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Glycosphingolipid-Binding Protein of *Borrelia burgdorferi* Sensu Lato

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The binding of *Borrelia burgdorferi*, the causative agent of Lyme disease, to glycosphingolipids present in various types of cells was examined. *B. burgdorferi* bound specifically to galactosylceramide (GalCer) and glucosylceramide (GlcCer) but not to other glycosphingolipids, as determined by a thin-layer chromatography (TLC) overlay assay. The binding specificity of *B. burgdorferi* to various glycosphingolipids suggested that the binding receptor in this species is ceramide monohexoside. The levels of binding of *B. burgdorferi* virulent strain 297 to GlcCer, sulfatide, lactosylceramide, and galactosylgloboside were 56.2, 1.6, 15.9, and 9.7%, respectively, relative to that to GalCer. Virulent low-passage strains of *B. burgdorferi* were serially subcultured in BSK II medium, and the resultant high-passage strains were not capable of infecting mice and did not induce footpad swelling. The levels of binding of the low-passage strains to GalCer on TLC plates and to CHO-K1 cells in vitro were threefold higher than those of high-passage strains. Binding was not affected by pretreatment of *Borrelia* with monospecific anti-outer surface protein C (OspC) antiserum. These results indicated that the binding of *Borrelia* to glycosphingolipid expressed on the cell surface plays an essential role in infection of mammalian hosts. However, OspC was not associated with binding. The necessity of the sugar and *N*-acyl moieties in GalCer for the binding of *Borrelia* was shown by a TLC overlay assay using chemically modified GalCer. Furthermore, three proteins, 67-kDa protein, 62-kDa Hsp60, and 41-kDa flagellin, were involved in binding of *B. burgdorferi* to GalCer, as shown by blotting assay using biotinylated GalCer as a probe.

Lyme borreliosis is a tick-borne zoonosis caused by *Borrelia burgdorferi* sensu lato (5). *B. burgdorferi* sensu lato is transmitted primarily by ticks of the genus *Ixodes* (5), and multisystemic infection can affect skin, joints, heart, and the central nervous system (35). The attachment of *B. burgdorferi* to host tissues is the first stage in the initiation of infection. A number of studies have indicated that cell surface molecules in the host act as attachment sites for microbial ligands (20).

B. burgdorferi has been shown to bind to platelets via integrin $\alpha_{IIb}\beta_3$ (7) and possess integrin-mediated hemagglutination activity (23). Isaacs (18) demonstrated that *B. burgdorferi* bound to heparin of epithelial cell glycosaminoglycans (GAG). Further, *B. burgdorferi* adheres to proteoglycans such as decorin (16) and fibronectin (21). Proteoglycans are expressed on the cell surface as well as extracellular matrix and play a role in cellular adhesion and migration. Garcia-Monco et al. (15) reported that *Borrelia* adhered to Schwann cells and bound galactosylceramide (GalCer) specifically in an assay based on the enzyme-linked immunosorbent assay. GalCer is present in various types of cells as a surface glycosphingolipid. In addition, Backenson et al. (2) demonstrated that outer surface proteins A and B (OspA and OspB) are not directly involved in adherence of *B. burgdorferi* to GalCer. On the other hand, OspA and the 70-kDa protein of *B. burgdorferi* bound human plasminogen which produced plasmin (12, 17). Plasmin is a host-derived protease (37) and thus might participate in borrelial dissemination in local skin lesions. Coleman et al. (8) showed that plasmin-coated *B. burgdorferi* can penetrate endothelial cell monolayers. Therefore, OspA and the 70-kDa protein may be involved in the initial step of infection by *B. burgdorferi* (12, 17).

In this study, we demonstrated the relationship between infectivity and glycosphingolipid binding activity of *B. burgdorferi* and characterized the glycosphingolipid-binding protein of *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains and in vitro cultivation. *B. burgdorferi* sensu stricto 297, originally isolated from human spinal fluid in the United States, *B. garinii* HP1 and NP81, isolated from *Ixodes persulcatus* in Japan, and *B. afzelii* PGau, isolated from human spinal fluid in Germany, were used. *Borrelia* strains were cultivated at 34°C in Barbour-Stoenner-Kelley II (BSK II) medium (3). *Borrelia* strains cloned by the limiting-dilution method in BSK II medium were inoculated into ddY mice. Reisolates from the bladders of infected mice were used as virulent strains (denoted by the suffix "vi") and stored at −80°C until use. The virulent strain was passaged less than three times in BSK II medium. Passaged strains were prepared by serial subculture of the virulent strain in BSK II medium at 34°C. Briefly, 3 μl of a log-phase culture was transferred into 5 ml of fresh medium every 7 to 10 days, and serial passage was continued for 1 year. The high-passage *Borrelia* strains used were strain 297 passed 63 times (297P63), strain HP1 passaged 53 times (HP1P53), strain NP81 passaged 56 times (NP81P56), and strain PGau passaged 61 times (PGauP61). When spirochetes reached the log phase of growth, the cells were harvested by centrifugation at 8,000 rpm for 15 min and gently washed three times in phosphate-buffered saline (PBS). Spirochete suspension (ca. 10^9 cells/ml) in PBS was sonicated three times for 20 s each time for the thin-layer chromatography (TLC) overlay assay and stored at 4°C until use.

SDS-PAGE and Western blotting analysis. The borrelial isolates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The procedures for SDS-PAGE and Western blotting analysis were as described previously (25). Virulent and high-passage strains were subjected to SDS-PAGE and Western blotting analysis with anti-OspC monoclonal antibodies (MAbs) G7, L22 1F8, and L22 2B8 (27, 39). MAb p62a against Hsp60 was prepared from mice immunized with strain NT24 isolated from *I. persulcatus* (26). MAb H9724 against flagellin was a gift from A. G. Barbour, University of Texas Health Science Center (4).

Inoculation of *Borrelia* and measurement of footpad swelling in ddY mice. Infectivity and virulence of *B. burgdorferi* were evaluated as described by Masuzawa et al. (26). Briefly, 5-week-old ddY mice were inoculated subcutaneously into the right hind footpad with 10^5 *Borrelia* cells. Footpad thickness was measured with a thickness gauge at 14 days after inoculation. Since 2 standard deviations for mean thickness in the footpad of nontreated mice was less than 5% of the mean thickness, an increase of over 5% in the thickness of the inoculated footpad in comparison with another nontreated footpad was considered significant. After 14 days, bladder tissues were removed from each mouse and incu-

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TABLE 1. Glycosphingolipids used in this study

Glycosphingolipid	Structure	Source
GalCer	Galα1→1′ Cer[a]	Bovine brain
GlcCer	Glcβ1→1′ Cer	Gaucher disease patient's spleen
Sulfatide	HSO_3→3′ Galβ1→1′ Cer	Bovine brain
LacCer	Galβ1→4Glcβ1→1′ Cer	Bovine erythrocytes
Galgloboside	Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′ Cer	Bovine erythrocytes
GM1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′ Cer 3 ↑ 2αNeuAc	Bovine brain
GD1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′ Cer 3 3 ↑ ↑ 2αNeuAc 2αNeuAc	Bovine brain
GD1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′ Cer 3 ↑ 2αNeuAc8←2αNeuAc	Bovine brain
GT1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′ Cer 3 3 ↑ ↑ 2αNeuAc 2αNeuAc8←2αNeuAc	Bovine brain

[a] Ceramide (Cer) contained α-hydroxy- or nonhydroxy fatty acid.

bated in BSK II medium. Serum collected from mice infected with strain 297vi was used for preparation of polyclonal anti-OspC antibody.

TLC overlay assay. The glycosphingolipids used in this study (Table 1) were prepared from the sources indicated in the table by a method described previously (36). Phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, and lysophosphatidylcholine were purchased from Sigma (St. Louis, Mo.); α-glycerophosphoric acid was purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan). The TLC overlay assay was performed as described by Suzuki et al. (36). Briefly, the glycosphingolipids in methanol solution and phospholipids in ethanol solution were spotted on TLC plates (Polygram Sil-G; Macherey-Nagel, Düren, Germany) and developed with chloroform–methanol–0.02% aqueous $CaCl_2$ (65:25:4, vol/vol; solvent A) and chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol), respectively. The plates were dried and then blocked by incubation with 1% ovalbumin (OVA; Kanto Chemical Co., Tokyo, Japan) in 1% polyvinylpyrrolidone (PVP)–PBS (1% OVA solution). After incubation for 3 h at room temperature, the plates were incubated with sonicated *Borrelia* preparations in PBS (approximately 10^8 organisms/ml) overnight at 37°C. After being washed twice with PBS, the plates were incubated with a 1:1,000 dilution of homologous anti-*Borrelia* rabbit serum in 3% PVP–PBS for 2 h at 37°C. Anti-*Borrelia* rabbit sera used in this study were prepared as described by Masuzawa et al. (27). They were again washed twice with PBS and then blocked with 1% OVA solution for 30 min at room temperature. The plates were washed again and incubated with a 1:1,000 dilution of protein A conjugated with horseradish peroxidase (Organon Teknika Co., Durham, N.C.) for 2 h at 37°C. The bound conjugate was detected colorimetrically with 4-chloro-1-naphthol, *N,N*-diethyl-*p*-phenylenediamine, and H_2O_2 as the substrate for 10 min at 37°C. The enzymatic reaction was stopped by adding water. Subsequently, the intensity of staining was measured densitometrically with a Flying-Spot scanner (model CS-9000; Shimadzu, Kyoto, Japan) at 540 nm.

Chemical modification of glycosphingolipid. The glycosphingolipids were spotted onto TLC plates and then developed with solvent A. Periodate oxidation was performed by incubating the TLC plates with 10 mM $NaIO_4$ at 37°C for 30 min in the dark. After incubation, the TLC plates were washed 10 times with 0.05% Tween 20–PBS and then five times with PBS. N-deacylated GalCer (lysoGalCer) was prepared by treatment with a strong base as described by Neuenhofer et al. (29). GalCer (3.8 μmol) was dissolved in 2 ml of 1 M KOH in methanol, sealed under nitrogen in glass tubes, and heated at 102°C for 6 h. After neutralization with acetic acid, the solvent was evaporated. The residue was resuspended in 10 ml of water and dialyzed against water for 2 days. The solution, containing the lysoGalCer, was lyophilized and subjected to the TLC overlay assay. LysoGalCer on TLC plates was detected by orcinol and ninhydrin reaction.

Cell attachment assay and attachment inhibition assay. The spirochetes were radiolabeled metabolically by addition of 6 MBq (10 μl) of EXPRE^{35}S^{35}S labeling mix (70:23 mixture of [^{35}S]methionine and [^{35}S]cysteine; NEN Research Products, Wilmington, Del.) to early-log-phase cultures in 5 ml of BSK II medium followed by incubation at 34°C. ^{35}S-labeled organisms were centrifuged and washed with PBS three times to remove unincorporated radioactivity. These attachment assays were performed with Chinese hamster ovary cell (CHO-K1) monolayers grown in 24-well tissue culture dishes. CHO-K1 cells, supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, Kan.) and 1% L-glutamine without antibiotics at 37°C in 5% CO_2. Radiolabeled *B. burgdorferi* was coincubated with confluent CHO-K1 cell monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum without antibiotics for 6 h at 37°C under 5% CO_2. The monolayers were washed three times with prewarmed PBS at 37°C to remove unattached organisms, and attached *Borrelia* cells were solubilized by adding 0.5% (wt/vol) SDS. The solubilized cells were analyzed with a scintillation counter, and the proportion of the attached organisms was calculated. The attachment inhibition assay was performed by using a modification of the previously described protocol in which *B. burgdorferi* were preincubated with heparin (Sigma), heparan sulfate (Seikagaku Co., Tokyo, Japan) in RPMI 1640 medium, and GalCer in dimethyl sulfoxide–RPMI 1640 medium (160 μl:1 ml) as inhibitors at various dilutions for 1 h at 37°C before coincubation with the cell monolayers. The inhibitory activity of *Borrelia* attachment was calculated as $(B_{total} - B_{inhibit})/B_{total}$, where B_{total} and $B_{inhibit}$ represent binding with and without the inhibitors, respectively.

Preparation of monospecific polyclonal anti-OspC antibody from infected mouse serum. The monospecific anti-OspC antibody was prepared as described by Skare et al. (34). Pooled serum from ddY mice infected with *B. burgdorferi* virulent strain 297vi was absorbed with avirulent strain 297P63, which did not express OspC, as confirmed by Western blotting with MAbs G7, L22 1F8, and L22 2B8 (27, 39) against OspC. Avirulent 297P63 cells were added to 500 μl of the serum and incubated at 37°C for 1 h. After incubation, borreliae were removed by centrifugation at 15,000 × *g* for 10 min, and the supernatant was transferred to a new tube containing fresh avirulent 297P63 cells. This procedure was repeated 10 times. The reactivity and specificity of anti-OspC antibody were confirmed by Western blotting.

Cell attachment inhibition assay and TLC overlay inhibition assay with monospecific polyclonal anti-OspC antibody. The cell attachment inhibition assay and TLC overlay inhibition assay were performed by using modifications of protocols in which *B. burgdorferi* were preincubated with inhibitors (GalCer, heparin, and heparan sulfate) before coincubation with cell monolayers or TLC.

Preparation of biotinyl GalCer. Biotinyl GalCer [*N*-(6′-biotinamidohexanoyl) galactosylsphingosine] was prepared by treating lysoGalCer with NHS-X-biotin (biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide; Calbiochem-Novabiochem Co., La Jolla, Calif.). NHS-X-biotin (3.6 mg, 8 μmol) in 1 ml of chloroform-methanol (1:1, vol/vol) containing 2 drops of triethylamine was mixed with lysoGalCer (2.5 mg, 4.4 μmol) and stirred at room temperature overnight. The solvents were evaporated, and the residue was suspended in 500 μl of chloroform-methanol (1:1). Biotinyl GalCer was purified by preparative TLC using solvent A.

Extraction of detergent-soluble fraction from *B. burgdorferi* and binding of biotinyl GalCer to detergent-soluble fraction. *B. burgdorferi* 297vi cells were suspended in 1% octyl-β-D-thioglucoside and then centrifuged at 1,500 rpm for 10 min. The supernatant was precipitated with 5% $(NH_4)_2SO_4$–PBS and then centrifuged at 15,000 rpm for 20 min. The pellet was subjected to two-dimensional gel electrophoresis as described by O'Farrell (30).

Isoelectric focusing was carried out in polyacrylamide tube gels containing 2%

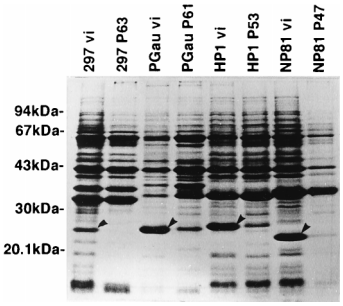

FIG. 1. SDS-PAGE profiles of *Borrelia* strains used in this study. Strains were subjected to electrophoresis on 12.5% polyacrylamide gels and stained with Coomassie brilliant blue. OspC in virulent strains reactive with specific MAbs is indicated with arrowheads.

pH 5 to 7 and 0.8% pH 3 to 10 ampholines (Pharmacia, Uppsala, Sweden). SDS-PAGE in the second dimension was carried out as described previously (22). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, Calif.), which were then blocked with 3% nonfat dry milk–PBS at room temperature for 1 h and incubated with a 1:5,000 dilution of biotinyl GalCer in methanol. The membranes were subsequently incubated with a 1:4,000 dilution of alkaline phosphatase streptavidin (Vector Laboratories, Burlingame, Calif.) and developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Sigma).

RESULTS

Infectivity and virulence of low- and high-passage strains of *B. burgdorferi* sensu lato in ddY mice. Protein profiles of low- and high-passage *Borrelia* strains used in this study are shown in Fig. 1. The low-passage *Borrelia* strains expressed higher levels of OspC in comparison with high-passage strains. All mice were infected and developed footpad swelling following inoculation of the virulent low-passage strains into the footpad (Table 2). In contrast, high-passage strains 297, PGau, and NP81 did not infect the mice and did not induce footpad swelling. However, two of five mice inoculated with high-passage strain HP1 were infected, and one of these two infected mice showed significant footpad swelling.

Binding specificity of *B. burgdorferi* 297 to glycosphingolipids. The binding specificity of strain 297 to glycosphingolipids is shown in Fig. 2. Strain 297 showed no binding to TLC plates without glycosphingolipid under our experimental conditions and bound strongly to the ceramide monohexosides GalCer and glucosylceramide (GlcCer) rather than to lactosylceramide (LacCer) or galactosylgloboside (galgloboside). Virulent strain 297vi bound better to these glycosphingolipids than the high-passage strain 297P63. Six phospholipids (phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, lysophosphatidylcholine, and α-glycerophosphoric acid) did not bind with strain 297vi (data not shown).

Binding of virulent and avirulent *B. burgdorferi* sensu lato to GalCer. Binding of *B. burgdorferi* sensu lato to GalCer is shown in Fig. 3. Virulent strains showed higher binding activities than avirulent strains, and binding to GalCer was dose dependent.

Inhibition adherence of *B. burgdorferi* to CHO-K1 cells by GAGs and GalCer. Adherence of *B. burgdorferi* to CHO-K1 cells was inhibited by GalCer in a dose-dependent manner (Fig. 4). The inhibitory activity of GalCer was lower than that of heparin but was higher than that of heparan sulfate in terms of receptor activity. RPMI 1640 medium containing dimethyl sulfoxide as the solvent for GalCer did not affect adhesion of *Borrelia* to CHO-K1 cells.

GalCer-binding epitope on *Borrelia*. To identify the GalCer-binding epitope, we performed the TLC overlay assay using the $NaIO_4$ oxidation product and N-deacylation product (lysoGal Cer) of GalCer. Figure 5 shows GalCer and lysoGalCer detected with orcinol reagent (Fig. 5A) and binding of *Borrelia* to GalCer (Fig. 5B) on TLC plates. *Borrelia* bound to α-hydroxy-fatty acid-containing GalCer (lower band) and non-hydroxy-fatty acid-containing GalCer (higher band). On the other hand, lysoGalCer did not bind to *Borrelia*. $NaIO_4$-treated GalCer could be detected with orcinol reagent (Fig. 6A) but did not bind to *Borrelia* (Fig. 6B, lane 2).

Participation of OspC in binding of *B. burgdorferi* to GalCer. Monospecificity of the anti-OspC serum was confirmed by Western blotting. Nonabsorbed serum from infected mice inhibited the binding of *Borrelia* to GalCer, whereas absorbed serum (monospecific anti-OspC serum) had no inhibitory effect (Table 3). Furthermore, the monospecific anti-OspC serum had less of an inhibitory effect on binding of *B. burgdorferi* to CHO-K1 cells in vitro than did nonabsorbed mouse serum.

Detection of glycosphingolipid-binding protein on an octyl-thioglucoside extract of *B. burgdorferi* by biotinyl GalCer. Fractons of *B. burgdorferi* 297vi solubilized with 1% octyl-β-D-thioglucoside were separated by two-dimensional PAGE, electroblotted onto PVDF membranes, and then probed with biotinyl GalCer (Fig. 7). Biotinyl GalCer bound to flagellin, Hsp60, and the 67-kDa protein, and the binding was inhibited by addition of unlabeled GalCer (data not shown). The reactive spots were identified by reactivity with MAb H9724, specific to flagellin, and MAb p62, specific to Hsp60 of *B. burgdorferi* (data not shown).

DISCUSSION

It has been shown that *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* induce arthritis, neuritis, and acrodermatitis chronica atrophicans, respectively (38). Infection by *B. garinii* in particular is frequently associated with neurological manifestations (1). Adherence of the etiological agent *B. burgdorferi* to cells of primary cultures of rat brain and of the C6 glioma cell line was demonstrated previously (14). However, *B. burgdorferi* sensu stricto did not bind to sulfatide, which is abundant on nerve cells (13, 15).

Generally, substituents of the lactosyl moieties of glycosphingolipid act as receptors for many bacteria, yeasts, and fungi (19, 20). However, *B. burgdorferi* bound weakly to LacCer containing the Galβ1→4Glc sequence. *B. burgdorferi* sensu lato bound specifically to GalCer and GlcCer as determined by

TABLE 2. Infectivity and virulence of low- and high-passage strains in ddY mice[a]

Challenge strain	No. of mice with swollen footpad/no. of mice used	No. of isolates/ no. of mice used
297vi	5/5	5/5
297P63	0/5	0/5
PGauvi	5/5	5/5
PGauP61	0/5	0/5
HP1vi	5/5	5/5
HP1P53	1/5	2/5
NP81vi	5/5	5/5
NP81P56	0/4	0/4

[a] Mice were inoculated subcutaneously into the hind footpad with live *Borrelia* culture (10^5 cells/mouse). After 14 days, bladder tissues were removed from each mouse and incubated in BSK II medium. Since 2 standard deviations for mean thickness in the footpad of nontreated mice was less than 5% of mean thickness, an increase of over 5% in the thickness of the inoculated footpad was considered significant swelling.

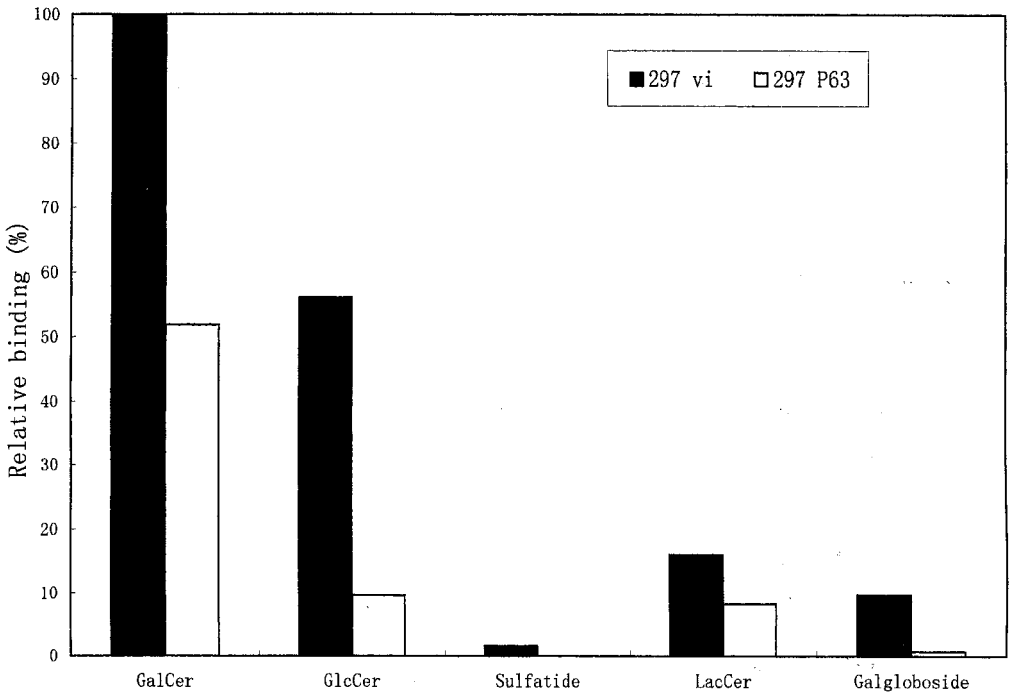

FIG. 2. Relative binding of *B. burgdorferi* 297 to glycosphingolipids (GalCer, GlcCer, sulfatide, LacCer, and galgloboside). Glycosphingolipids were spotted onto TLC plates at 10 nmol/spot. The variability of binding intensity in triplicate samples was less than 5%.

the TLC overlay assay. It was previously reported that *B. burgdorferi* bound to heparin (18) and decorin (16) with sulfated sugars. However, *B. burgdorferi* showed little binding to sulfatide (GalCer sulfated at the 3′ OH of galactose). Heparin was not involved in competitive inhibition to GalCer as determined by the TLC overlay assay (data not shown). These findings suggest that heparin-binding protein and glycosphingolipid-binding protein are different. The sugar moiety and the nonsubstituted terminal galactosyl or glucosyl residue of glycosphingolipid are required for binding to *Borrelia*. Low-pas-

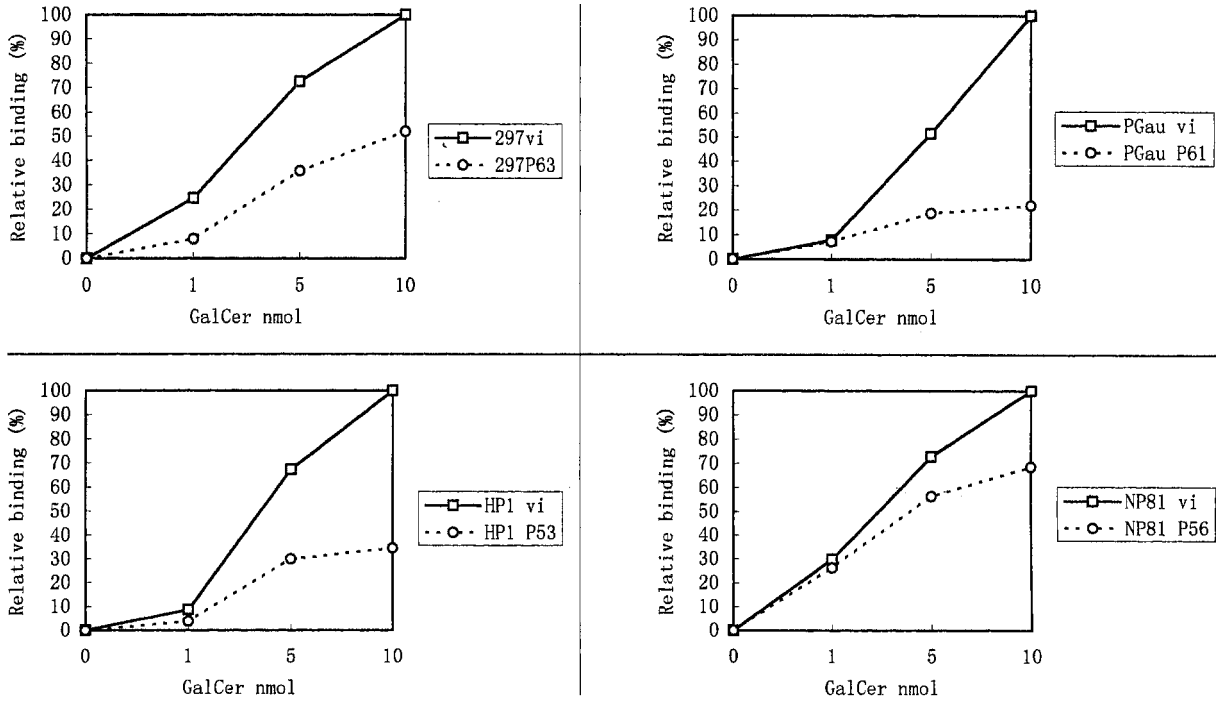

FIG. 3. Relative binding activity of *B. burgdorferi* 297, *B. garinii* HP1 and NP81, and *B. afzelii* PGau to GalCer. Data are expressed as relative binding of each virulent strain to 10 nmol of GalCer. The variability of binding intensity was less than 5%.

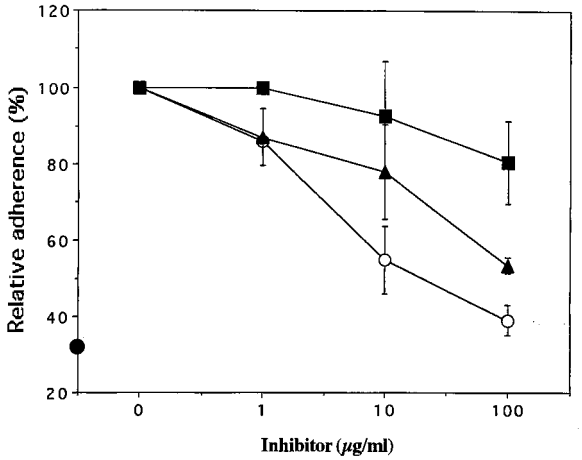

FIG. 4. Adherence inhibition by GAGs and GalCer of *B. burgdorferi* 297vi to CHO-K1 cells. Curves represent heparin (○), heparan sulfate (■), and GalCer (▲); the black dot shows relative adherence of 297P63.

sage and virulent strains showed greater ability to bind to GalCer in the TLC overlay assay and to CHO-K1 cells in the adherence assay in vitro than high-passage and avirulent strains. The adherence of *Borrelia* to CHO-K1 cells was specifically inhibited by preincubation of *Borrelia* with GalCer. Furthermore, it was confirmed that *Borrelia* bound to glycosphingolipid identified as LacCer extracted from CHO-K1 cells but not protein derived by the binding assay on PVDF membranes. These results suggested that infectivity of *Borrelia* may be associated with binding to glycosphingolipids such as Gal Cer and LacCer on the cell surface.

The strains used in this study were able to bind to GalCer, GlcCer, and LacCer, with higher affinity to GalCer. No binding was observed with sulfatide or ganglioside mixtures containing GM1b, GD1a, GD1b, and GT1b (data not shown). Furthermore, the six phospholipids used in this study did not bind with strain 297vi. The binding of *Borrelia* to GalCer was affected by the structure of the ceramide moiety. *Borrelia* did not bind to deacylated GalCer (lysoGalCer) or sugar-decomposed GalCer prepared by periodate oxidation. These observations indicate that borreliae recognize not only the *N*-acyl group but also the sugar moiety as the binding epitope. α-Hydroxyfatty acid-containing GalCer is localized in natural cell membranes, and the carbohydrate moiety in glycosphingolipid contained α-hydroxyfatty acid- rather than nonhydroxyfatty acid-exposed outer membrane. Garcia-Monco et al. (15) previously reported that borreliae bind to GalCer and GlcCer but not sulfatide or deacylated ceramide. The findings of this and previous studies suggested that the binding of *Borrelia* to GalCer was affected by the structure of the sugar and the ceramide moiety.

Early studies found that the OspA and OspB of *B. burgdorferi* play important roles in the host-bacterium interactions in Lyme borreliosis (9, 31). It was suggested that the expression of OspA, OspB, and OspC in *Borrelia* was regulated by environmental conditions such as those in the tick midgut and in the host. In unfed ticks, *Borrelia* produces OspA but not OspC (10, 11, 32), while OspC is abundantly expressed in the host (26). Furthermore, *Borrelia* in the midgut of ticks that had fully engorged on mice expressed OspC at 32 to 37°C but not at 24°C (32). These findings suggested that OspC may play an essential role in the infectivity of *Borrelia* to mammalian hosts. It has been reported that OspA and OspB did not participate in binding to glycosphingolipid of *B. burgdorferi* (2). Since low-passage *Borrelia* strains expressed high levels of OspC (Fig. 1) and showed stronger binding than high-passage strains to the glycosphingolipids, the binding of OspC to GalCer was examined by using a monospecific anti-OspC antiserum. The inhibitory activity of the serum suggested that OspC, like OspA and OspB, was not involved in the binding of *Borrelia* to Gal Cer.

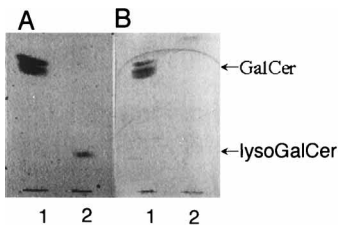

FIG. 5. Binding of *B. burgdorferi* 297vi to GalCer (lane 1) and lysoGalCer (lane 2). (A) Orcinol reagent; (B) TLC overlay assay.

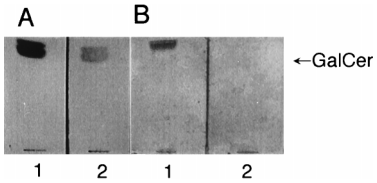

FIG. 6. Binding of *B. burgdorferi* 297vi to GalCer (lane 1) and $NaIO_4$-treated GalCer (lane 2). (A) Orcinol reagent; (B) TLC overlay assay.

Three molecules capable of binding to GalCer, flagellin, Hsp60, and 67-kDa protein, were detected in the binding assay with biotinyl GalCer as a probe. Recently, it was reported that 39-kDa heparin-binding protein (18), 19- and 20-kDa decorin-binding proteins (16), OspA and 70-kDa plasminogen-binding proteins (17), and 28-kDa transferrin-binding protein (6) are expressed on the *Borrelia* cell surface. The molecules which bound to GalCer in this study differed from these proteins with respect to molecular mass. The flagella are located in the periplasmic space, wrapped around the cell, and part of the distal end of the flagella is exposed in spirochetes. Therefore, flagella do not seem to contain glycosphingolipid-binding protein. Hsp60 is a major immunodominant antigen (24). Since antibodies to Hsp60 were detected in synovial fluid of Lyme arthritis patients (28, 33), Hsp60 may participate as a virulence factor, but its functions remain unknown. The 67-kDa protein of *B. burgdorferi* identified here has not been described previously.

Binding to glycosphingolipid may contribute to the mechanism of tissue-specific adhesion of *B. burgdorferi*. The interaction between *B. burgdorferi* and glycosphingolipid may affect the inflammatory process in Lyme borreliosis. Purification and characterization of GalCer-binding protein may facilitate understanding of the pathogenesis of Lyme disease borreliae.

TABLE 3. Inhibition of cell adhesion and GalCer binding of *Borrelia* by monospecific anti-OspC serum[a]

Serum	Inhibition of cell adhesion (%)	Inhibition of binding to GalCer (%)
Control (normal mouse serum)	0	0
From mice infected with 297vi	41.2	42.7
Absorbed with 297P63 (monospecific anti-OspC serum)	6.8	1.2

[a] Dose/serum dilution, 1:100.

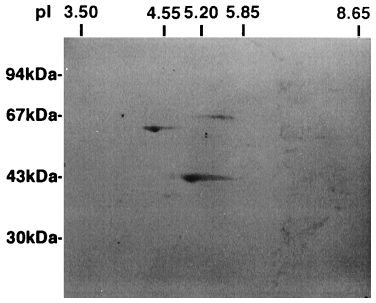

FIG. 7. Immunodetection of glycosphingolipid-binding protein of *B. burgdorferi* 297vi, using biotinyl GalCer. Separation of protein in the first dimension was by isoelectric focusing between pH 3.9 and 10.0, and separation in the second dimension was by SDS-PAGE (10% gel). pH values are shown at the top.

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