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Interleukin 1β up-regulates the expression of sulfoglucuronosyl paragloboside, a ligand for L-selectin, in brain microvascular endothelial cells

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ABSTRACT **Treatment of cultured bovine brain microvascular endothelial cells (BMECs) with interleukin 1β (IL-1β), an inflammatory cytokine, was shown to induce the accumulation of sulfoglucuronosyl paragloboside (SGPG), a glycolipid bearing the HNK-1 epitope. This resulted in the attachment of a greater number of human lymphocytes to the treated than to the untreated BMEC monolayers. Attachment of human lymphocytes to the IL-1β-activated BMEC cells could be blocked either by incubation of the human lymphocytes with an anti-L-selectin antibody or by application of an anti-SGPG antibody to the BMECs. These results suggest that SGPG may act as an important ligand for L-selectin for the regulation of the attachment of activated lymphocytes and their subsequent invasion into the nervous system parenchyma in inflammatory disorders of the central and peripheral nervous systems.**

The endothelial cells (ECs) of brain microvascular origin (BMECs) are highly specialized cells and are believed to make up the structural basis of the blood–brain barrier (BBB) and blood–nerve barrier (BNB). BMECs are the only cell groups in the nervous system that are continuously exposed to blood constituents including leukocytes and soluble cytokines. For this reason, information that is 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 (CNS and PNS) is believed to be conveyed via the surface receptors on BMECs. Recently, a number of activation-dependent adhesion molecules that mediate leukocyte/EC attachment are identified (1–4). These adhesion molecules are presumed to play an important role for the development of inflammatory neuro-immunological disorders of the CNS/PNS such as multiple sclerosis, Guillain-Barré syndrome, and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP); the initial key steps of these disorders include transmigration of activated leukocytes and/or leakage of humoral factors such as immunoglobulins into the nervous system parenchyma across the BBB/BNB. However, very little information has been obtained concerning the adhesion molecules in BMECs so far, presumably because of the difficulty in acquiring sufficient quantities of BMECs for biochemical analyses.

Previous studies from this laboratory have demonstrated the presence of a family of sulfated glucuronic acid-containing glycolipids (SGGLs), including sulfoglucuronosyl paragloboside (SGPG) and sulfoglucuronosyllactosaminyl paragloboside (SGLPG), both of which bear the HNK-1 epitope, in rat brain microvessels (5); cultured human umbilical vein ECs (5); and bovine BMECs (6). Evidence has been presented that these glycolipids may play critical roles in the immunopathogenesis of such neurological disorders as Guillain-Barré syndrome and CIDP (6–8). In addition, we demonstrated that immunological insults against BMEC-bound glycolipid antigens could induce the destruction or malfunction of the BBB, resulting in the penetration of circulating immunoglobulins into the nerve parenchyma (6, 7). In this investigation, we further showed that interleukin 1β (IL-1β), an inflammatory cytokine, could induce the increased expression of SGGLs in BMECs; this resulted in an enhanced attachment of human lymphocytes on IL-1β-activated BMEC monolayer surface.

MATERIALS AND METHODS

Cell Culture. BMECs were isolated from adult bovine brain as described (6). Briefly, the cortex of fresh bovine brain tissue, obtained from a local slaughterhouse, was homogenized, incubated in 0.005% dispase (grade I; Boehringer Mannheim) for 2 h, and then centrifuged in 15% dextran to separate the microvascular fraction. The isolated microvessels were redigested with 0.035% collagenase/dispase (Boehringer Mannheim) solution for 12 h and then seeded on collagen-coated 60-mm-diameter plastic Petri dishes in Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with 15% (vol/vol) equine plasma-derived serum (Sigma), 4% (vol/vol) fetal bovine serum (BioWhittaker), penicillin (GIBCO/BRL) at 100 units/ml, streptomycin (Sigma) at 100 μg/ml, 1 mM sodium pyruvate (Sigma), heparin (Sigma) at 50 μg/ml, and recombinant human basic fibroblast growth factor (Collaborative Biomedical Products, Bedford, MA) at 1 ng/ml. After about 10 days, colonies with pure BMECs were cloned and reseeded in another collagen-coated Petri dish. BMECs were passaged weekly. The purity of BMECs was judged as >99% based on several morphological and biochemical criteria, including the incorporation of DiI-Ac-LDL (1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyliodocarbocyanine perchlorate)-conjugated, acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA) (6). BMECs cultured for 21–35 days *in vitro* were used for the experiments described below. At this time, the SGPG content in BMEC was substantially decreased [<2 ng per mg of protein, as described previously (6)].

Quantitation of SGPG. Total lipids were extracted from the homogenized cells and the glycosphingolipids (GSLs) therein were isolated from Sephadex LH-20 column chromatography as described (9, 10). The total GSL fraction, including both acidic and neutral GSLs, was applied directly to a high-performance TLC plate. Quantitative analysis of SGPG was

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Abbreviations: BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; BNB, blood–nerve barrier; EC, endothelial cell; IL-1β, interleukin 1β; SGGL, sulfated glucuronic acid-containing glycolipid; SGPG, sulfoglucuronosyl paragloboside; SGLPG, sulfoglucuronosyllactosaminyl paragloboside; CNS, central nervous system; PNS, peripheral nervous system; GSLs, glycosphingolipids; sialyl-Le^x, sialylated Lewis^x.

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achieved by the TLC–immunooverlay method described previously (9, 10). Briefly, after the GSL fraction and authentic SGPG (from 5 to 40 ng) were chromotographed in the solvent system of 50:45:10 (vol/vol) chloroform/methanol/water containing 0.22% $CaCl_2 \cdot 2H_2O$, the plate was dipped in 0.2% poly(isobutyl methacrylate) (Aldrich) solution in *n*-hexane for 30 s and air-dried. Then the plate was incubated with LT serum from a patient with demyelinative polyneuropathy and IgM paraproteinemia (9) at a dilution of 1:1000 in phosphate-buffered saline (PBS) overnight. After the excess antiserum was rinsed off with PBS, the plate was incubated with peroxidase-conjugated rabbit anti-human IgM (μ-chain specific, 1:1000; Cappel). The plate was further washed with PBS, followed by incubation with a cyclic diacylhydrazide solution (Amersham) 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 (10).

Lymphocyte Isolation. Blood mononuclear cells were isolated by Ficoll–Histopaque density gradient centrifugation of Na_2EDTA–treated blood from healthy volunteers. Lymphocytes were further isolated by using Mono-Poly resolving medium (Flow Laboratories), followed by lysis of erythrocytes with Gay's hemolytic solution. The cells were kept on ice until use.

Monoclonal Antibodies. A mouse anti-SGPG monoclonal antibody was prepared with the collaboration of T. Tai (Department of Tumor Immunology, Tokyo Metropolitan Institute of Medical Science, Tokyo) and used as a diluted (1:10) culture medium (M.Y., unpublished data). Mouse anti-disialoganglioside-GD1a and anti-monosialoganglioside-GM3 monoclonal antibodies were provided by T. Tai (11). A mouse anti-sialylated Lewis^x (sialyl-Le^x) monoclonal antibody [clone FH6 (12)] was provided by S. Hakomori (The Biomembrane Institute, University of Washington, Seattle). These monoclonal antibodies were used as diluted (1:10) culture supernatants. Anti-L-selection monoclonal antibody [clone DREG 56, which is known to inhibit human lymphocyte binding to peripheral lymph node high endothelial venules (13)] was purchased from Endogen (Cambridge, MA), and anti-P-selectin monoclonal antibody (clone AC1.2) was purchased from Becton Dickinson.

Immunostaining of IL-1β-Activated and Nonactivated BMECs with LT Serum. BMECs grown on collagen-coated 35-mm Petri dishes were stimulated with 5 ng of recombinant human IL-1β (Collaborative Biomedical Products, Bedford, MA) per ml for 4 or 24 h and fixed with 4% paraformaldehyde for 30 min. After rinsing three times with cold PBS, cells were incubated with LT serum (1:100 dilution in PBS) overnight at 4°C and then incubated with fluorescein isothiocyanate-conjugated rabbit anti-human IgM (μ-chain specific, 1:50 dilution, Dako) for 30 min. Control cultures did not receive IL-1β and were used as nonstimulated BMECs.

BMEC–Lymphocyte Attachment Assay. BMECs (2 or 3 passages after isolation) were grown confluently on collagen-coated 24-well plates (Falcon) and stimulated with 5 ng of IL-1β (Collaborative Biomedical Products) per ml for 24 h. Anti-SGPG antibody was applied on the stimulated BMECs for 30 min just before the administration of human lymphocytes. As controls, monoclonal antibodies against GD1a and GM3 were also applied to the stimulated BMECs for 30 min. In parallel, lymphocytes were incubated at 4°C in 50 ml of EC medium containing monoclonal antibodies against L-selectin or P-selectin at 25 μg/ml or against sialyl-Le^x (clone FH6, 1:10 diluted supernatant) for 30 min. After rinsing twice with DMEM, 2.5×10^4 human lymphocytes (0.5 ml per well) were added on top of the BMEC monolayer and incubated at 4°C with rotation (64 rpm). After a 17-min incubation period, the cells were fixed with 2.5% glutaraldehyde overnight and stained with hematoxylin. Four microscopic fields, located at half-radius distances from the center of the culture well, were photographed (1.37 mm^2 per field), and the number of attached cells was counted.

RESULTS

The SGPG content in BMECs (21 days in culture) increased from 3.1 ± 0.5 (n = 3) ng/mg of protein to 24 ± 1.1 (n = 3) and 68 ± 5.0 (n = 3) ng/mg of protein, or 8- and 22-fold, after stimulation for 4 and 24 h with IL-1β, respectively (Fig. 1). In older cultures the increase was even more dramatic. Thus, in 35-day-old BMEC cultures, the amount of SGPG increased from 0.1 ng/mg of protein for unstimulated cells to 40 and 50 ng/mg of protein after stimulation for 4 and 24 hr, respectively (data not shown). SGLPG, an analog of SGPG, was not normally detected in unstimulated BMECs (6), but it usually remained barely detectable even after the cells were treated with IL-1β (Fig. 1). Because its level was below the detection limit, further quantitation of SGLPG was not carried out. Significantly larger numbers of BMECs were stained with anti-SGPG monoclonal antibody after stimulation with IL-1β (Fig. 2 *b* and *c*). A significant amount of the immunoreactivity resided intracellularly within the first 12 h of stimulation, shifting to the cell surface after 24 h (Fig. 2 *b* and *c*). In addition, a significantly larger number of human lymphocytes became attached to the IL-1β-stimulated BMECs than to the nonstimulated BMECs ($P < 0.01$) (Fig. 3*a*), and this adherence was effectively blocked by preincubation of the lymphocytes with anti-L-selectin antibody or pretreatment of the BMEC monolayer with anti-SGPG antibody (Figs. 3*a* and 4). None of the other monoclonal antibodies (anti-GD1a, anti-GM3, anti-P-selectin, and anti-sialyl-Le^x) could significantly interfere with the attachment of human lymphocytes on the BMEC monolayer (Fig. 3*b*).

DISCUSSION

The selectins [lectin, EGF, complement binding-cell adhesion molecules (LEC-CAMs)] are a family of mammalian receptors participating in the initial interactions between leukocytes and vascular endothelia—namely, "rolling" of the leukocytes (3, 4, 14–17). All three selectins (E-, P-, and L-selectins) are known to recognize sialyl-Le^x and sialyl-Le^a oligosaccharides as the common ligands (15, 17–20). In addition to the above, the putative ligands for L-selectin have also been described as sulfated glycoproteins of M_r ≈50,000 and 90,000, termed Sgp50 and Sgp90, respectively (21, 22), and sulfated heparin-like molecules (23). Sgp50, which has recently been cloned and

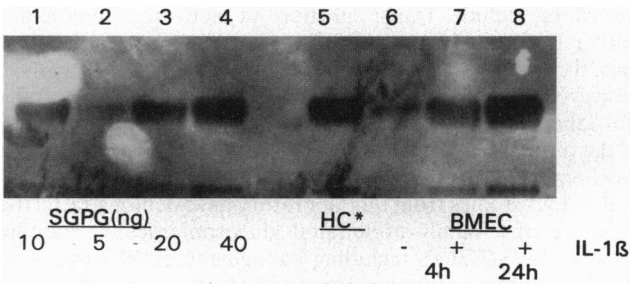

FIG. 1. Quantitation of SGPG in BMECs. Lanes: 1–4, standard SGPG (10, 5, 20, and 40 ng, respectively); 5, total acidic glycolipid fraction of human cauda equina (HC); 6–8, glycolipid fraction from BMECs before (lane 6) and after 4 h (lane 7) and 24 h (lane 8) of stimulation with human recombinant IL-1β. The plate was immunostained with the LT serum. The SGPG content in BMEC was significantly increased after stimulation with IL-1β from 3.1 to 24 (4-h stimulation) and 68 (24-h stimulation) ng/mg of protein ($P < 0.01$; three independent determinations). See the text for other experimental conditions.

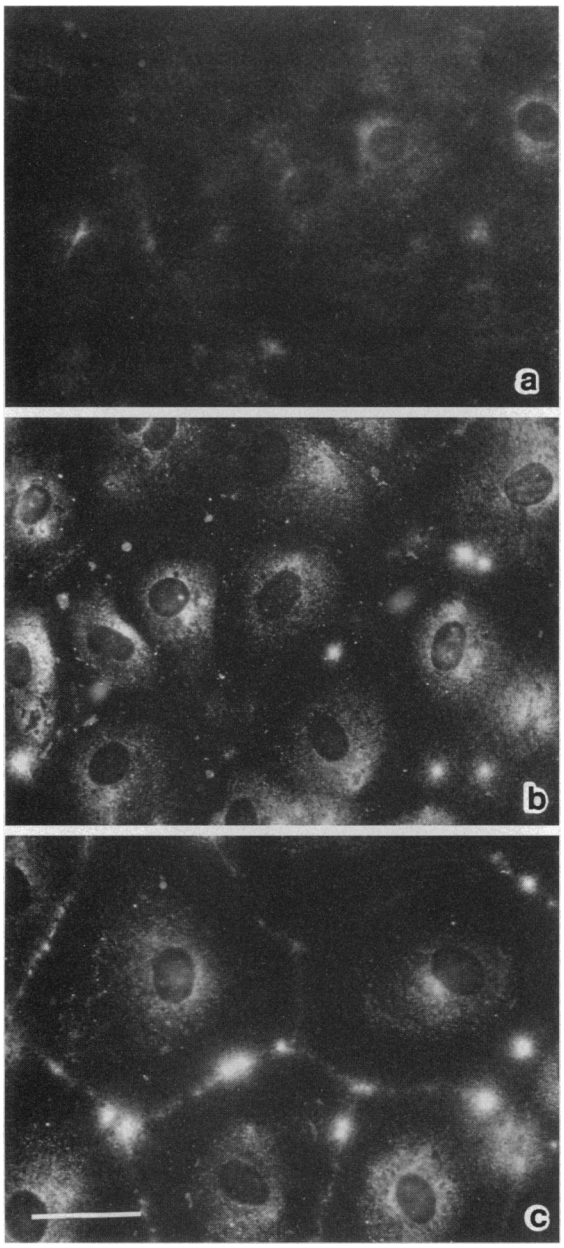

FIG. 2. Immunostaining of fixed BMEC monolayer with anti-SGPG monoclonal antibody before (*a*) and after 4 hr (*b*) and 24 hr (*c*) of stimulation with human recombinant IL-1β. The immunoreactivity of the BMEC monolayer was apparently increased after IL-1β administration. Note the immunoreactivity at the junction of adjacent cells in the 24-hr specimen (*c*). (Bar = 100 μm.)

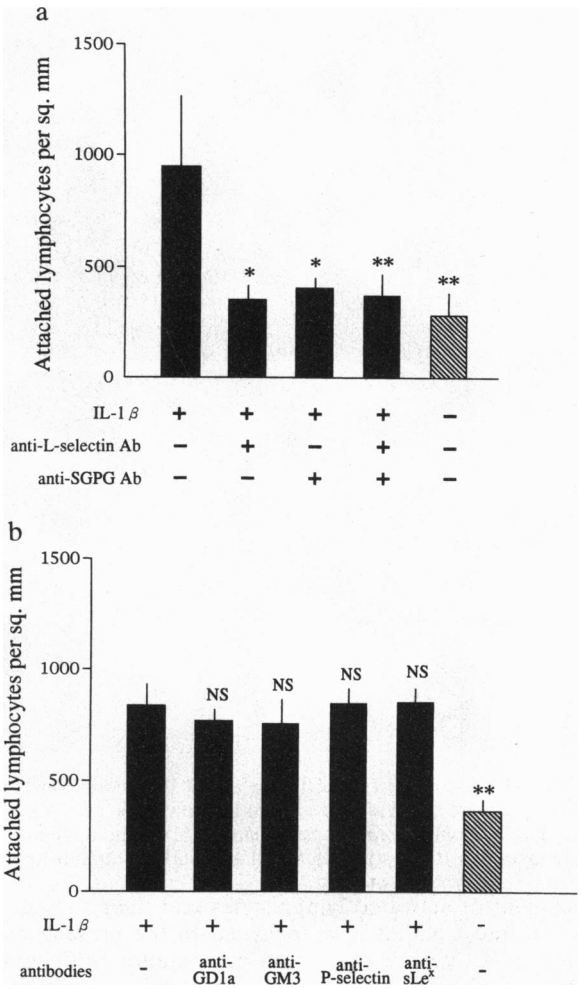

FIG. 3. (*a*) Effect of anti-L-selectin and anti-SGPG antibodies on the adhesion of human lymphocytes. Filled bars represent IL-1β-activated BMECs and the hatched bar represents the nonactivated BMEC monolayer. Preincubation with anti-L-selectin and/or anti-SGPG antibodies significantly reduced the attachment of human lymphocytes onto the BMEC monolayer (*, $P < 0.05$; **, $P < 0.01$; at least four independent determinations). Error bars indicate the SD. (*b*) Effect of anti-GD1a, anti-GM3, anti-P-selectin, and anti-sialyl Le^x (anti-sLe^x) monoclonal antibodies on the adhesion of human lymphocytes to BMEC monolayers. Filled bars represent IL-1β–activated BMECs, and the hatched bar represents nonactivated, antibody-untreated BMECs. None of the four monoclonal antibodies effectively reduced the attachment of human lymphocytes to the activated BMEC monolayer. NS, not significant; **, as in *A*. Error bars indicate the SD.

is now designated as GlyCAM-1 (glycosylation-dependent cell adhesion molecule), has been found to require sulfation for its ligand activity (21). Baumhueter *et al.* (22) identified CD34 as a ligand for an L-selectin chimeric molecule. In addition to these sulfated mucin-like glycoconjugates, sulfated GSLs, including sulfatide and SGGLs, are also implicated as ligands for L-selectin (24–26) and P-selectin (26).

Recently we provided evidence that SGPG and its analogues were integral components in isolated brain microvessels (5) and cultured BMECs (6). Among the various GSLs in BMECs, SGPG is the only glycolipid whose concentration shows a wide fluctuation depending upon culture conditions and age (6). Thus, its concentration ranges from 65 ng/mg of protein for cells grown 7 days in culture to as little as 0.1 ng/mg of protein for cells grown 35 days in culture (6). Such a dramatic fluctuation in glycolipid expression may suggest a functional regulation by humoral factors or culture conditions. It is well known that BMECs lose the activity of a BMEC-specific marker enzyme, γ-glutamyl transpeptidase, in older cultures and that the activity is recovered when the cultures are treated with astrocyte-conditioned medium or are cultured with astrocytes (27). However, the functional role of these glycolipids remains a subject of speculation. In this investigation, we found that the SGPG content in BMECs increased dramatically after stimulation with IL-1β, and the IL-1β-dependent lymphocyte attachment was partially blocked by treatment of the lymphocytes with an anti-L-selectin monoclonal antibody or by treatment of the BMECs with an anti-SGPG monoclonal antibody. Up-regulation of various adhesion molecules after stimulation with inflammatory cytokines has been reported in ECs as well as human fetal astrocytes (28). Thus, our results strongly suggest that SGPG acts as one of the ligands for L-selectin in inflammatory disorders of CNS/PNS and regulates the

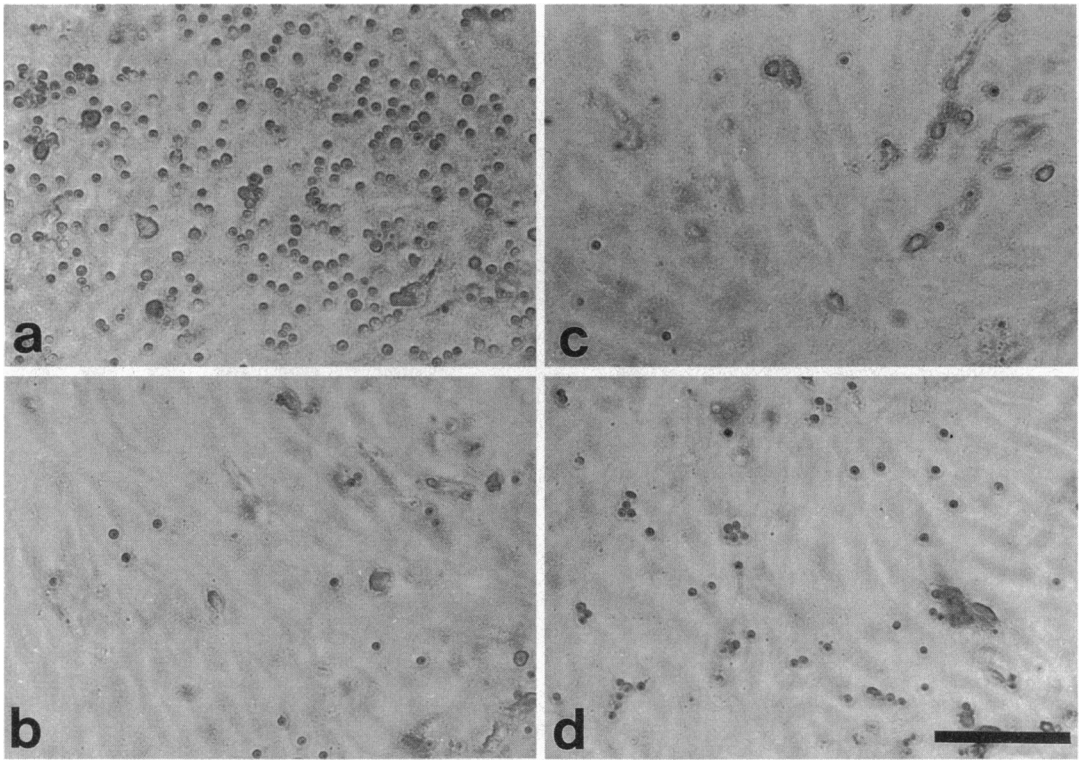

FIG. 4. Effect of anti-L-selectin and anti-SGPG monoclonal antibodies on the adhesion of human lymphocytes on the BMEC monolayer. The photographs were taken after overnight fixation of the cells with 2.5% glutaraldehyde and staining with hematoxylin. (*a*) IL-1β-stimulated BMEC (no antibodies applied). (*b*) Nonstimulated BMEC (no antibodies applied). (*c*) IL-1β-stimulated BMEC + anti-L-selectin antibody-preincubated lymphocytes. (*d*) IL-1β-stimulated and anti-SGPG antibody-applied BMECs. (Bar = 100 μm.)

attachment of activated lymphocytes and their subsequent invasion into CNS/PNS parenchyma. In the present study, bovine BMECs were utilized in combination with human lymphocytes because of difficulties encountered in obtaining fresh tissues for preparing pure human BMECs in culture (T.K., unpublished observation). However, L-selectin ligands expressed by bovine BMECs have been reported to be similar to their human counterparts (29), and the structure of L-selectin is known to be highly conserved during mammalian evolution (30).

The mechanism underlying the induction of SGGL expression by IL-1β is likely to be complex, which involves the initiation of the signal-transduction pathways and the subsequent up-regulation of the activities of the rate-limiting glycosyltransferases and sulfotransferase and/or the synthesis of these enzymes. It is interesting to note, however, that the SGGL immunoreactivity resides initially more intensely in intracellular locations following stimulation of the cells by IL-1β. This suggests active *de novo* synthesis, most likely in the Golgi apparatus as the result of activation by IL-1β. More interestingly, the intensity of the immunoreactivity shifted to the "edge" of neighboring cells with increasing time. Since a number of glycoconjugates possessing the HNK-1 epitope, including the glycoproteins MAG (31), J1, L1, N-CAM (32, 33), and neurothelin (34), and the glycolipid SGPG (35), have been implicated to play an important role in cellular adhesion, it is possible that SGGLs may also be involved in intercellular adhesions of BMECs for the formation of BBB/BNB and the maintenance of the barrier function. Our recent demonstration that anti-SGGL monoclonal antibody can disrupt the barrier function in a complement-independent manner is certainly consistent with the above view (6).

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