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Novel Anti-idiotypic Antibody Therapy for Lipooligosaccharide-Induced Experimental Autoimmune Neuritis: Use Relevant to Guillain-Barré Syndrome

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Abstract

Campylobacteriosis is a frequent antecedent event in Guillain-Barré syndrome (GBS), inducing high-titer serum antibodies for ganglioside antigens in the peripheral nervous system (PNS). Molecular mimicry between the lipooligosaccharide (LOS) component of *Campylobacter jejuni* and human peripheral nerve gangliosides is believed to play an important role in the pathogenesis of GBS. Conventional treatment strategies for patients with GBS include plasmapheresis, intravenous immunoglobulin (IVIG), and immunosuppression, which are invasive or relatively ineffective. In this study, we used our animal model of GBS, in which Lewis rats were immunized with GD3-like LOS isolated from *C. jejuni*. The animals developed anti-GD3 ganglioside antibodies and manifested neuromuscular dysfunction. To develop novel therapeutic strategies, we treated the animals by intraperitoneal administration of an anti-GD3 antiidiotypic monoclonal antibody (BEC2) that specifically interacts with the pathogenic antibody. The treated animals had a remarkable reduction of anti-GD3 antibody titers and improvement of motor nerve functions. The results suggest that ganglioside mimics, such as antiidiotypic antibodies, may be powerful reagents for therapeutic intervention in GBS by neutralizing specific pathogenic antiganglioside antibodies.

Keywords

ganglioside; *Campylobacter jejuni*; Guillain-Barré; syndrome; lipooligosaccharide; GD3 ganglioside; anti-idiotypic antibody

Guillain-Barré syndrome (GBS) is an immune-mediated peripheral neuropathy characterized by neuromuscular weakness and frequently accompanied by flaccid paralysis and may occasionally lead to death. The major pathological hallmarks involve demyelination, axonal degeneration, and/or impairment of neurotransmission by ion channel blockage (Rinaldi and Willison, 2008; van Doorn et al., 2008; Kaida et al., 2009). The etiology of GBS is complex and is not fully known. A growing body of evidence, however, indicates that aberrant

immune responses triggered by an infectious agent or vaccination allow disease development and the underlying pathogenetic mechanisms (Langmuir et al., 1984; Kuwabara, 2004; Souayah et al., 2007). The most commonly identified microbial agents are *Campylobacter jejuni* (*C. jejuni*), *Haemophilus influenzae*, cytomegalovirus (CMV), Epstein-Barr virus, and *Mycoplasma pneumoniae* (Hughes et al., 1999; Hadden et al., 2001; Sivadon-Tardy et al., 2006, 2009). A preceding infectious event and patient-related host factors also seem to be related to certain subtypes of GBS and may affect the severity of the disease (Geleijns et al., 2005; Caporale et al., 2006; Yuki, 2007). *C. jejuni* infection frequently induces antiganglioside antibodies in the patient's serum (Yuki et al., 1990; Usuki et al., 2006b). Thus, despite the possibility of other pathogenic mechanisms, an antibody-mediated process is one of the major insults to the nerve, causing both conduction block and velocity loss and the ensuing clinical symptoms (Rinaldi and Willison, 2008; van Doorn et al., 2008; Kaida et al., 2009).

The etiology of GBS has been not been fully clarified; one possibility is based on molecular mimicry and cross-reacting antiglycolipid antibody induction during the postinfectious phase (Yu et al., 2006; Yuki, 2007). For this reason, an elevated level of serum antiganglioside antibodies in GBS is the most important serological marker for disease diagnosis (Ariga and Yu, 2005; Kaida et al., 2009). Conventional treatment strategies rely heavily on removal of pathogenic antiglycolipid antibodies from the blood circulation. In practice, plasmapheresis and intravenous immunoglobulin (IVIG) have been used extensively for treatment (Buchwald et al., 2002; Kieseier et al., 2008). Both strategies, however, are invasive and remove both nonpathogenic and pathogenic antibodies from circulation, with attendant risk of undesirable side effects. One of IVIG's mechanisms is neutralization by antiidiotypic antibodies (Dalakas, 2004a). For this reason, we have devised a novel therapeutic strategy to remove specific pathogenic antiglycolipid antibodies by using antiidiotypic antibodies. This molecular mimic would serve as a specific competitive inhibitor for antiganglioside antibodies in the circulation.

According to the idiotype network in autoimmunity, antiidiotypic mAbs are produced in syngenic mice against a mouse mAb recognizing GD3 ganglioside (mAb R24; Chapman and Houghton, 1991). BEC2, an antiidiotypic mAb that recognizes the GD3 binding site of R24, mimics GD3 and can be used as an immunogen in mice and humans to induce anti-GD3 antibodies. Thus, the structure of BEC2 mirrors that of GD3.

Previously, we reported a model of GBS-like neuromuscular disorder by sensitizing rats with a crude lipooligosaccharide (LOS) fraction of *C. jejuni* (Usuki et al., 2006b). Interestingly, immunization produced polyclonal antibodies for GD3, GM1, GM2, GD1a, and GQ1b in response to ganglioside-like antigens found in the crude LOS. We have validated that the anti-GD3 antibody is one of the cross-reacting antibodies found in rats that have been sensitized by the crude LOS that contains many ganglioside-like carbohydrate epitopes (Usuki et al., 2006b). This finding was further confirmed by isolation of a purified GD3-like LOS (LOS_{GD3}) and a GM1-like LOS (LOS_{GM1}). Previously, we reported anti-GD3 antibody in two patients with acute and chronic inflammatory demyelinating polyneuropathy (AIDP/CIDP; Usuki et al., 2005). Anti-GD3 antibody is known to be elevated in rare cases of GBS (Yuki and Tagawa, 1998; Yuki et al., 2000) and Miller Fisher syndrome (Willison et al., 1994; Koga et al., 1999). In our current study, the LOS_{GD3} was used as an immunogen to induce neurodysfunction and concomitant serum anti-GD3 antibody activity in the rat. We previously demonstrated that the serum anti-GD3 antibody possesses neuromuscular junction (NMJ)-inhibitory activity (Usuki et al., 2005, 2006b). This model offers us an opportunity to test the concept of using an antiidiotypic antibody for GD3 (BEC2) as a novel agent for treating dysfunction in an LOS_{GD3}-induced animal model as a prototype for developing treatment of similar antiglycolipid-mediated neurological

disorders, including GBS, Miller Fisher syndrome, and related immune-mediated neurological disorders.

MATERIALS AND METHODS

The following items were purchased: high-performance thin-layer chromatography (HPTLC) plates coated with silica gel 60 (aluminum-based sheets) from E. Merck (Darmstadt, Germany). The hybridoma cell line for mAb R24 was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The hybridoma cell line producing antiidiotype mAb (BEC2) was supplied by Memorial Sloan-Kettering Cancer Center (New York, NY).

Preparation of BEC2 and R24 mAbs

The hybridoma cell lines for mAb R24 (IgG3) and BEC2 (IgG2b) were cultivated in serum-free cell cultures (BD Cell MAb Medium Serum Free; Becton Dickinson and Company, Sparks, MD). A small aliquot of the supernatant from routine cell culture was tested for IgG production using the IsoStrip Monoclonal Antibody Isotyping Kit (Santa Cruz Biotechnology, Santa Cruz, CA). Each of the collected conditioned media was concentrated about 20-fold using an Amicon concentrator (model No. 8200) with an ultrafiltration membrane YM10 (Millipore Corp., Bedford, MA). After concentration, the IgG fraction of mAb R24 or BEC2 was precipitated with a saturated solution of ammonium sulfate (80 g dissolved in 100 ml of hot water) and dialyzed against phosphate-buffered saline (PBS) in water for 4 days at 4°C, and the dialyzed IgG proteins were further purified by affinity column chromatography (HiTrap Protein G HP, 1 ml; Amersham Bioscience, Uppsala, Sweden). The IgG fraction was recovered from the column by eluting with a glycine buffer (0.1 M glycine-HCl, pH 2.7); the eluting solution was neutralized by addition of 1 M NaOH and then dialyzed against PBS for 3 days at 4°C. Finally, the dialyzed mAb R24-IgG protein was used for ELISA and constructing the mAb R24 affinity column. The dialyzed BEC2 IgG protein was biotinylated (see below), and the biotinylated BEC2 (bBEC2) was used for ELISA.

Biotinylation of BEC2

The BEC2-IgG protein was biotinylated by using an EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce, Rockford, IL). One to ten milligrams BEC2-IgG was dissolved in 0.5–2 ml PBS according to the instruction kit. After addition of the Sulfo-NHS-LC-Biotin solution, the reaction mixture was incubated at room temperature for 1 hr, and the biotinylated product was purified by a Zeba Desalt Spin Column. Estimation of biotin incorporation to BEC2-IgG was done using HABA (4'-hydroxyazobenzene-2-carboxylic acid) Biotin Quantitation Kit (Pierce, Rockford, IL) and expressed as mmole biotin/mmmole protein.

Preparation of LOS_{GD3}

C. jejuni ATCC-43446 (serotype HS:19) was grown in *Brucella* broth with gentle shaking (100–150 rpm) for 48 hr at 37°C under microaerobic conditions. The cells were recovered by centrifugation at 4,000 rpm for 30 min and washed twice with saline. The LOS fraction was extracted from the cell pellets by the hot phenol-water procedure (Westphal et al., 1952), and isolation of LOS_{GD3} was done as described previously (Usuki et al., 2006b). The protocol outline is shown in Figure 1. Treatment of the cell pellets of *C. jejuni* with a hot phenol solution yielded aqueous and phenolic phases. The aqueous phase was dialyzed against water and the dialysate treated with 2 vol of methanol and 1 vol of chloroform. The chloroform layer was recovered by a separatory funnel, and the aqueous layer was again partitioned with 1 vol of chloroform and 1 vol of water. The chloroform layer was recovered and combined with the previous chloroform layer. Most of the LOS was recovered in the

combined chloroform fraction. An additional minor amount of LOS was precipitated at 4°C overnight from the remaining phenolic phase by the addition of 9 vol of cold acetone. The two LOS fractions thus obtained were combined, dried, and subjected to alkaline hydrolysis with 25% ammonia at 56°C for 48 hr. The solution was then dialyzed against water, and the retentate was lyophilized.

The LOS fraction described above was further fractionated by stepwise elution from a silica gel column (13 × 1.5 cm i.d.; Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo, Japan) using the following solvents in succession: 1) 60 ml n-propanol, 2) 40 ml n-propanol:H₂O (75:20, v/v), 3) 100 ml n-propanol:H₂O:triethylamine (75:20:5, v/v/v), and 4) 100 ml n-propanol:H₂O:triethylamine (60:20:20, v/v/v). The fraction eluted with solvent 3 was collected, and the aliquot was tested by two separate TLC-immune overlays to detect LOS_{GD3} and LOS_{GM1}. A portion of the sample was developed on an HPTLC plate using the solvent system of n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v). After the plate was dried, the plate was overlaid with anti-GD3 mAb R24 (1:5 dilution). Another similarly developed plate was overlaid with cholera toxin B subunit (CTxb) as described previously (Usuki et al., 2006b, 2007).

For affinity purification, the purified R24 IgG protein mentioned above was dialyzed in coupling buffer (0.1 M NaHCO₃, pH 8.0) and coupled with affinity gel CNBr-sepharose 4B (Sigma, St. Louis, MO) using a previously described procedure. The column was preeluted with 3 M potassium thiocyanate in 0.5 M ammonium hydroxide and washed well with 1 mM PBS. The LOS fraction eluted with solvents 3 and 4 from the silica gel column was applied onto an affinity column (column volume 1 ml) and eluted stepwise with the following solvents (Fig. 1): 5) washing solution 1 (10 ml of 1 and 10 mM PBS); 6) washing solution 2 (50 mM PBS); 7) eluting solution 1 (5 ml of 50 mM diethylamine/HCl buffer, pH 8.6, containing 0.1 M NaCl); and 8) eluting solution 2 (5 ml of 50 mM diethylamine/HCl buffer, pH 10.5, containing 0.1 M NaCl). The bound LOS fraction was eluted with solvents 7 and 8, consecutively, of the affinity column and recovered in 0.5-ml fractions. R24-binding LOS (LOS_{GD3}) was present in the 10th to 15th tubes of the eluting solutions. These fractions were collected, combined, dialyzed against water, and lyophilized as LOS_{GD3}. LOS_{GD3} was stored at -20°C before use.

Pharmacokinetic Analysis

Each female rat was given a single dose of bBEC2 (1, 5, or 10 mg kg⁻¹; n = 3 per group). The total number (n) was 9 (body weight = 200 ± 20 g). Serial blood samples (~200 µl) were taken from tail vein for bBEC2 for predose (0 hr) and from the tail vein at serial time points (1, 3, 6, 12, 24, 48, 96, and 168 hr) postdose. Blood was centrifuged at room temperature, plasma harvested, and stored at -60°C to -80°C until analyzed by a streptavidin-coated ELISA plate assay for total concentration of bBEC2.

The plasma concentration–time data of bBEC2 were analyzed by a noncompartmental method of i.p. administration with first-order output as previously reported (Wahl et al., 1988; Barrett et al., 1997). The following parameters were calculated: maximum observed plasma concentration (C_{max}), area under the plasma concentration–time curve from zero to infinite time point (AUC), plasma clearance (CL), distribution half-life (t_{1/2α}), and elimination half-life (t_{1/2β}). Calculations of rate constants t_{1/2α} and t_{1/2β} were obtained by the curve-peeling method (Gibaldi and Perrier, 1982). AUC was estimated according to the method of the trapezoidal rule by including the remaining AUC after the last measurable time point (168 hr), extrapolating the curve from the last time point to infinity.

Streptavidin-Coated ELISA for bBEC2

bBEC2 was attached onto streptavidin-coated 96-well polystyrene plates, obtained from Pierce (Rockford, IL; No.15121), and ELISA was performed according to the manufacturer's instructions. The efficacy of binding of the anti-GD3 antibody to immobilized bBEC2 was determined by using mAb R24, followed by an anti-mouse horseradish peroxidase-conjugated secondary antibody and colorimetric development. Briefly, the plasma samples from single-dose administration of bBEC2 were applied to the streptavidin-coated ELISA plate at serial double dilutions in 1% BSA/PBS solution. The plate was subjected to incubation for 1 hr at room temperature, and, after washing with 1% BSA/PBS buffer, each well of the plate was treated with the mAb R24 (1 µg/ml in 1% BSA/PBS). After washing with 1% BSA/PBS buffer, each well was treated with an anti-mouse IgG horse-radish peroxidase-conjugated antibody (Jackson Immomeresearch, West Grove, PA). This secondary antibody was pretreated with rat IgG to eliminate contamination from any anti-rat IgG binding activity. Finally, the bound secondary antibody was visualized by a color-generating reagent (OPD Peroxidase Substrate in PBS; Sigma). Half-saturation absorbance values for plasma samples were estimated by serial plasma dilution curves of ELISA. The absorbances were then converted into values of plasma concentration using a standard serial dilution curve of rat serum containing an authentic bBEC2 sample.

ELISA for Gangliosides and LOS_{GD3}

Antiganglioside antibody activity was evaluated for GM1, GM2, GD1a, GD1b, GT1b, GQ1b, GD3, and LOS_{GD3} by our conventional ELISA method (Usuki et al., 2005). To measure half-maximal inhibitory concentrations (IC₅₀s) of BEC2 against mAb R24 binding to GD3, each well of the ELISA plate (Immunlon 1B; Lab System, Franklin, MA) was coated with 0.1 µg GD3. Before the ELISA plate assay, each well was treated with 1% BSA/PBS solution. The purified mAb R24 IgG (10 µg/ml by 1% BSA/PBS solution) was incubated with various concentrations of BEC2 for 30 min. After incubation, the reaction mixture was passed through a 0.22-µm syringe filter (Millipore Corp., Bedford, MA). Subsequently, 100 µl of the mixture was added to each well and incubated for 1 hr at room temperature. To each of the wells, a secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG, 1:5,000 dilution in 1% BSA /PBS solution) was added and incubated for 1 hr at room temperature. Finally, the bound secondary antibody was visualized by addition of a color-generating reagent (OPD Peroxidase Substrate). The absorbance was measured at 492 nm with a microplate spectrophotometer (Bio-Rad, Hemel Hempstead, United Kingdom). The nomenclature of gangliosides is based on that of Svennerholm (1964).

Animal Experimental Protocol

Immunization was performed according to our previously described procedure (Usuki et al., 2006b) and is shown in Figure 2. Twelve-week-old female Lewis rats weighing 200–250 g were used. One hundred micrograms LOS_{GD3} was dissolved in 50 mM PBS buffer with a vehicle of 0.05 ml keyhole limpet hemocyanin (KLH; 2 mg/ml) and emulsified with an equal volume of complete Freund's adjuvant (CFA). Rats were given a single subcutaneous injection of 0.1 ml inoculum (or vehicle) into the shoulders and the footpads of the hind limbs. In addition, booster injections were administered similarly to injections in LOS_{GD3} (or vehicle)-treated animals at 2-week intervals with 100 µg LOS_{GD3} (or no additive) and 2 mg/ml KLH in PBS emulsified with an equal volume of incomplete Freund's adjuvant (ICFA) 6 weeks thereafter. Sixteen experimental animals were divided into four groups (n = 4 each): 1) LOS_{GD3}, 2) LOS_{GD3}/BEC2, 3) vehicle, and 4) vehicle/BEC2. The remaining animals were included as the untreated control group (n = 4). As shown in the third and fourth stepladders of Figure 2, BEC2 (5 mg kg⁻¹) was administered to rats once per week from 8 to 15 weeks.

Before injection of BEC2 to the animals, any endotoxin included in the BEC2 was removed by ToxinEraser Endotoxin Removal Kit (GenScript Corp., Piscataway, NJ), and the endotoxin remaining after the passages was detected by the *Limulus* amoebocyte lysate (LAL) test (ToxinSensor™ Gel Clot Endotoxin Assay Kit; GenScript Corp.).

The blood samples were drawn retroorbitally by bleeding with a capillary tube at 0, 4, 8, 12, 16, and 18 weeks after the primary immunization. All experimental animals were weighed and assessed for clinical signs of peripheral nerve abnormalities. Electrophysiological measurements of the rats' nerve conduction velocity (NCV) were made at 0 and 16 weeks, with a rotarod test at 17 weeks after the primary immunization. All animals were allowed free access to water and food. The use of these animals had been approved by the Medical College of Georgia's Institutional Animal Care and Use Committee. Treatment of these animals was performed according to approved procedures.

NCVs

NCVs (m/sec) were measured in the rat tail nerve using a Nicolet VikingQuest EMG machine (NeuroCare Group, Madison, WI) according to the modified procedure of Andersen et al. (1994). In brief, the nerves were stimulated using external digital ring electrodes with twisted wires (Medtronic Functional Diagnostics, Skovlunde, Denmark) instead of needle electrodes (Usuki et al., 2006b). The electrodes were placed in segments proximal (5 cm) and distal (2 cm) to the recording position (7 cm from the rat tail joint). NCV was evaluated from four different waves generated from electrical stimulations; each wave showed a reproducible pattern and also showed the same amplitude level as the stimulator voltage was increased. During measurement, a constant surface temperature of the rat tail was maintained at 34–35°C. Each NCV value represents an average value of four nerve conduction wave measurements per animal.

Rotarod Motor Test

The rotarod motor test was performed according to the procedure of Geralai et al. (Dunham and Miya, 1957; Jones and Roberts, 1968). A rat was placed on a rotating roller (4 cm in diameter), and the time for which the rat remained on the roller was measured. The test consisted of eight trials; each trial was performed at an interval of 10 min; four trials were performed. An additional four trials were conducted after 24 hr. When the rat could stay on the rotating rod at a constant speed of 5 rpm for a cumulative duration of at least 2 sec, the rotation speed was increased by 0.2 rpm/sec.

NMJ Activity

Spinal cord–muscle coculture was performed according to the method of Taguchi et al. (2004). Briefly, muscle and spinal cord explants cells were prepared from muscle and spinal cord tissues (containing dorsal root ganglia), respectively, of 17-day-old fetal rats. Muscle cells and spinal cord explants were cocultured and maintained for up to 1 week in the medium (67% Dulbecco's modified Eagle's medium and 23% medium 199) containing 10% fetal calf serum supplemented with 25 ng/ml fibroblast growth factor and 20 µg/ml insulin. Functional NMJ formation was observed by an inverted microscope (IX-70; Olympus, Tokyo, Japan) with an experimental chamber on the stage. The preparation was perfused continuously with the medium at a rate of 1–2 ml/min. Spontaneous muscle action potential frequency was recorded by a glass microelectrode (Ag/AgCl, 30–40 MΩ) and a recording electrode (3 M KCl). The recording system consisted of a Microelectrode Amplifier MEZ-8301, Memory Oscilloscope VC-11 (Nihon Kohden, Tokyo, Japan), and an A-D Converter DigiData 1200 Interface (Axon Instruments, Union City, CA). The spontaneous muscle action potential was low-pass filtered at 1 kHz. An antiserum solution, 10 µl, was delivered directly near the innervated muscle cells with a micropipette.

Histological/Morphological Examination

Pathological examination was undertaken to correlate changes with clinical manifestations in the animals. At the endpoint (week 18) of animal experimentation (Fig. 2), the animals were sacrificed. After a blood sample was withdrawn from the heart, the animals were perfused with 4% paraformaldehyde in PBS buffer via the arcus aortae. After perfusion, the lumbar spinal cord was dissected and sectioned into four to six transverse segments spaced 1 mm apart. The right sciatic nerves also were carefully dissected from their origin (5 mm distal to the gluteus maximus) through the distal branch point at the peroneal and tibial nerves in order to avoid stretching. These nerve sections were placed at 4°C overnight in a fixative solution containing 4% paraformaldehyde, 2% glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.4). After the nerves were washed three times in cacodylate buffer (pH 7.4), they were postfixed at 4°C with 2% osmium tetroxide/0.1 M sodium cacodylate buffer (pH 7.4) for 60 min, dehydrated in graded ethanol, stained at 4°C with 2% uranyl acetate/70% ethanol for 30 min, and embedded in epoxy resin (Poly/Bed 812; Polysciences, Warrington, PA). One-micrometer-thick cross-sections of nerve were stained with toluidine blue for histological examination using an Axiophot photomicroscope equipped with an Axiocam (Carl Zeiss, Jena, Germany). Images were stored and analyzed using AxioVision. The total number of myelinated fibers in each nerve was assessed by visual counting. Myelination-to-myelinated area ratio was calculated in Scion Image software. The total fiber area and the total myelin area were masked and automatically quantitated by the program. In total five images were analyzed per cross-section, and the percentage of myelinated area was determined by using the following formula: myelin % = (myelinated area/total area) × 100. For electron microscopy, ultrathin sections were prepared and examined by a high-performance, high-contrast, 40–120-kV transmission electron microscope (JEOL JEM-1230).

Immunofluorescence Analysis of NMJ

Adult rats were anesthetized with pentobarbital and then perfused with 2% paraformaldehyde in 0.01 M PBS buffer. Diaphragms were excised and frozen in liquid nitrogen. Cryosections (10 µm) were double immunostained with mAb R24 and antineurofilament polyclonal rabbit antibody (50–100 µg/ml) in a blocking buffer containing 10% BlockAce (Dainippon Pharmaceutical, Osaka, Japan) in normal goat serum at 50–100 µg/ml for 30 min at room temperature and developed with the following secondary antibodies for 60 min at 4°C: anti-mouse IgG conjugated to Alexa Fluor 488 (1:20; Sigma) and anti-rabbit IgG conjugated to Alexa Fluor 350 (1:100; Vector, Burlingame, CA). Motor end-plates were simultaneously labeled with α -bungarotoxin conjugated to Alexa Fluor 594 (0.5 µg/ml; Molecular Probes, Eugene, OR). Immunostained sections were mounted on slides, covered with microslips, and observed with an Olympus laser scanning confocal microscope (Fluoview BX50; Olympus) at a wavelength of 488 nm or 543 nm.

Statistical Analysis

Statistical analyses were performed in the GraphPad Prism 2.01 software package (GraphPad, San Diego, CA). One-way ANOVA was performed for data from the experimental animal groups, followed by Tukey's multiple-comparisons test. Differences in the control group vs. the LOS_{GD3}-treated group were analyzed by Dunnett's multiple-comparisons test.

RESULTS

Purification of LOS_{GD3} From the LOS Fraction of *C. jejuni*

LOS_{GD3} was purified from a cell pellet of *C. jejuni*, strain HS19 (30.5 g wet weight) according to the procedure outlined in Figure 1. After silica gel column chromatography, a total of 1.72 g of the LS fraction was recovered in fractions 3 and 4. The crude LS fractions were combined and further fractionated by an mAb R24 affinity column. Each of the column fractions from the silica gel and the mAb R24 affinity chromatography was examined by TLC-immuno-overlay with mAb R24 and CTxb (Fig. 3). LOS_{GD3} was associated with LOS_{GM1}, as shown in fractions 3 and 4 of the silica gel column as revealed on the TLC-immuno-overlay plate (Fig. 3A, lanes 4 and 5). mAb R24 affinity chromatography successfully allowed elution of LOS_{GD3} into fractions 7 and 8 (Fig. 1) and separation of LOS_{GD3} from LOS_{GM1} (Fig. 3B, lanes 9 and 10; CTxb overlay). Finally, 140 mg LOS_{GD3} was purified and used for further experiments.

Competitive Inhibition Assay

BEC2 was tested for competitive inhibition by ELISA. BEC2 was inhibitory at an IC₅₀ of 10 µg/ml, and there was full inhibition at 60 µg/ml (Fig. 4A).

Pharmacokinetic Study

Dosage schedule of BEC2 for animal experiments was designed by pharmacokinetic analysis based on a single dose to the animal. BEC2 was successfully biotinylated. The specific activity for biotin labeling was determined as 0.9 for bBEC2 (mole of biotin per mole of protein).

The purified bBEC2 was administered via i.p. to animals as described in Materials and Methods. The plasma concentration vs. time data resulting from single doses of 1, 5, and 10 mg kg⁻¹ for BEC2 are plotted in Figure 4B. None of the experimental rats died or experienced any observable toxicity as a result of the drug administration.

Table I lists the parameter values obtained from a noncompartmental analysis of bBEC2. The concentration vs. time data showed a two-phase attenuation of distribution and elimination expressed as $t_{1/2\alpha}$ and $t_{1/2\beta}$. There was no change in parameter values for clearance, $t_{1/2\alpha}$, or $t_{1/2\beta}$ in the single-dose range of 1.0–10.0 mg kg⁻¹ for bBEC2. According to linearity parameters, bBEC2 exhibits linear pharmacokinetics after multiple doses. In consideration of $t_{1/2\beta}$ and minimum effective plasma concentrations from a single-dose data point, we adopted chronic i.p. administration of 5 mg kg⁻¹ bBEC2 (Fig. 4B). We processed the dosing simulation by multiple (5 mg kg⁻¹ once weekly for 8 weeks) i.p. dose administration of bBEC2 (Fig. 4C). The results derived from the multiple-dosing simulation suggested that average and minimum concentrations were 87 µg/ml and 50 µg/ml of BEC2, respectively.

Before administration, the BEC2 was subjected to purification by passing through an endotoxin removal column. The final endotoxin level was below 0.1 EU/ml after three or four repeated endotoxin-removal processes.

Experimental Animals

Consistent with our previous findings (Usuki et al., 2006b), the LOS_{GD3} treatment showed mild clinical signs of neurological dysfunctions, including a remarkable slowness of motion and noticeable weight loss during 13–18 weeks compared with vehicle treatment. Despite a significant lowering of NCVs, the LOS_{GD3}-treated rats did not show any flaccid limb paralysis or standing still during the same period. However, they were judged as having mild

nerve dysfunctions from their rotarod performance. Furthermore, nerve dysfunctions were substantiated by pathological examination with loss of myelin in myelinated fibers (see below). At the endpoint of the experiment, all animals were sacrificed and dissected. There was no evidence of adhesive peritonitis or abdominal dropsy resulting from infection in animals with administration by multiple i.p. doses of BEC2.

Time Course of Anti-GD3 and Anti-LOS_{GD3} Antibody Production

Anti-GD3 and anti-LOS_{GD3} antibody responses for sensitization by LOS_{GD3} were examined chronically by testing for ELISA absorbance of rat serum samples (Fig. 5). Antibodies for GM1, GM2, GD1a, GD1b, GT1b, and GQ1b were not detected in these sera during the entire course of the animal experiment (data not shown). The anti-GD3 IgG antibody titer was elevated 8 weeks postinoculation, in parallel with elevation of the anti-LOS_{GD3} antibody titer. The anti-GD3 IgG antibody titer was maintained at a plateau during 8–12 weeks and decreased slightly after 12–16 weeks (Fig. 5A). The titer of serum anti-GD3 antibody was elevated (8–12 weeks; Fig. 5A), and this period was involved with BEC2 treatment, which extended to 15 weeks (Fig. 2, bottom stepladder). There were no IgG antibody responses for GD3 in the vehicle-treated group or the untreated group (Fig. 5B). The LOS_{GD3}/BEC2 group showed a remarkable suppression of anti-LOS_{GD3} and anti-GD3 antibodies after chronic treatment with BEC2 (Fig. 5C), whereas vehicle/BEC2 treatment was ineffective (Fig. 5D).

NCV Changes

NCVs were measured at the beginning and the endpoint according to the experimental schedule of treatments. As shown in Figure 6A, according to the treatment schedule, control rats showed increased NCV within the experimental period, presumably resulting from an age-related change (Birren and Wall, 1956). At 16 weeks, the NCV of the group sensitized by LOS_{GD3} decreased significantly compared with the vehicle group ($P < 0.01$). BEC2 showed improved relative attenuation of NCV, and a statistically significant difference was observed between LOS_{GD3} and LOS_{GD3}/BEC2. No effect on NCV, other than the age-dependent change, was observed for animals treated with vehicle or BEC2 alone (Fig. 6A).

Rotarod Test

Animals underwent a rotarod test at the endpoint according to the experimental schedule of treatments. There was no statistically significant difference in retention time between the vehicle and untreated control groups (Fig. 6B). LOS_{GD3}-treated animals had muscle weakness, with significantly shorter retention in all the experimental rats ($P < 0.01$). There was a statistically significant difference in improved rotarod performance between the BEC2 treatment group and the LOS_{GD3} treatment group.

Morphological Analysis of Motor Neurons and Motor Axons

We examined animals for structural alterations and pathological changes of the motor spinal cord neurons and sciatic nerves in LOS_{GD3}-treated rats. Within the lumbar spinal cord, there were no overt structural differences between the anterior horn cells in the untreated control group and the LOS_{GD3}-treated rats (Fig. 7Aa,b). LOS treatment resulted in changes in toluidine blue staining, which revealed myelin. The sciatic nerve change was detected by EM analysis. Treatment with BEC2 caused clear improvement in demyelination based on morphological analysis (data not shown). Profile counts (numbers of large cells in the anterior horn/cross-section) indicated similar densities of lumbar motor neurons between the control (nontreatment) and the LOS_{GD3}-treated rats (31 ± 5 vs. 34 ± 4 neurons/section, $N = 4$, respectively). There was no difference between the control and the LOS_{GD3}-treated rats in the neuronal profile area ($302 \pm 15 \mu\text{m}^2$ vs. $298 \pm 21 \mu\text{m}^2$, $N = 4$, respectively).

To assess for morphologic alterations corresponding to motor dysfunction or muscle weakness evaluated by NCV measurement and rotarod tests, we examined distal motor nerves near the tibial branch in the sciatic nerve. This nerve contains predominantly myelinated motor fibers that serve skeletal muscle fibers of the lower distal leg. There was a noticeable change in myelin thickness and a statistically significant difference between the control and the LOS_{GD3}-treated groups in the percentage of myelinated area in the myelinated fiber (control, $36 \pm 5.2\%$; LOS_{GD3}, $12.8 \pm 4.8\%$; $P < 0.01$; Fig. 7Ac–f).

NMJ immunostaining

As shown in Figure 7, mAb R24 (anti-GD3) immunostained motor nerve terminals. GD3 was localized in the presynaptic area of the neurons together with NF (Fig. 7Ba,c), and the staining of α -bungarotoxin was localized in postsynaptic membrane (Fig. 7Bb). Despite a generally diffuse staining of the presynaptic area by anti-GD3, a condensed localization of GD3 at the NMJ was shown in the white-colored segment, which was costained by α -bungarotoxin, anti-NF, and anti-GD3 (Fig. 7Bd).

Inhibition of NMJ Activity

To determine the nature of the anti-GD3 antibody produced in LOS_{GD3}-sensitized rats with neuromuscular weakness, spontaneous muscle action potential frequencies were examined by addition of the serum to a coculture system of spinal cord–muscle cells. Those treated with LOS_{GD3} showed a strong blockade of NMJ action potential frequencies immediately after addition of the serum (Fig. 8A). This inhibitory activity, however, could be abolished from the serum after immunoabsorption with GD3 as shown by LOS_{GD3} (+absorption; Fig. 8B). The LOS_{GD3}/BEC2 serum showed an immediate strong blockade of NMJ action potential frequencies, but the blockade was reversibly removed by the washing-out procedure, and the NMJ activity recovered (Fig. 8C).

DISCUSSION

Campylobacteriosis is a frequent antecedent event in GBS and induces high titers of serum antibodies for glycolipid antigens of the peripheral nervous system (Ariga and Yu, 2005; Kaida et al., 2009). *Campylobacter*-induced hyperimmunity incites an autoimmune response that facilitates the production of autoantibodies by host factors and bacterial components such as Toll-like receptors (TLRs) and lipid A. Studies on TLR polymorphism in GBS, however, have failed to show a direct correlation between the two (Geleijns et al., 2004, 2005). On the other hand, activation of TLR2 has been shown to be involved in a rat experimental autoimmune neuritis (Zhang et al., 2009). TLRs recognize bacterial components and activate various signaling pathways, some of which may lead to subsequent initiation of the autoimmune response, such as the neuromuscular dysfunctions of GBS.

The etiology of GBS is likely complex, and the disease is manifested differently clinically (Rinaldi and Willison, 2008; Kaida et al., 2009). One of the characteristic features, however, is the elevated antiglycolipid antibody titers in circulation. Currently, treatment strategies include plasmapheresis, intravenous administration of immunoglobulins (IVIG; Kieseier et al., 2008), and antiinflammatory steroids. Those treatments can ameliorate and attenuate GBS in patients by removing high-titer antiglycolipid antibodies but are invasive and frequently ineffective. All those treatment strategies affect pathogenic antibodies but also those that are beneficial. IVIG has the potential risk of causing anaphylactic shock, renal failure, virus infection, and thromboembolism (Dalakas, 2004b). For those reasons, it would be desirable to eliminate or reduce only those pathogenic serum antiglycolipid antibodies. We have used an agent that interferes with the binding of the pathogenic antibodies to tissue

antigens and thereby directly neutralizes the neurotoxicity of circulating antiglycolipid antibodies.

We have established a disease model by sensitizing rats with injections of crude LOS fractions from a strain HS:19 of *C. jejuni* (Usuki et al., 2006b). The rats develop high-titer serum polyclonal antiganglioside antibodies and subsequent muscle weakness. The serum causes in vitro inhibition of depolarization at the NMJ junction, i.e., reduction of spontaneous muscle action potentials (Goodyear et al., 1999; Buchwald et al., 2002, 2007; Taguchi et al., 2004). In the present investigation, we found that anti-GD3 antibodies could be induced as reflected by anti-LOS_{GD3} production in rats sensitized by LOS_{GD3}. The anti-GD3 antibody is thought to be produced via a cross-reacting epitope structure of LOS_{GD3}. Then, we used an anti-idiotypic antibody, BEC2, to treat our rat model bearing anti-GD3 antibody, anticipating that it would react directly with the circulating antibody. It should be noted that the anti-idiotypic antibody, BEC2, has been employed to mount humoral- and tumor-protective immune responses in patients and animals with melanoma (Chapman et al., 2004). For the immunotherapy of melanoma, the GD3 mimic elicited an anti-GD3 antibody production that was followed by an attack on melanoma cells via an anti-GD3 antibody interaction with the GD3 antigen on the cell surface of melanoma (Chapman, 2003). In the current study, our animal model already has been developed with high-titer anti-GD3 antibodies. We anticipated that the antibodies could be inhibited by a specific GD3 mimic. Although GD3 itself should also be effective for neutralizing the anti-GD3 antibody titer, it was not used for a number of reasons. First, GD3 is cytotoxic in high concentrations. Second, it is difficult to prepare in pure form in sufficient quantities for clinical use. Third, as an antigen, it may induce adverse immune responses; thus, it may actually exacerbate GBS.

The LOS fraction bears GD3- and GM1-like carbohydrate epitopes, and we were successful in separating LOS_{GD3} from LOS_{GM1} and using the highly purified LOS_{GD3} as the immunogen (Usuki et al., 2006b). Analysis of the oligosaccharide portion of LOS_{GD3} confirmed the presence of a GD3-like epitope with the following tetrasaccharide structure: NeuAc–NeuAc–Gal–Hep (Usuki et al., 2006b). Our results bear out the expectation that LOS_{GD3} induces neuromuscular weakness and generates NMJ-inhibitory anti-GD3 antibodies in the serum of experimental animals (Fig. 8A). BEC2 proved to be a potent inhibitor for anti-GD3 with IC₅₀ values (10 µg/ml) as shown by competitive inhibition ELISA (Fig. 4A).

For pharmacokinetic analysis of BEC2, chronic animal experiments were designed to determine IC₅₀ values and effective plasma concentrations over a chronic course. A BEC2 response in rats was readily obtained, likely because of the long half-life ($t_{1/2\beta}$) in circulation and the accompanying maintenance of an effective serum concentration of BEC2. Thus, BEC2 was proved effective in “neutralizing” the activity of anti-GD3 antibodies, thus inhibiting its pathophysiological effects.

In the present investigation, we found motor axonal degeneration but no motor neuronal degeneration in the sciatic nerve of rats sensitized by LOS_{GD3} (Fig. 7Ad,f), suggesting that neuromuscular weakness is caused by structural deterioration of the distal nerve, NMJ, and myelin. A plausible reason is that LOS_{GD3} stimulates the TLR-related inflammatory system, leading to the release of inflammatory cytokines and blood–nerve barrier injury. LOS_{GD3} also serves as an immunogen that elicits anti-GD3 antibody; the circulating antibody leaks and penetrates to the blood–nerve barrier of the peripheral nerve microvasculature and enters the endoneurial space as well as the NMJ. Anti-GD3 antibody inhibits spontaneous muscle action potentials in an in vitro NMJ system, as shown in Figure 8A and in our previous reports (Usuki et al., 2005, 2006b). In our present study, it takes a few minutes

before the NMJ action potential frequency is blocked after application of these serum samples. This time delay may reflect the antibody-diffusion time or the antibody-inducing trans-signaling time necessary to reveal a functional blockade of NMJs. Immunoabsorption by GD3 removed the blocking activity included in serum, showing instead a gradual decrease in residual blockade (Fig. 8B). Moreover, BEC2 treatment still leaves the blocking activity intact before washing out (Fig. 8C). In consideration of these data, we are mindful of the evidence that anti-LOS_{GD3}/GD3 antibody is implicated as a polyclonal antibody in the rat serum and BEC2 is a monoclonal antibody. A small portion of the anti-LOS_{GD3}/GD3 antibody may remain in the serum even after immunoabsorption by GD3 or after BEC2 treatment. This residual antibody may have low reactivity to LOS_{GD3} or GD3, which accounts for the functional blockade of NMJs. Another possibility is that anti-lipid A antibody activity was also generated in the rat by LOS_{GD3} immunization as a result of the presence of bacterial adjuvant in the immunogen. The anti-lipid A activity is causing the functional blockade of NMJs, as seems possible from a previous study (Usuki et al., 2006b, 2008). This anti-lipid A antibody activity would be expected to remain in the serum even after immunoabsorption with gangliosides (Usuki et al., 2006a).

By confocal imaging, most of the GD3 immunopositivities are diffusely localized in the presynaptic axonal area by the involvement of Schwann cells (Ogawa-Goto et al., 1992; Ribeiro-Resende et al., 2007). Some of the GD3 immunoreactivities, however, are condensed in a restricted area on the NMJs (Fig. 7Bd). Collectively, these findings indicate that distal nerve degeneration in the LOS_{GD3}-treated rats occurs at NMJs without motor neuron cell death in the spinal cord.

In conclusion, we have established a rat model of peripheral nerve dysfunction induced by antiganglioside antibodies via sensitization by the LOS of *C. jejuni*. We have shown further that it is possible to utilize an antiidiotype antibody design, on the basis of molecular mimicry, to ameliorate the neurological dysfunction in this animal model. The model demonstrates the feasibility of inhibition of monospecific antibodies, which could serve as a prototype for similar therapeutic strategies to treat GBS effectively. This work thus opens up the possibility of novel treatments for GBS, for which at present there is no effective treatment.

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Fig. 1.

Isolation scheme for LOS_{GD3} from crude LOS fraction. The steps involved in the isolation and purification of LOS_{GD3} are shown. For details of each step, see Materials and Methods. LS, a major LOS component characterized as a slow-migrating band based on the TLC of CTxb overlay; LF, a minor LOS component characterized as a fast-migrating band based on the TLC of CTxb overlay. Fractions 1–4: Stepwise elution from a silica gel column with the following solvents: 1) n-propanol, 2) n-propanol:H₂O (75:20 v/v), 3) n-propanol: H₂O:triethylamine (75:20:5 v/v/v), and 4) n-propanol: H₂O:triethylamine (60:20:20 v/v/v). Fractions 5–8: Stepwise eluting from an mAb R24 affinity column with the following solvents: 5) washing solution 1 (10 mol of 1 and 10 mM PBS), 6) washing solution 2 (50 mM PBS), 7) eluting solution 1 (5 ml of 50 mM diethylamine/HCl buffer, pH 8.6, containing 0.1 M NaCl), and 8) eluting solution 2 (5 ml of 50 mM diethylamine/HCl buffer, pH 10.5, containing 0.1 M NaCl).

Experimental schedule for animal treatments

Fig. 2.

Experimental schedule of animal treatments. The timing of treatment and performance is shown between 0 and 18 weeks in the experimental period. Sixteen experimental animals were divided into three inoculum-treated groups ($n = 4$, each): 1) LOS_{GD3} , 2) $LOS_{GD3}/BEC2$, 3) vehicle, and 4) vehicle/ $BEC2$. The remaining four animals were included as the untreated control group ($n = 4$).

Fig. 3.

TLC of purified LOS_{GD3} from silica gel column and mAb R24 affinity column chromatography. **A:** LOS_{GD3} was detected by TLC overlay at 10 μg/ml of mAb R24 IgG; lane 1, GD3 standard; lane 2, fraction 1; lane 3, fraction 2; lane 4, fraction 3; lane 5, fraction 4, lane 6, GD3 standard; lane 7, fraction 5; lane 8, fraction 6; lane 9, fraction 7; lane 10, fraction 8; lane 11, GD3 standard; lane 12, purified LOS_{GD3}. **B:** LS and LF were detected by TLC overlay at 1:1,000 dilution of CTxb; lane 1, GM1 standard; lane 2, fraction 1; lane 3, fraction 2; lane 4, fraction 3; lane 5, fraction 4, lane 6, GD3 standard; lane 7, fraction 5; lane 8, fraction 6; lane 9, fraction 7; lane 10, fraction 8; lane 11, GM1 standard; lane 12, purified LOS_{GD3}. Fractions 1–8 are shown in Figure 1.

Fig. 4.

Dose-dependent inhibition of binding of mAb R24 to GD3 by BEC2. **A:** BEC2 was first incubated with MAb R24 at room temperature for 30 min at various concentrations as indicated on the horizontal axis. Subsequently, an aliquot (100 μl) was added to each well, and each well of the ELISA plate was coated with 0.1 μg GD3. The remaining binding activity of mAb R24 was determined by ELISA, as shown in Materials and Methods. Values are means $\pm$ SD, $n = 6$ individual experiments. **B:** Mean plasma concentration–time profiles following i.p. single dosing with 1 mg kg⁻¹, 5 mg kg⁻¹, or 10 mg kg⁻¹ bBEC2. The experiment at each dose was performed on three rats, and values are means $\pm$ SD. **C:** Simulation of concentration–time course of 5 mg kg⁻¹ bBEC2 once per week for 8 weeks. The simulation was performed with values of $t_{1/2\beta}$ and AUC for 5 mg kg⁻¹ bBEC2, as shown in Table I.

Fig. 5.

A–D: Changes of serum level of the anti-GD3 antibody and the anti-LOS_{GD3} antibody of rats. According to the experimental schedule shown in Figure 2, animal serum samples were obtained at the specified times to test activities of anti-GD3 antibody (solid circles) and anti-LOS GD3 antibody (open circles) using ELISA. Values are means $\pm$ SD for four animals.

Fig. 6.

Effect of BEC2 on NCV and rotarod performance of animals undergoing treatment. **A:** Effect of BEC2 on NCV at week 0 and week 16 after primary immunization by LOS_{GD3}. NCVs were measured at week 0 and at week 16 for each group of animals; LOS_{GD3}, LOS_{GD3}/BEC2, vehicle, vehicle/BEC2, and control (nontreatment). Values are means $\pm$ SD for four animals. The values were analyzed by one-way ANOVA followed by Tukey's multiple-comparison tests (<0.01). The asterisk denotes statistical significance ($*P < 0.01$) estimated by Dunnett's multiple-comparisons test. **B:** Effect of BEC2 on motor performance on the rotarod test. At 17 weeks after primary immunization, the rotarod test was performed for five experimental animal groups: LOS_{GD3}, LOS_{GD3}/BEC2, vehicle, vehicle/BEC2, and control (nontreatment). Values are means $\pm$ SD for four animals. The data values were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test (<0.01). The asterisk denotes statistical significance by Dunnett's multiple-comparisons test ($*P < 0.01$).

Fig. 7.

A: Examples of toluidine blue-stained lumbar motor neurons (a,b) and sciatic nerves (c,d). a and c are from a control rat (non-treatment); b and d are from an LOS_{GD3}-treated rat. EM is from sciatic nerves from a control rat (e) and an LOS_{GD3}-treated rat (f). Arrows show the large cells in the anterior horn. **B:** Immunofluorescence imaging of NMJ was performed by following primary antibodies and BTx. mAb R24 (a), BTx (b), or NF (c), each diluted appropriately by 1% BSA/PBS buffer containing 0.1% Triton X-100 at 4°C, and (d) merging of R24 mAb and BTx images. Scale bars = 100 μm in Aa,b; 10 μm in Ac,d; 2 μm in Ae,f.

Fig. 8.

Time course of inhibitory effect on spontaneous muscle action potential frequencies in spinal cord-muscle cocultured cells. The frequencies were recorded every 5 sec. Arrows show addition of 10 μ l rat serum and washing out. Each serum sample was obtained from four rats, in three groups. **A:** LOS_{GD3} . **B:** LOS_{GD3} serum as shown in A was subjected to immunoabsorption by GD3. **C:** $LOS_{GD3}/BEC2$ at the endpoint of the experiment. One of the four sera was used for addition to the assay system for spontaneous muscle action potential activity. This absorbed serum (B) was examined in the assay system for spontaneous muscle action potential activity.

TABLE I

Pharmacokinetic Parameters After i.p. Administration of BEC2 to Normal Lewis Rats

Parameter		bBEC2 dose group		
		n = 3	n = 3	n = 3
Dose	(mg kg ⁻¹)	1.0	5.0	10.0
t _{max}	(hr)	3.0	3.0	3.0
C _{max}	(µg ml ⁻¹)	15.5 (0.53)	74.7 (4.7)	141.4 (9.5)
t _{1/2α}	(hr)	7.3 (1.4)	7.1 (1.0)	7.5 (0.2)
t _{1/2β}	(hr)	169.3 (8.3)	161.3 (6.4)	167.8 (6.1)
CL	(ml hr ⁻¹ kg ⁻¹)	0.37 (0.06)	0.47 (0.06)	0.46 (0.04)
Vdss	(ml)	ND	ND	ND
AUC	(µg ml h ⁻¹ kg ⁻¹)	2,757 (434)	10,750 (1,329)	22,940 (2,041)

The values are the means ± (SD).

ND, not determined.

Vdss, steady-state volume of distribution.