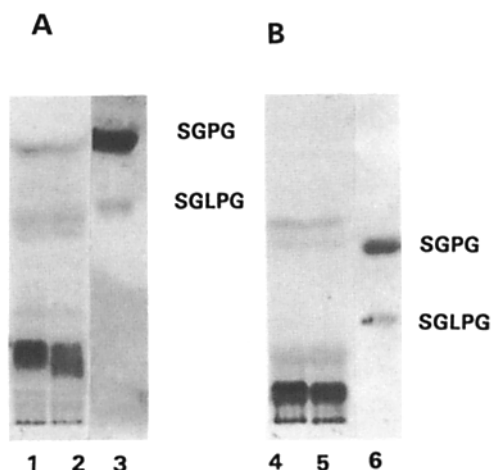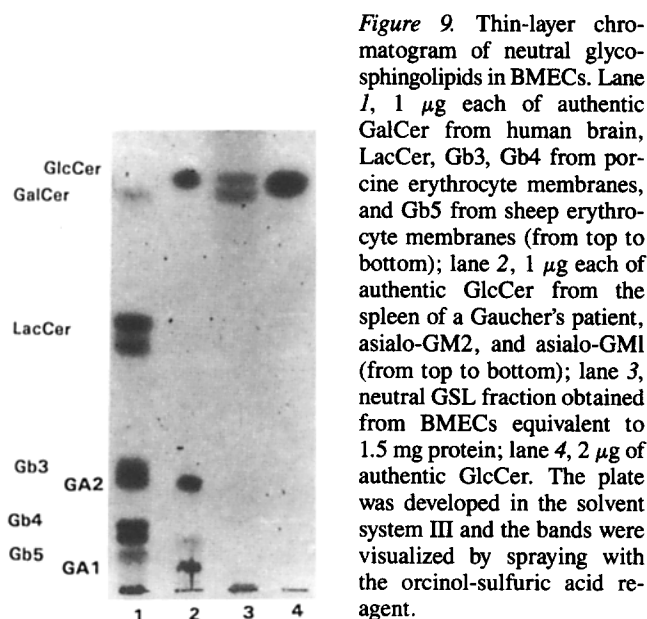

GlcCer
GalCer

LacCer

Gb3
GA2

Gb4

Gb5
GA1

1 2 3 4

Table II. Clearance of Inulin across BMEC Monolayer

	n	[carboxy-^{14}C]-inulin clearance rate (μl/min)
Empty insert	3	12.338 ± 1.081***
Cocultured insert		
LT-IgM	5	0.811 ± 0.084**
LT-IgM + complement	6	0.685 ± 0.260**
IgM (control)	4	0.213 ± 0.129
IgM (control) + complement	4	0.220 ± 0.116

[Carboxyl-^{14}C]-inulin clearance (μl/min) across the empty insert and the BMEC/astrocyte cocultured insert was measured following 3 h of 0.1% IgM (LT and control, with or without complements) challenge. Asterisk represents a significant increase in [carboxyl-^{14}C]-inulin clearance across membrane over controls (**P <0.01, ***P <0.001). Values were expressed as mean ± SD. n, number of experiments.

tions with and without complements (Fig. 10 b). After incubation with 0.1% LT-IgM, [^{125}I]-labeled LT-IgM also showed an enhanced leakage as compared with the condition where incubation was carried out in 0.1% normal IgM with [^{125}I]-labeled normal IgM as a tracer (Fig. 10 c).

Fig. 11 shows the specific release of ^{51}Cr from BMEC monolayer after incubation with 0.5 and 0.1% of IgM (LT and normal), with or without complements. Specific release of ^{51}Cr was significantly increased when 0.5% LT-IgM (9.16 ± 2.16% with complements, 8.48 ± 1.91% without complements) was applied. It was also significantly increased when incubated with 0.1% LT-IgM (8.54 ± 2.86% with complements, 11.79 ± 3.83% without complements). These data suggest that the toxic effect of LT-IgM against BMECs was independent of the complements. The cytotoxicity of LT-IgM was completely suppressed after incubation with a 10-fold molar excess of SGPG (Fig. 12 and 13), but the same amount of sulfatide had no effect (Fig. 13). Fig. 14 shows the immunostaining of living single endothelial cell with LT-IgM, further demonstrating binding of this antibody to intact endothelial cells. Fig. 15 shows the morphology of the BMEC monolayer after incubation with LT-IgM. During the incubation period with LT-IgM, some of the BMECs became round in shape and the monolayer was disrupted at places. A few of them even showed evidence of degeneration, which coincided with the increase release of ^{51}Cr in LT-IgM treated cells. The addition of complements did not affect this morphological change.

Discussion

Because of the low amount of GSLs in BMECs and the difficulty in obtaining large quantities of "pure" cell populations, the GSL composition of BMECs has not been carefully scrutinized before. In our culture methods, which is a modification of the methods of Meresse et al. (1989) and Gordon et al. (1991), we started with colonies formed by pure BMECs and the purity of these cells was maintained after 6-8 passages. In the presence of bFGF and absence of contaminating cells (e.g., fibroblasts, astrocytes, pericytes, etc.), bovine BMECs multiplied rapidly (10 to 15 times per week) to afford sufficient quantities of cells for chemical analyses. Endothelial cells obtained from human brain microvessels would be a better choice for research of human diseases, but there are still technical difficulties in obtaining sufficient quantities of pure human BMECs for this purpose. The exclusion of non-EC contaminants is crucial in this kind
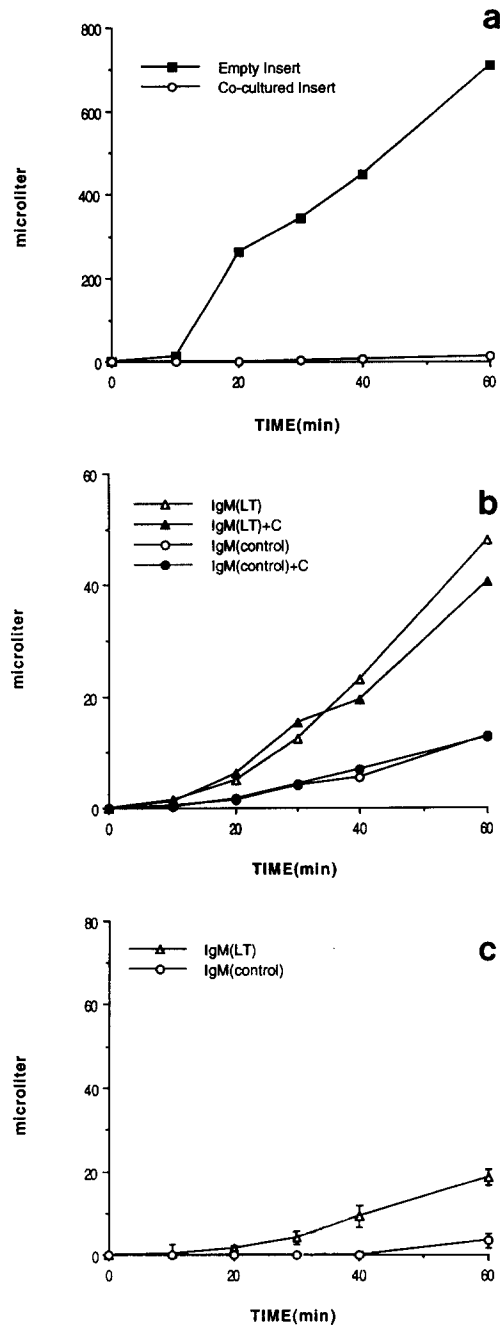

Figure 10. Effect of immunoglobulin M on the permeability of BMEC monolayers. (a) Permeability to [carboxyl-^{14}C]-inulin of empty insert (*closed square*) and BMEC/astrocyte cocultured insert (*open circle*; as designated in Fig. 3). Empty insert showed ∼60 times high value of [carboxyl-^{14}C]-inulin influx as compared to cocultured insert, which suggested that the cocultured insert provided an excellent barrier for inulin passage. (b) Changes of [carboxyl-^{14}C]-inulin clearance after incubation with 0.1% LT-IgM (with or without complement, *closed* and *open triangles*, respectively) as compared with those with 0.1% control IgM (with or without complement, *closed* and *open circles*, respectively). Incubation with LT-IgM resulted in higher [carboxyl-^{14}C]-inulin clearance at all times (p <0.05), regardless of the coincubation of complements. (c) Changes of [^{125}I]-IgM clearance after incubation with 0.1% LT-IgM (*open triangle*; [^{125}I]-conjugated LT-IgM as tracer) and with 0.1% control IgM (*open circle*; [^{125}I]-conjugated normal IgM as tracer). Slight but steady IgM leakage was observed after LT-IgM challenge, whose value was ∼⅓ of [carboxyl-^{14}C]-inulin leakage (see Fig. 10 b). On the other hand, no penetration of IgM was observed until 40 min under normal conditions. Error bars indicate SEM.
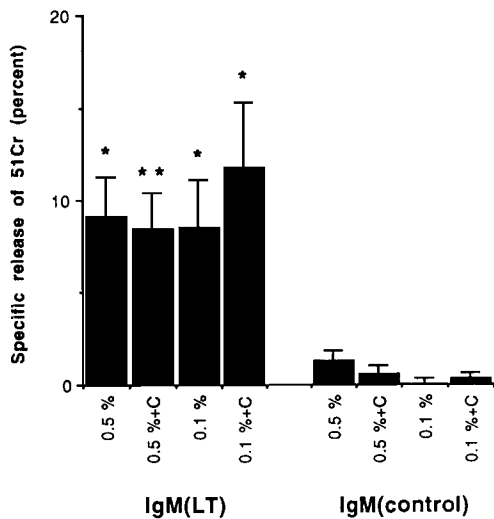
Figure 13. Effect of preincubation of IgM with sulfatide on chromium-51 release from BMECs. Suppression of the cytotoxic effect of patient's IgM by preincubation with sulfoglycolipids. A 0.05% LT-IgM solution was incubated with 10-fold molar excess of pure sulfoglycolipids for 3 h and was applied to the [^{51}Cr]-incorporated BMEC monolayers. Although SGPG suppressed the toxic effect almost completely (91.1 ± 4.1%, p <0.001), sulfatide did not have any effect (8.4 ± 28.6%, not significant). Error bars represent SEM.

Figure 11. Chromium-51 release from BMECs. BMECs incubated with LT-IgM showed a significant increase of ^{51}Cr release in all four conditions, suggesting a complement independent BMEC cytotoxicity in patient's IgM. Cells incubated with 0.5% normal IgM (1.24 ± 0.58% with complements; 0.58 ± 0.44% without complements) shows slight but insignificant increase of ^{51}Cr release over those with 0.1% normal IgM (0.00 ± 0.30% with complements; 0.34 ± 0.25% without complements). This may imply the possibility of nonspecific IgM toxicity against BMECs in this concentration range, but the value is lower than 1/7 of that challenged by patient's IgM and it is almost negligible. Asterix represents a significant increase in specific release of ^{51}Cr over its respective control (*P <0.05, **P <0.01). Error bars represent SEM.

of study because the GSL content of EC is so low that only a small proportion of non-EC contamination may influence the quantitative results, especially in the case of neutral GSLs.

Prior to our study, there were only few reports (Gillard et al., 1987, 1992) presenting the ganglioside composition of endothelial cells from human umbilical cord. These investi-
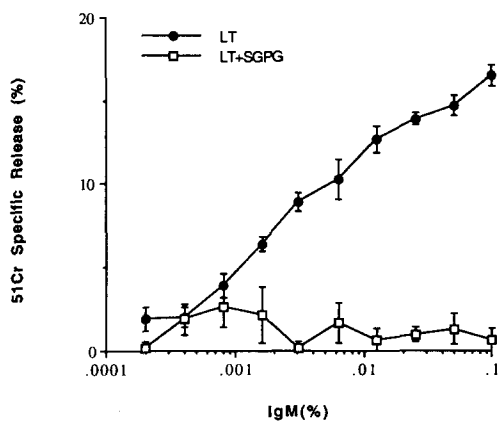
gators also reported high amounts of GM3 and SPG, but not GM1 nor SGPG. Relatively high amounts of neutral GSLs were also reported; the amount of gangliosides and neutral GSLs are almost the same (71 nmol sialic acid vs. 53 nmol neutral GSL). They also reported the presence of LacCer, Gb3, Gb4, paragloboside, and Gb5 as the major neutral GSLs in addition to a smaller amount of GalCer. In contrast, we could detect only GlcCer but not other neutral GSLs based on TLC and GLC analyses. The amount of total GlcCer in BMECs was extremely low, about 1/5 of the amount of gangliosides. The GlcCer in BMECs appeared as doublets which might be due to differences in fatty acid compositions (Rauvala et al., 1976; Ariga et al., 1982). The differences between the GSL compositions of bovine BMECs and human umbilical cord ECs may be attributable

Figure 12. Relationship between chromium-51 release and IgM concentration. Linear correlation between the ^{51}Cr-specific release and the concentration of LT-IgM in the incubation media (*closed circles*). Open squares denote the ^{51}Cr-specific release after exposure to LT-IgM solution, which was preincubated with a 10-fold molar excess of SGPG for 3 h. The toxicity of LT-IgM against BMECs was almost completely abolished. Error bars represent SEM.
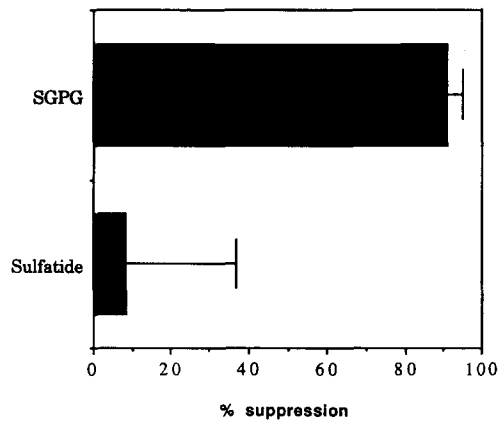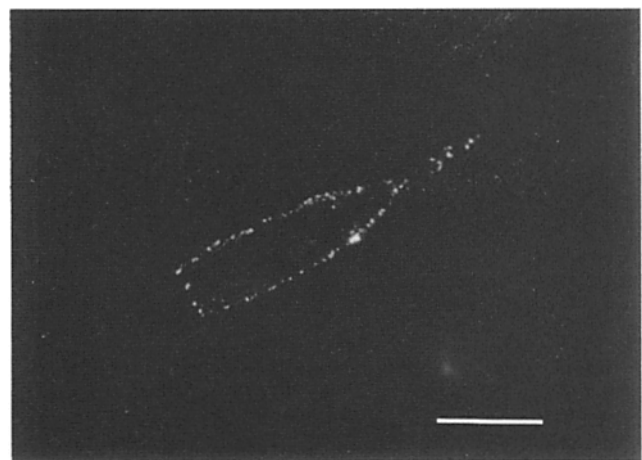

Figure 14. Immunofluorescent staining of BMEC with anti-SGGL antibody. Fluorescent micrograph of a single, living BMEC (in vitro 16 d) stained with LT-IgM. Bar, 50 μm.
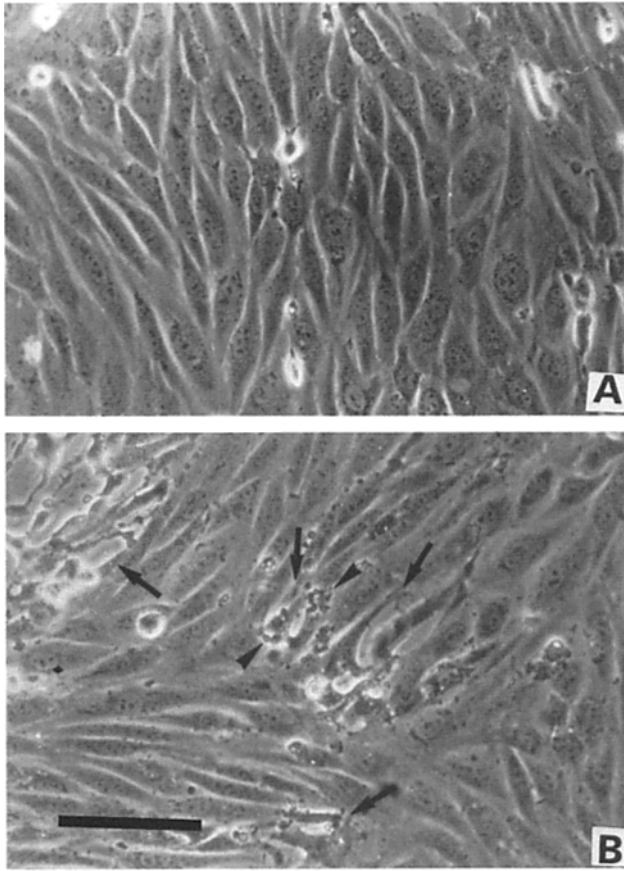
Figure 15. Light microscopy of BMEC monolayers. Phase contrast micrograph of BMEC monolayer before (A) and after (B) incubation with EC media (heat-inactivated) plus 0.5% LT IgM for 120 min. (A) Typical cobblestone pattern was observed. (B) Some cells shrank and the BMEC monolayer showed disruption of cellular interconnection at places (arrows). Some cells showed vesicular appearances (arrowheads). These morphological changes are well correlated with the data shown in Figs. 10, 11, and 12. Control IgM did not cause any morphological changes. Bar, 100 μm.

to differences in animal species, site of EC origin, or degree of non-EC contaminations.

The physiological significance of GM3 in BMEC is unknown. However, GM3 inhibits EGF receptor-associated tryosine kinase (Bremer et al., 1986) and is supposed to act as a modulator of transmembrane signal transducers in several kinds of cells (Hakomori, 1990). Recently we found that exogenously administered GM3(NeuAc) promoted the cyclic AMP-dependent protein kinase and inhibited protein kinase C in BMEC (Kanda et al., 1993). Since the barrier function of ECs is considered to be enhanced by cyclic AMP-dependent protein kinase and diminished by protein kinase C (Stelzner et al., 1989; Oliver, 1990; Rubin et al., 1991), GM3 may act as a potent mediator of BBB/BNB function via these two kinase systems.

Another interesting aspect of the present study is the decrease of the amount of SGPG with the age of culture. Earlier studies have indicated that SGGLs belong to a novel class of acidic lipids present primarily in the peripheral nervous tissues (Chou et al., 1986; Ariga et al., 1987; Kohriyama et al., 1987). In the mammalian CNS, SGGLs are known to be developmentally regulated, being expressed in embryonic fore-

brain and disappeared during the postnatal period (Schwarting et al., 1987) except in murine cerebellum (Chou and Jungalwala, 1988) and human optic nerve (Yoshino et al., 1993). Recent studies, however, indicated that they are also present in nonneural cells or tissues. Thus, SGGLs were found in dura mater (Kohriyama et al., 1987). In addition, we recently reported the presence of SGPG in the microvessel fraction derived from rat cerebral hemisphere (Miyatani et al., 1990 b). This latter observation suggests the presence of SGPG in BMECs. Since the cerebral microvessel-enriched fraction inevitably contains nonendothelial components (e.g., pericytes, smooth muscle cells, nerve endings, glial cells), further confirmation of the presence of SGPG using pure populations of endothelial cells of brain microvascular origin seems warranted.

The concentration of SGPG fell below the detection limit of HPTLC-immunostaining after 21 d in vitro. SGPG could only be revealed by [35S]-incorporation study in cultures as old as 38 d old (Fig. 7 B). Such an age-dependent expression was not observed for other BMEC glycolipids. It is well known that BMECs lose the activity of a BMEC-specific marker enzyme, gamma-glutamyl transpeptidase, in older cultures and they recover it when treated with astrocyte-conditioned media or cocultured with astrocyte (De Bault and Cancilla, 1980). The age-dependent expression of SGPG might also be regulated by similar humoral factors.

Our present results may also shed light on the role of GSLs in the pathogenesis of various autoimmune-mediated neurological disorders. It is well known that four gangliotetraose-series gangliosides, GM1, GD1a, GD1b, and GT1b, are especially abundant in the central and peripheral nervous systems (Yu and Saito, 1989). In the peripheral nervous system, four gangliotetraose-series ganglioside as well as SPG (LM1) occur in relative abundance (Chou et al., 1985; Ariga et al., 1990; Svennerholm and Fredman, 1990). SGGLs including SGPG and SGLPG are also present in small concentrations (Ariga et al., 1987; Chou and Jungalwala, 1988; Yu et al., 1990). Recently the presence of various anti-GSL autoantibodies have been reported in certain neurologic disorders, including neuropathy associated with monoclonal gammopathy (Ariga et al., 1987; Miyatani et al., 1987; Duane et al., 1992), motor neuron disease (Latov et al., 1988; Yoshino et al., 1992; Sanders et al., 1993), polyneuropathy with multifocal conduction block (Pestronk et al., 1988; Adams et al., 1991), Guillain-Barré syndrome (Ilyas et al., 1988, 1992a; Yiki et al., 1990), etc. The pathogenicity of these antibodies have been advocated in these disorders; however, the question about how the large molecule like immunoglobulins can traverse BBB or BNB has always been raised (Yu et at., 1990, 1993). Our present study revealed that BMECs and the peripheral nervous tissues shared many GSLs including SGPG as common antigens; hence, the presumed cascade of pathological processes—immunological attack of microvascular endothelial cells, destruction or malfunction of BNB, increased permeability across BNB, leakage of immunoglobulins into the endoneurial space, and the subsequent immunological attack of peripheral myelin and axons leading to their destruction—can be logically explained in those patients with anti-glycolipid antibody. In rats sensitized with SGPG, Maeda et al. (1991) described fenestration of the capillaries in the posterior horn of the lumbar spinal cord.

To investigate this possibility, we purified LT-IgM and demonstrated that this anti-SGGL IgM antibody facilitated the leakage of small as well as large molecules through EC/astrocyte membrane, and was directly toxic against BMECs in vitro based on the [carboxyl-^{14}C]-inulin and [^{125}I]-IgM leakage studies and the ^{51}Cr release study. The cytotoxic effect of this antibody depended on its concentration in the incubation media and was completely blocked by preincubation with a 10-fold molar excess of SGPG. Although cross reactivity of these IgM antibodies with sulfatide was also reported recently (Ilyas et al., 1992*b*), preincubation with the same amount of sulfatide did not inhibit the cytotoxic effect of this antibody.

At present, it is not clear whether and how immunological insults against BMECs actually occur in vivo. There have been only a few studies on the pathological changes of endothelial cells in IgM neuropathy. Meier et al. (1984) described the gaps between the adjacent endoneurial cells in a case of IgM neuropathy, and Powell et al. (1984) showed microvascular changes including the endothelial cytoplasmic enlargement occasionally obliterating the vessel lumen and intracytoplasmic actin-like filaments in dysglobulinemic neuropathies. Detailed characterization of the antibodies was not performed. In both reports, pathological changes in endothelial cells were minimal and are completely different from those of acute inflammatory changes presumably due to the deposition of circulating immune complexes, typically observed in vessels in collagen diseases with vasculitis such as periarteritis nodosa (Conn and Dyck, 1984). In the present investigation, no qualitative or quantitative difference of BMEC damages were observed between the conditions with complements and those without complements. These in vivo and in vitro observations suggest that immunological attack against BMECs occurs independent of the presence of complements in this disorder, and the underlying immunological mechanism is different from those usually encountered in humoral immunity, namely, a complement-mediated cell-lytic process or deposition of circulating immune complexes.

In view of a certain similarity in the function for glycoproteins possessing the HNK-1 epitope, namely, N-CAM, J1, and L1 for nervous system cell adhesion and neurothelin (Schlosshauer and Herzog, 1990) for endothelial cell adhesion with BBB formation, it is possible that SGPG is related to the adhesion of BMECs including BBB/BNB formation. SGGLs were also found to bind laminin, an extracellular matrix glycoprotein involved in cellular adhesion (Mohan et al., 1990). Hence, immunological block of SGPG epitope by IgM antibody may result in the loss of cellular interconnection among BMECs and finally cell death, as shown in our present experiments. Recently, Needham and Schnaar (1993) reported that SGGLs act as ligands for L- and P-selectins, but not for E-selectin. Because selections are involved in the interaction between leukocytes and vascular endothelial cells leading to lymphocyte homing, platelet binding, and neutrophil extravasation (Lawrence and Springer, 1991; Foxall et al., 1992), SGPG on the surface of BMECs might play an important role in inflammatory or demyelinating diseases of the central and peripheral nervous systems. It may support the selective adhesion of a desired subpopulation of leukocytes expressing L- or P-selectin (Needham and Schnaar, 1993), but this mechanism can be harmful in pathological conditions such as inflammatory or demyelinating

neurological disorders because it will allow the cytotoxic leukocytes to enter into the CNS or PNS parenchyma. L-selectin–mediated attachment of lymphocytes to myelinated tracts of CNS was also reported (Huang et al., 1991). In this regard, blockage of leukocyte/endothelial cell connection by applying excess amount of polysaccharides (Willenborg and Parish, 1988) or by administration of monoclonal antibodies to block these oligosaccharide epitopes might be an effective therapeutic strategy. However, the toxic effect of anti-SGPG antibody against BMECs as shown in this study should also be noted. Further studies are now in progress to clarify the functional aspect of SGPG in BMECs.

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