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Topology and Patch-clamp Analysis of the Sodium Channel in Relationship to the Anti-Lipid A Antibody in Campylobacteriosis

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Abstract

An infecting strain VLA2/18 of *Campylobacter jejuni* was obtained from an individual with campylobacteriosis and used to prepare chicken sera by experimental infection to investigate the role of serum anti-ganglioside antibodies in Guillain-Barré syndrome. Both sera of the patient and chicken contained anti-ganglioside antibodies and anti-Lipid A (anti-Kdo2-Lipid A) antibodies directed against the lipid A portion of the bacterial lipooligosaccharide. The anti-Kdo2-Lipid A activities inhibited voltage-gated Na (Nav) channel of NSC-34 cells in culture. We hypothesized that anti-Kdo2-Lipid A antibody acts on the functional inhibition of Nav1.4. To test this possibility, a rabbit peptide antibody (anti-Nav1.4 pAb) against a 19-mer peptide (KELKDNHILNHVGLTDGPR) on the α subunit of Nav1.4 was produced. Anti-Nav1.4 pAb was cross-reactive to Kdo2-Lipid A. Anti-Kdo2-lipid A antibody activity in the chicken serum was tested for the Na⁺ current inhibition in NSC-34 cells in combination with μ -Conotoxin and tetrodotoxin. Contrary to our expectations, the anti-Kdo2-Lipid A antibody activity was extended to Nav channels other than Nav1.4. By overlapping structural analysis, it was found that there might be multiple peptide epitopes containing certain dipeptides showing a structural similarity with v-Lipid A. Thus, our study suggests the possibility that there are multiple epitopic peptides on the extracellular domains of Nav1.1 to 1.9, and some of them may represent target sites for anti-Kdo2-Lipid A antibody, to induce neurophysiological changes in GBS by disrupting the normal function of the Nav channels.

Keywords

ganglioside; sodium channel; *Campylobacter jejuni*; Guillain-Barré syndrome; lipooligosaccharide; chicken

Bacterial infection, especially from *Campylobacter jejuni* (*C. jejuni*), is a frequent antecedent event in peripheral neuropathy (Hughes et al., 1999; Yu et al., 2006). Interspecies transmission of *C. jejuni* from chicken to human can occur, resulting occasionally in the development of Guillain-Barré syndrome (GBS) (Li et al., 1996; Newell and Wagenaar, 2000). Recently, we isolated a strain of *C. jejuni* (VLA2/18) from a patient who had developed high-titer serum anti-GM1 antibodies (Usuki et al., 2006a), but did not subsequently develop clinically distinct

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GBS. Thus, this represents a unique case of interspecies transmission in which the patient suffered only severe gastroenteritis without neuritis, though the patient serum contained a high titer of anti-GM1. In a previous report, we found high titers of anti-GM1 and anti-GD1a polyclonal antibodies in rabbits immunized with purified antigens; however, they too lacked an apparent neurological disability (Dasgupta et al., 2004). Anti-GM1 antibody was also detected in chickens subjected to experimental infection with strain VLA2/18 (Usuki et al., 2006a). However, this chicken antibody was demonstrated to induce an inhibitory effect of neuromuscular junctions using an in vitro system of spinal cord–muscle coculture (Taguchi et al., 2004; Usuki et al., 2005, 2006a). The present study investigated the antibody effect on voltage-gated ion channels. Inhibition of Na⁺ currents by anti-GM1 has been shown in isolated myelinated rat nerve fibers (Hartung et al., 1995; Hirota et al., 1997; Benatar et al., 1999; Paparounas et al., 1999; Susuki et al., 2007; Takigawa et al., 1995).

Molecular mimicry of carbohydrate structures between GM1 and the O-antigen of lipooligosaccharide (LOS) is well known as a mechanism of antibody-mediated neuropathies (Aspinall et al., 1992, 1994; Yuki et al., 1993, 2004). Little is known about the other antigenic determinants of the LOS, e.g., Kdo and Lipid A. Surprisingly, we found anti-Kdo2-Lipid A antibodies in these chicken and human sera in addition to anti-ganglioside antibodies. These sera showed a strong depression of Na⁺ currents. This effect may be due to a Kdo2-Lipid A-like epitope of the Nav channel protein. The results suggest a novel molecular mimicry between Kdo2-Lipid A and a particular peptide portion of Nav channel protein, which can contribute to the pathophysiology of GBS-like disorders.

In 9 gene subfamilies of Nav, Nav1.2 and Nav1.6 are relevant to peripheral nervous system (PNS) remyelination (Schafer et al., 2006). On the contrary, Nav1.4 is generally known to be expressed in skeletal muscles, although we recently found expression of functional Nav1.4 protein in one of the motor neuron-like cell lines, NSC-34. We hypothesized that Nav 1.4 might be an important target for anti-Kdo2-Lipid A antibody. To test this hypothesis, we generated a polyclonal rabbit antibody for a 19-mer peptide that is unique in the Nav1.4 channel protein and that possesses mimicry with Kdo2-Lipid A. This antibody was tested for its cross-reactivity between this peptide portion in the Nav1.4 channel and Kdo2-Lipid A. Anti-Kdo2-Lipid A antibody was also examined using a specific inhibitor for Nav1.4, μ -Conotoxin (μ -Conx).

MATERIALS AND METHODS

Materials

The following items were purchased: Dulbecco modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY); fetal bovine serum (Roche, Mannheim, Germany); high-performance thin-layer chromatographic (HPTLC) plates coated with silica gel 60 (E. Merck, Darmstadt, Germany); o-phenylenediamine dihydrochloride tablet set (OPD Peroxidase Substrate, Sigma, St. Louis, MO); complete Freund's adjuvant (CFA, Sigma); incomplete Freund's adjuvant (IFA, Sigma); Lipid A, diphosphoryl (LPA2, from *Escherichia coli* F583, Rd mutant, Sigma); Lipid A, monophosphoryl (LPA1, from *E. coli* F583, Rd mutant, Sigma); tetrodotoxin (TTX, from Sigma); μ -conotoxin GIIIB (μ -Conx, from Almone Labs, Jerusalem, Israel); Kdo2-Lipid A (Avanti Polar Lipids Inc., Alabaster, AL); and affinity-purified anti-Nav1.6 and -Nav1.7 rabbit antibodies (Almone Labs, Jerusalem, Israel). Anti-GD3 mouse monoclonal antibodies (mAbs) R24 (IgG) and DMAb-8 (IgM) antibodies were from the conditioned medium of hybridoma R24 (Knuth et al., 1984) and DMAb-8 (Hedberg et al., 2000), respectively. Authentic ganglioside standards were prepared from bovine brain as previously described (Ledeen and Yu, 1982). The nomenclature of gangliosides is based on that of Svennerholm (1964).

Antibodies

C. jejuni strain VLA 2/18 (HS type unknown) was isolated from a United Kingdom laboratory worker who became ill with diarrhea. The *C. jejuni* strain was recovered from a fecal swab obtained 1 day after the onset of stomach cramps. Blood samples were taken on that day (day 1) and again on day 22 after isolation (Table I), and serum was isolated and stored frozen. To obtain chicken sera for comparison, the strain was used to colonize chicken in an oral model according to the procedure of Wassenaar et al. (1993). Briefly, 1-day-old specific-pathogen-free chickens (Lohmann Animal Health, Cuxhaven, Germany) were dosed by oral gavage with approximately 10^6 CFU of *C. jejuni* strain VLA 2/18. The colonized birds were killed after 6 weeks, and blood samples were obtained. All birds were maximally colonized ($>10^7$ CFU/g fecal content) at the time of death. All previous work has shown that detectable systemic anti-*C. jejuni* antibodies were induced in colonized chickens in this time period (Cawthraw et al., 1994). To further study *C. jejuni*-associated anti-ganglioside antibodies in chickens, antisera were raised in a similar manner in chickens colonized with strains 81116 (heat-stable antigen [HS] 6), isolated from a human during a waterborne outbreak (Palmer et al., 1983), and 99/419 (HS21) isolated from a naturally colonized chicken flock.

In addition to the anti-*C. jejuni* antiserum, sera were obtained from chickens hyperimmunized with purified gangliosides GM1, GM2, and GM3 (Table I). Antisera for GM1, GM2, and GM3 were prepared as described previously (Miyoshi et al., 1982). Briefly, 1 mg of purified ganglioside was mixed with 1 mg of bovine serum albumin (BSA) and CFA, and injected intramuscularly into chickens, followed by a booster injection with IFA 4 weeks later. The birds were killed 4 weeks after the booster injection, and blood samples were obtained.

A 19-mer peptide corresponding to 880–898 (KELKDNHILNHVGLTDGPR) of the amino acid sequence of mouse Nav1.4 channel α subunit was selected as the specific sequence to produce antibody. The peptide was synthesized and conjugated to keyhole limpet hemocyanin (KLH) (Kohjin Bio Co., Ltd., Saitama, Japan). The peptide–KLH complex was assessed by high-performance liquid chromatography as more than 90% homogenous. Sixty micrograms of the peptide–KLH was dissolved in 1 mL of phosphate-buffered saline (PBS). One milliliter of CFA was added, and the mixture was emulsified. The emulsion was subcutaneously injected into a female New Zealand rabbit. Booster sensitization with IFA was done three times with a 2-week interval between each injection. The titer of antiserum was determined by enzyme-linked immunosorbent assay (ELISA) for the peptide–KLH. The cross-reactive antibody to KLH was removed by immunoabsorption onto a column of KLH coupled to CNBr-activated Sepharose 4B (Amersham-Pharmacia, Les Ulis, France) (Farboud et al., 1999).

In the United Kingdom, all experiments involving infected chickens were performed in Veterinary Laboratories Agency under license according to Home Office requirements and were approved by the local ethical review process. In the United States, the experiments for chicken polyclonal antibody production against gangliosides were performed in Medical College of Georgia and were approved by Medical College of Georgia's Institutional Animal Care and Use Committee. In Japan, the experiment for producing a rabbit polyclonal antibody was carried out in accordance with the guidelines for animal care of Showa Pharmaceutical University, as well as the guidelines for animal use published by the National Institutes of Health, Bethesda, Maryland.

ELISA for Anti-ganglioside and Anti-Lipid A Antibodies

The reactivity (titer) of anti-ganglioside antibodies in human and chicken sera was determined by a conventional ELISA as modified in our laboratory (Usuki et al., 2006a). Briefly, 96-well flat-bottom polystyrene microtiter plates were coated with 0.1 μ g of each of the following gangliosides in each well: GM1, GM2, GM3, GD1a, GD1b, and Kdo2-Lipid A in 50 μ L ethanol

(Table I). After the solvent was evaporated at room temperature, nonspecific binding sites in the wells were blocked by incubation for 1 hr with 250 μ L of 1% BSA in PBS solution. After decanting the blocking solution, the plate was filled with antisera at doubling dilutions (1:100 to 1:12,800 in 1% BSA/PBS) and incubated for 1 hr. Control wells contained only 1% BSA in PBS. The plate was then washed five times with 1% BSA in PBS, followed by the addition of an appropriate secondary antibody (horseradish peroxidase–conjugated anti-chicken IgY or anti-human IgG, 1:5,000 dilution in 1% BSA/PBS). The plate was incubated for 1 hr and washed as described above, followed by the addition of a coloring reagent (100 μ L of OPD Peroxidase Substrate in PBS). After 5 min in the dark, the reaction was stopped by addition of 100 μ L of 3 N sulfuric acid. The absorbance of the contents of each well was measured at 492 nm with a micro-plate spectrophotometer (BioRad, Hemel Hempstead, United Kingdom). The antibody titers were assigned as the highest dilution in which the absorbance was 0.1 or less.

Preparation of LOS

C. jejuni ATCC-43446 (serotype HS19) was grown in *Brucella* broth with gentle shaking (100–150 rpm) at 37°C for 48 hr 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 cell pellets by the hot phenol–water procedure (Westphal et al., 1952), followed by chloroform partitioning and mild alkaline hydrolysis as described previously (Usuki et al., 2006b, 2007). After dialysis against water, the dialysate was lyophilized and used as the LOS fraction of HS19.

Thin-layer Chromatography (TLC)-Immunooverlay

Antisera for chicken and human were analyzed by TLC–immunooverlay (Usuki et al., 2006b). Briefly, the LOS 2fraction, authentic gangliosides (GM1, GM2, and GM3) and the LOS fraction were applied onto a high-performance TLC (HPTLC) plate. The plate was developed with the solvent system of n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v). After drying, the plate was soaked with 0.1% polyisobutylmethacrylate in n-hexane for 1 min. The plate was overlaid with the serum (1:100 dilution) and held at room temperature for 1 hr. After washing with 1% BSA/PBS solution containing 0.05% Tween-20, the plate was then incubated with an appropriate secondary antibody solution (horseradish peroxidase–conjugated anti-chicken IgY or anti-human IgG, 1:5,000 dilution in 1% BSA/PBS). TLC–immunooverlay with anti-Nav1.4 polyclonal antibody (pAb) was performed for Lipid A antigens, including Kdo2-Lipid A, LPA1, and LPA2, by the same procedure as described above, but instead the plate was incubated with horseradish peroxidase–conjugated anti-rabbit IgG antibody. The plate was incubated for 1 hr and then washed as described above, followed by detection with OPD Peroxidase Substrate. Bands were recorded by an imaging scanner.

Immunoabsorption

In order to remove anti-ganglioside or anti-Kdo2-Lipid A antibodies from the antisera, immunoabsorption was performed by a modified procedure of Koga et al. (1999). Briefly, antisera were diluted to 1:500 in 1% BSA/PBS solution. Each diluted serum sample was absorbed three times at 4°C overnight on a microtiter plate coated with 0.1 μ g of the candidate ganglioside or Kdo2-Lipid A. Finally, the immunoabsorbed sera were concentrated by Centricon Centrifugal Filter Unit (Amicon Inc., Beverly, MA) and used for patch-clamp analysis. The titers of nonabsorbed antibodies were determined by ELISA.

Cell Culture

NSC-34, NSC-19, and N18TG2 cells were cultured at 37°C in 5% CO₂–95% air in DMEM supplemented with 10% fetal bovine serum. Cells were subcultured routinely by harvesting

monolayers with 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS solution. Confluent cultures were split 1 to 5 twice a week (Usuki et al., 1999).

Whole-cell Patch-clamp Analysis

Na⁺ currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981; Taguchi et al., 2004). NSC-34 cells were plated onto poly-L-lysine-coated micro-glass coverslips (25 mm in diameter) in a petri dish and subsequently incubated in a 5% CO₂ incubator at 37°C. Cells were grown in DMEM containing a 10% fetal bovine serum–1% penicillin and streptomycin solution (Sigma, St. Louis, MO). The microglass coverslip was placed in an experimental chamber on the stage of an inverted microscope. The pH of the external solution, composed of 143 mM NaCl, 1.0 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), and 11 mM glucose, was adjusted to 7.4 with 1 M NaOH. Patch electrodes were pulled in two stages from borosilicate glass on a vertical patch pipette puller. The pipette solution used for the Na⁺ current recordings contained 144 mM CsCl, 5.8 mM NaCl, 10 mM HEPES, and 10 mM ethyleneglycol-bis (2-aminoethylether)-N, N'-tetra-acetic acid, and was adjusted to pH 7.2 with 1 M CsOH. The electrode resistance was 4–8 MΩ when the electrode filling with the pipette solution was touched to the external solution. Na⁺ ion currents were recorded at room temperature with an Axopatch 1D amplifier using a whole-cell patch-clamp technique. To evoke the inward Na⁺ current, the cell was kept at a holding potential of –80 mV and given command pulses with 10 mV steps, ranging from –80 mV to +40 mV. The duration of the depolarizing pulses was 30 msec, and the amplitude of the inward Na⁺ current was measured as the peak current during the depolarizing pulse. Na⁺ currents were sampled at 10 kHz after low-pass filtering at 2 kHz (–3 db). Data were stored and analyzed by pCLAMP 8.2 software (Axon Instruments, Union City, CA).

Tunicamycin Treatment

NSC-34 cells were treated with an effective concentration of tunicamycin for at least 18 hr without loss of cell viability or change in morphology (Maruo et al., 2006). N-glycosylation in NSC-34 cells was inhibited with 0.1 μg/mL tunicamycin after 18 hr of culturing. Patch-clamp analysis was performed after exchange to fresh DMEM medium without tunicamycin.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the transfected cells using Isogen (Nippon Gene, Tokyo, Japan). RT-PCR was performed using a Superscript One-step RT-PCR with a Platinum *Taq* Kit (Invitrogen, CA). RNA was reverse transcribed for 30 min at 50°C. Amplification conditions were as follows: for Nav1.1 to Nav1.9, and for hamster glyceraldehyde-3-phosphate dehydrogenase (GAPDH), initial denaturation at 94°C for 2 min, followed by three temperature steps consisting of 21 cycles (1 min at 94°C, 1.5 min at 60°C and 2 min at 72°C). The PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide using 100-bp or 1,000-kb markers as reference (both Promega, Southampton, United Kingdom). The primer pairs used for GAPDH were the following: forward 5'-GTCAT CATCTCCGCCCTTCTGC3' and reverse 5'-GATGCC TGCTTCACCACTTCTTG3-3'. The following primers for α subunit were used for mouse Nav channels: Nav1.1, forward 5'-CAAAAAGCCACAAAAGCCT-3' and reverse 5'-TTAGCTCCGCAAGAAACATC-3'; Nav1.2, forward 5'-CTCGCCCTGCTAACAAGTTT-3' and reverse 5'-CCAGG TTGATCCAATACAGG-3'; Nav1.3, forward 5'-TTGGCT CCAAAAACCTCAG-3' and reverse 5'-TTGAGAGAATC ACCACCACA-3'; Nav1.4, forward 5'-GTCTTCAAGCTG GCCAAGTC-3' and reverse 5'-ACAGGAGAGCCAGGAAC AGA-3'; Nav1.5, forward 5'-TTCTAGCTCGAGGCTTCT GC-3' and reverse 5'-TCAGAGCTGTCCCCACACAG-3'; Nav1.6, forward 5'-

AGGGAACCTGGTGTTCCTG-3' and reverse 5'-TCAGAAGCAAGGCCAGAAAT-3'; Nav1.7, forward 5'-CAGCAAAGAGAGACGGAACC-3' and reverse 5'-GCGTTGACCACTACCCTCAT-3'; Nav1.8, forward 5'-GAACCTGACAAAGCCAGCTC-3' and reverse 5'-AATGCGGTAACAGGTTTTGC-3'; and Nav1.9, forward 5'-AGCAGCG GAGCACTGATATT-3' and reverse 5'-GCAGACCAGCAA GACATTGA-3'.

Western Blot Testing

Subconfluent NSC-34, NSC-19, or N18TG2 cells (1×10^7) cultured in 75-cm² plates were rinsed twice with cold PBS solution and then in a lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 0.5% deoxycholate). The plates were rocked gently at room temperature for 5 min. Total cell lysates were prepared using lysis buffer, and nuclei were removed by centrifugation at 15,000g for 20 sec. To prepare membrane proteins, cells (3×10^7) were harvested and washed three times with PBS, homogenized using a Teflon-glass homogenizer in 10 mL of 20 mM Tris-HCl, pH 7.4, containing 2 mM KCl and a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Nuclei were removed from the homogenate by centrifugation for 8 min at 800g. The resulting supernatant was subjected to ultracentrifugation at 100,000g for 40 min. A membrane protein preparation was obtained by solubilizing the pellet for 1 hr at 4°C in 180 μ L of native buffer (200 mM KCl, 20 mM Tris-HCl, pH 7.4, 2% CHAPS, and a protease inhibitor cocktail). The protein concentration was determined by the Bradford method (Bradford, 1976) using a Bio-Rad Protein Microassay Kit (Bio-Rad, Hercules, CA).

Total cell lysate and membrane proteins (5 μ g protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 hr in PBS solution containing 5% nonfat dry milk and 0.1% Tween-20 (PBST). The membranes were then incubated overnight with PBST solution containing 1:10,000 affinity-purified anti-Nav1.6, Nav1.7, and anti-Nav1.4 pAbs. After several washings in PBST, the membranes were incubated with 1:2,000 affinity-purified horseradish peroxidase–conjugated anti-rabbit IgG, washed with PBST, and visualized with the enhanced chemiluminescence reagent (Amersham Pharmacia UK Limited, Buckinghamshire, United Kingdom), and the protein bands were detected with BioMax film (Kodak, Rochester, NY). The apparent molecular weights of the α subunits of Nav1.6, Nav1.7, and Nav1.4 were estimated by comparing their migratory rates on SDS-PAGE with those of SigmaMarker molecular weight standards (Sigma). For tunicamycin-treated cells, the cell lysates were similarly prepared and analyzed by the Western blot procedure described above.

Immunocytochemistry

NSC-34 cells cultured on glass coverslips were fixed for 20 min in PBS solution containing 4% paraformaldehyde and then permeabilized for 5 min in PBS. Next, cells were exposed for 20 min to PBS containing 4% BSA to block nonspecific binding, followed by overnight incubation at 4°C with PBS containing 1:200 antibody solution with C1.4 or C1.5 chicken antisera. After several washings with cold PBS, the cells were incubated for 1 hr at 4°C with PBS containing 1:100 secondary antibody solution of tetramethylrhodamine isothiocyanate–conjugated goat anti-chicken IgY. Double immunostaining was used to examine the localization of anti-GM2 antibody with anti-Nav1.4 pAb after NSC-34 cells were fixed with 4% paraformaldehyde and then permeabilized with PBS containing 0.2% Triton X-100. After incubation with the primary anti-GM2 chicken antibody, followed by treatment of the secondary fluorescein isothiocyanate–conjugated goat anti-chicken IgY antibody (diluted 1:100) in 1% BSA/PBS, the cells were exposed overnight at 4°C to anti-Nav1.4 pAb (diluted 1:100) and were incubated for 1 hr at 4°C with the secondary tetramethylrhodamine

isothiocyanate–conjugated goat anti-rabbit IgG (diluted 1:100) in 1% BSA/PBS. The cells were then washed with cold PBS and mounted in Aqua-Poly/mount (Polysciences Inc., Warrington, PA). Immunofluorescent images were obtained and analyzed using a Fluoview BW50 laser-scanning confocal microscope (Olympus, Tokyo, Japan) equipped with a 60× oil immersion objective and excitation at 488 and 568 nm.

To determine whether GM2 and 19-mer peptide of Nav1.4 were localized inside or outside the cell membrane, NSC-34 cells were subjected to immunostaining at 4°C overnight with or without 0.2% Triton X-100, and washed with PBS before treatment with the respective primary antibody.

Identification of the Extracellular Domains of the α Subunit of Nav Channel

Sequencing alignment of mouse Nav1.1 to Nav1.9 α subunit proteins was performed by CLUSTALW (Thompson et al., 1994). The mouse Nav family shows a highly conserved sequence in the transmembrane domain and P-loop. With that sequence alignment, the sequence of transmembrane domains with mouse P-loops was determined. The sequences between the transmembrane domain S5 and P loop were identified as the extracellular loop of the Nav subunit (PubMed ID Nav1.1, NP_061203; Nav1.2, XP_001001633; Nav1.3, NP_061202; Nav1.4, NP_573462; Nav1.5, NP_067519; Nav1.6, AAC52242; Nav1.7, Q62205; Nav1.8, Q6QIY3; Nav1.9, AAD53403).

Identification of Structural Similarity

With HHpred in Bioinformatics Tool Kit (Department of Protein Evolution, Max-Planck Institute, Hanover, Germany), a fragment (amino acid residues 75 to 91) of mouse ubiquitin-like protein (SB132, Protein Data Bank ID 1×1m_A) was chosen as a homologue template of the 19-mer peptide (KELKDNHILNHVGLTDGPR). The tertiary structure of the 19-mer peptide was built by Modeller 7.0 (Proteins 23:318–26, 1995) and energy minimized by ChemOffice 10 (CambridgeSoft, Austin, TX). The structural similarity between the 19-mer peptide and virtual lipid A (v-Lipid A as illustrated in Fig. 8C and defined in Table II) was investigated by the method of structural overlapping using ChemOffice 10. Kdo2-lipidA cannot be used for structural overlapping because the Kdo moiety and fatty acid chains are not intrinsic to the Lipid A common structure. Both Kdo2 and fatty acid chains disturb the structural overlapping and give nonsense results for Kdo2-Lipid A. The α subunit of Nav1.4 is illustrated in Figure 9. The roman numerals denote the transmembrane domains of the α subunit involving the extracellular loops after the S5 segment. With an ab initio method, the tertiary structure of each loop was created using SSPro package (Cheng et al., 2005). Structural overlapping between loops of the Nav1.4 α subunit and v-Lipid A was performed on each loop by ChemOffice 10. Structural similarity to v-Lipid A was detected on the tertiary structure of the extracellular loops of the Nav1.4 molecule, and is shown as dipeptides in the sequences of extracellular loops.

RESULTS

Antibody Titration

Titers of anti-ganglioside and anti-Kdo2-Lipid A antibodies in the sera of the laboratory worker and in chickens are summarized in Table I. The antibody reactivity was examined by a TLC–immunooverlay method as shown in Figure 1A,C. In serum H1.1 on day 1 after isolation of the *C. jejuni* strain VLA2/18, there were no detectable IgG antibodies to gangliosides or Kdo2-Lipid A. H1.1 taken at 1 day after isolation had a weak titer (less than 1:200) of anti-GM3 antibody activity. However, anti-GM1, -GM3, and -GD1b, and -Kdo2-Lipid A antibody activities were detected in the follow-up serum (H1.1; day 22), as shown in Table I. Immunoabsorption of H1.2 serum with GM1 abolished anti-GM1 antibody activity with a

partial loss of anti-GM3 and anti-GD1b antibody activities, but did not abolish anti-Kdo2-lipid A antibody activity (H1.2). Further immunoabsorption of the serum by Kdo2-lipid A revealed a complete elimination of anti-Lipid A, anti-GM3, and anti-GD1b antibody activities (H1.3).

Chicken sera containing anti-ganglioside antibodies were obtained by experimental colonization of chickens with *C. jejuni* (strains HS 6, HS 21, and VLA2/18) or by immunization with each of the following purified gangliosides: GM1, GM2, and GM3 (Table I). Chicken serum A1.1 had low-titer antibody activities against GM1, GM3, and Kdo2-Lipid A. After immunoabsorption with Kdo2-Lipid A, the anti-Kdo2-Lipid A and some of the anti-GM3 antibody activities disappeared as indicated in the A1.2 serum. The high-titer antibody to GM2 had a slight cross-reactivity against GM3 and Kdo2-Lipid A in chicken serum B1.1; the activities could be abolished by immunoabsorption using Kdo2-Lipid A. High-titer anti-GM1, -GM3, and -Kdo2-Lipid A antibodies observed in serum C1.1 were produced in chickens colonized with strain VLA2/18. Immunoabsorption by GM1 and GM3 eliminated both antibody activities from serum C1.4, but partially from anti-Kdo2-Lipid A antibody activities. The latter could be abolished completely by absorption using Kdo2-Lipid A, as seen in serum C1.5. Immunization of chickens with purified gangliosides GM1, GM2, or GM3 resulted in sera D1.1, E1.1, and F1.1, respectively, with high-titer specific antibodies, together with anti-Kdo2-Lipid A antibodies. Anti-ganglioside antibodies in sera could be eliminated by absorption with the respective ganglioside, whereas anti-Kdo2-Lipid A antibody activities could be abolished completely only by immunoabsorption by Kdo2-Lipid A.

TLC–Immunooverlay of LOS and Gangliosides

The antibody reactivities in sera obtained by experimental infection of chickens with *C. jejuni* (strains HS 6, HS 21, and VLA2/18) or by immunization with a purified ganglioside, GM1, GM2, or GM3, were examined by the method of TLC–immunooverlay. The results are shown in Figure 1A,B. A crude LOS fraction (from strain HS 21 of *C. jejuni*), containing both LOS and Lipid A, was also applied to the HPTLC plate for comparison. Both components were easily separated on the plate and identified as two discrete bands (Usuki et al., 2007): the upper band corresponded to Lipid A and the lower band consisted of ganglioside-like LOS. Cross-reactive antibodies between GM1 and GM1-like LOS were detected in the serum derived from strain HS 6 or VLA2/18, but were not detected in strain HS 21 (Fig. 1A). Anti-Kdo2-Lipid A antibody activities were detected, however, in all of the chicken sera as shown by ELISA (Table I). Thus, immunization of chickens with purified GM1, GM2, or GM3 resulted in eliciting specific high-titer anti-ganglioside antibodies, as well as anti-Kdo2-Lipid A antibodies (Fig. 1B). Authentic gangliosides GM1, GM2, and GM3 migrated near the same location as Lipid A with the developing solvent system n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v). Samples of the human sera (H1.1) taken on days 1 and 22 after isolation of VLA2/18 were also tested and revealed no antibody activities on day 1, but some activities for anti-Lipid A and anti-GM1-like LOS on day 22 (Fig. 1C).

Effect of Immunoabsorption on Nav Channel Current

NSC-34 cells were subjected to whole cell patch-clamping for Nav channel current activities and tested for the effect of antibody activities using chick sera before and after immunoabsorption. Figure 2A shows the voltage-dependent activation curve of the Na⁺ current and the voltage-independence of inhibition in the presence of anti-GM1 chicken antibody (serum D1.1), implying that the antibody-induced suppression occurred with all of the applied test potentials. Anti-GM1 or -GM2 chicken sera (D1.1 or E1.1) also showed a current-dependent inhibitory effect and a sluggish reduction of the relative Na⁺ current (Figs. 2C(1) and 2C(2)). Chicken anti-VLA2/18 antiserum also was inhibitory but reversible after washing out (Fig. 2C(3)). To test which antibody activity in the C1.1 serum sample was responsible for the inhibitory effect on the Na⁺ current, we eliminated anti-ganglioside or anti-Kdo2-Lipid A

antibody activities from the C1.1 serum by immunoabsorption (Fig. 2B(1)). The inhibitory effect of the anti-VLA2/18 antiserum (C1.4) was not affected, despite the removal of anti-GM1 and -GM3 antibodies. A complete elimination of the inhibition required the removal of anti-Kdo2-Lipid A antibody from the serum (see the bar for C1.5). Antisera D1.1, E1.1, or F1.1 obtained from chickens immunized with purified ganglioside GM1, GM2, or GM3, respectively, showed that removing anti-ganglioside antibodies D1.2, E1.2, or F1.2 (left closed-columns in Fig. 2B(2)) was insufficient to abolish the inhibition of Nav channel activities. Similar to the finding above, it is also essential for C1.4 (Fig. 2B(1)) to remove the anti-Kdo2-Lipid A antibody activities in order to abolish the inhibitory effect. It is also noteworthy that mouse anti-GD3 IgG and IgM mAbs, which were derived from a mouse hybridoma without CFA as an adjuvant and were devoid of anti-Kdo2-Lipid A activity, did not have any effect on Na^+ currents (Fig. 2C(4) and 2C(5)), suggesting that the anti-Kdo2-Lipid A activity appeared with a high degree of specificity for the chicken sera. The chicken sera, as indicated earlier, were generated in the presence of CFA and exhibited anti-Kdo2-Lipid A antibody activities.

Expression of Nav Channels

Figure 3A shows RT-PCR of Nav1.1 to Nav1.9 in N18TG2, NSC-34, and NSC-19 cells. There was selective expression of the α subunit genes of Nav channels in all three cell lines; the Nav1.6 and Nav1.7 transcripts appeared to be the most abundant, being more than 8-fold higher than that of the Nav1.3 transcript (Fig. 3A). The difference in expression between NSC-34 and NSC-19 indicated that the α subunit genes of Nav1.2, Nav1.3, and Nav1.4 were expressed in NSC-34 cells, but were not expressed in NSC-19 cells. This pattern of α subunit gene expression is consistent with the protein expression levels of Nav1.6 and Nav1.7 (Fig. 3B). There was no detectable level of Nav1.2 or Nav1.3 in N18TG2 and NSC-34 cells by Western blot analysis (data not shown).

Characterization of Anti-Nav1.4pAb

The polyclonal peptide antibody (anti-Nav1.4 pAb) against the 19-mer peptide (KELKDNHILNHVGLTD GPR) of Nav1.4 α subunit was produced by immunization of rabbit. The rabbit antiserum showed a high titer (1:80,000) of binding reactivity with the peptide-KLH conjugate by ELISA. The anti-KLH antibody could be removed from the antiserum by immunoabsorption (Farboud et al., 1999). After absorption by KLH, the residual anti-Nav1.4 pAb had a titer of 1:40,000. Interestingly, TLC-immunooverlay of this antibody revealed that it was cross-reactive to various species of Lipid A: Kdo2-Lipid A, LA1 (Lipid A, monophosphoryl), and LA2 (Lipid A, diphosphoryl) (Fig. 4A). This observation provides convincing evidence that there is a common “epitope,” or molecular mimicry, between Nav1.4 and Kdo2-Lipid A that can be recognized immunologically on the basis of cross-reactivity. Anti-Nav1.4 pAb, however, did not have any inhibitory effect on Na^+ current in NSC-34 cells (Fig. 4B).

Localization of Binding Sites in NSC-34 Cells by Anti-Kdo2-Lipid A Antibody

Immunocytochemical analysis using the procedure of Fujita et al. (2006) revealed the localization of GM2 and the 19-mer peptide epitope of Nav1.4 in NSC-34 cells (Fig. 5A(1,2)). GM2 was found to localize on the cell surface and did not colocalize with the 19-mer peptide epitope that was situated in the intracellular domain of the α subunit of the Nav1.4 channel (Fig. 5A(2,3)). Localization of the 19-mer peptide epitope was demonstrated in the inside membrane of NSC-34 using the anti-Nav1.4pAb applied to the cells treated with and without 0.2% Triton X-100 (Fig. 5A(5) and (4)). Without Triton X-100 treatment, only a few cells were immunostained with anti-Nav1.4pAb; on the other hand, all cells showed strong staining with Triton X-100-treatment. In contrast, anti-GM2 staining revealed strong staining of the cell-membrane in the absence of Triton X-100 (Fig. 5A(6)). These data support the notion that

anti-Nav1.4 pAb did not inhibit Na⁺ current, as this antibody could not reach the 19-mer peptide epitope of Nav1.4. The inability of anti-Nav14 pAb to inhibit the Na⁺ current also corroborates this conclusion (Fig. 4B).

To further examine the binding specificity of the chicken sera, we used chicken serum C1.4 that had been immunoabsorbed to remove anti-GM1-antibody and anti-GM3-antibody activities but not the anti-Kdo2-Lipid A activity (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁺). For comparison, we also used chicken serum C1.5 that had been immunoabsorbed by the appropriate antigens (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁻). We found that C1.4 immunostained NSC-34 cells but C1.5 did not, indicating that the cells possessed binding sites for anti-Kdo2-Lipid A antibody (Fig. 5B,C).

Tunicamycin Treatment

We treated NSC-34 cells with tunicamycin followed by treatment with immunoabsorbed chicken sera to investigate the relationship between the Na⁺ current-inhibitory effect of C1.4 chicken serum (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁺) and C1.5 chicken serum (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁻). The Na⁺ current was reduced by treatment with 0.1 µg/mL of tunicamycin at 37° C for 18 hr. After treatment of the cells with tunicamycin, the C1.4 serum (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁺) induced a slight reduction in Na⁺ current levels compared with cells without tunicamycin treatment (Fig. 6A, column 1 vs. 2). In comparison, C1.5 serum (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁻) showed no difference in the inhibition levels of Na⁺ currents with or without tunicamycin treatment (Fig. 6A, column 3 vs. 1, respectively). In contrast, C1.4 serum (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁺) showed the Na⁺ current more depressed with tunicamycin treatment than without treatment (Fig. 6A, column 4 vs. 2, respectively).

Tunicamycin treatment caused NSC-34 cells to detach and float from the bottom of the cultured dishes at a concentration of > 0.1 µg/mL in medium. It is entirely likely that tunicamycin caused deglycosylation of adhesion molecule and led to loss of scaffolding. The floating cells are not available for the patch clamping. Moreover, the floating cells had decreased viability. In the culture condition of 0.1 µg/mL for 18 hr, tunicamycin did not alter the amount of protein in NSC-34 cells (data not shown), and neither did it alter the subunit of Nav1.4 as revealed by Western blot analysis (Fig. 6B, lane 3). Although few Nav channels were devoid of glycosylation at 0.1 µg/mL of tunicamycin, this alteration is sufficient to reflect the changes of the resulting Na⁺ current. Thus, the Nav channel inhibitory activity due to anti-Kdo2-Lipid A antibodies was clearly not associated with the N-glycan of the Nav channel.

Treatment With µ-Conx and TTX

To test the possibility that anti-Kdo2-Lipid A antibody was a specific inhibitor for Nav1.4, NSC-34 cells were treated with µ-Conx and TTX and examined for preoccupied sites for binding. NSC-34 cells showed that Na⁺ currents of Nav1.4 were suppressed up to 76 to 72% of the control current by addition of 2 to 5 µM of µ-Conx. Subsequently, addition of chicken serum C1.3 (anti-GM3⁻, anti-GM1⁺, anti-Kdo2-Lipid A⁺) or C1.4 (anti-GM3⁻, anti-GM1⁻, anti-Kdo2-Lipid A⁺) showed further suppression of Na⁺ currents to 8 to 5% of the control current, respectively (Figs. 7A(3) and 7A(6)). The time course and response curves of this experiment are shown in Figures 7A(2) and 7A(5). The inhibitory effect of anti-Kdo2-Lipid A antibody continued and the recovery was sluggish after washing out. To investigate the binding sites of anti-Kdo2-Lipid A antibody on Nav channels, NSC-34 cells were subjected to TTX treatment followed by wash-out. The total Nav-derived currents showed a strong inhibition by the addition of TTX and a reversible recovery by washout (Fig. 7B). When TTX was used to suppress Nav channel activities, C1.4 treatment did not alter the inhibition, and the washout only caused a sluggish recovery. This finding suggests that antiserum C1.4

treatment did not produce any competitive inhibition on the binding site of Nav channels. Subsequent to the wash-out, an additional treatment with μ -Conx induced a further inhibition of Na^+ currents. This observation suggests that parts of the binding sites in Nav1.4 by anti-Kdo2-Lipid A may be shared by μ -Conx.

Structure Overlapping of v-Lipid A and 19-Mer Peptide

The tertiary structure of the 19-mer peptide KELKDNHILNHVGLTDGPR was examined by molecular modeling that was optimized by energy minimization. The structural mimicry between the 19-mer peptide and v-Lipid A (Fig. 8C) was assessed by the method of structural overlapping (Fig. 8A). The dipeptide (Leu³-Lys⁴) revealed a high degree of structural mimicry to a part of the reducing terminal glucosamine moiety of the v-Lipid A molecule (Fig. 8A). The results indicate that the glucosamine residue overlaps with the side chain of Lys⁴ and that the N-acyl chain overlaps with the side chain of Leu³. The phosphate residue of v-Lipid A is seen out of this overlapping structure (Fig. 8B). The rabbit polyclonal antibody, anti-Nav1.4 pAb, produced using the 19-mer peptide as the immunogen, however, showed cross-reactivity with Kdo2-Lipid A.

Schematic Structure of α Subunit of Nav Channel

The α subunit of Nav1.4 is illustrated in Figure 9. The roman numerals indicate the transmembrane domains involving the extracellular loops of the S5–S6 segment. The pore loop region (P-loop) is located in the extracellular loops ahead of segment 6 (not drawn in illustration, Fig. 9). The 19-mer peptide is located in an intracellular loop, where the peptide sequence is specific for Nav1.4. This peptide is localized in the inner membrane, as confirmed by lack of immunostaining (Fig. 5A(4)) and lack of suppression of Nav current by anti-Nav1.4 pAb (Fig. 4B).

Overlapping Structure of v-Lipid A and Extracellular Domains of Nav 1.4

Each of the loop domains of Nav 1.4 shown in Figure 9 was analyzed for overlap with v-Lipid A (Fig. 8B and Table II) by structure overlapping. The structural element that is similar to that of v-lipid A is shown as a dipeptide: D1, Ser-Trp; D2, Ser-Tyr; D3, Ser-Glu; D4, Lys-Lys, on the secondary structure of the extracellular loops (domains I to IV), as indicated with asterisks (Table III). The tertiary structures of these four domains are also shown in Figure 10A. The N-terminal Ser residue is the common amino acid residue in three of the four dipeptides in domains I to III, and this residue contributes to the structural similarity of the channel protein to v-Lipid A.

Overlapping Structure of Dipeptides and v-Lipid A

Analysis of the overlapping structure of v-Lipid A with each of the dipeptides (Ser-Trp, Ser-Tyr, Ser-Glu, and Lys-Lys) in the α subunit of Nav1.4 is shown in Figure 10B. The first Ser residue contributes to structural mimicry with v-Lipid A, showing a high degree of homology against the N-acyl group of the reducing terminal glucosamine residue. On the other hand, the second amino acid residue has an aromatic residue or a long side chain.

Molecular mimicry of v-Lipid A with these peptides in the four extracellular domains of Nav1.4 was investigated for Nav1.1 to Nav1.3 and Nav1.5 to Nav1.9 (Table IV). It is essential that the first Ser residue be homologous to v-Lipid A in domains I to III of all of the Nav channel protein family. However, domain IV requires a Lys residue on the N terminus of the dipeptides.

DISCUSSION

Axonal degeneration and demyelination of the PNS are the classical pathological hallmarks in patients with GBS and similar peripheral neuropathies, which in most cases account for the loss of motor and sensory functions in the PNS. The mechanisms of neuropathogenesis, however, are not fully understood (Ariga and Yu, 2005). The etiology of GBS is very complex; however, it is known that *C. jejuni* infection elicits anti-ganglioside antibodies by a molecular mimicry mechanism causing neuropathological changes in the PNS (Jacobs et al., 1996; Yuki et al., 1990; Quarles and Weiss, 1999; Yu et al., 2006).

In the present study, we investigated the *C. jejuni* strain VLA2/18 with the intention of elucidating GBS subsequent to interspecies transmission of *C. jejuni* from chicken. VLA2/18 is not a GBS neuritis strain, but it elicits high titers of anti-GM1 and anti-Lipid A antibodies. Anti-GM1 antibody itself did not produce blockade of Nav channels in NSC-34 cells. Interestingly, we detected anti-Lipid A antibody against Kdo2-Lipid A produced in both the human and chicken sera, of which the activity was associated with the blockade of Nav. Lipid A is a component in the outer membrane of Gram-negative bacteria, to which *C. jejuni* belongs. Consequently, it is not surprising that, as a part of the LOS, anti-Kdo2-Lipid A antibodies were also elicited in the sera of both the infected individual and colonized chickens. In addition, immunization of chickens with GM1, GM2, or GM3 resulted in the appearance of anti-Kdo2-Lipid A antibodies as well as anti-ganglioside antibodies. The anti-Lipid A activity in the latter cases comes presently unknown origin, and may arise from the generation of cross-reactivity by the lipid component of *Mycobacterium tuberculosis* (*M. tuberculosis*) in the CFA used for sensitization of animals for antibody production. As we recently reported (Usuki et al., 2007), cholera toxin B-subunit-binding Lipid A of *C. jejuni* is indeed composed of disaccharides with long-chain fatty acids, e.g., 3-(2-hydroxytetracosanoyl) octadecanoic acid. Support for this assertion is that there is cross-reactivity between anti-Lipid A and i antigen (polylactosamine), even though the anti-Lipid A mAb is generated by cloned hybridoma cells (Bhat et al., 1993).

As expected, chicken anti-VLA2/18 antiserum (C1.4) immunostained the surface of NSC-34 cells by binding of the anti-Kdo2-Lipid A antibody. Gangliosides GM1 and GD1a contribute to the stability of paranodal junctions and Nav channel clusters in myelinated nerve fibers (Susuki et al., 2007). Colocalization of the Nav channel and GM1 is an interesting possibility to elucidate the possible role of anti-Kdo2-Lipid A antibody inducing blockade of Nav channels. However, we consider the possibility of colocalization unlikely in NSC-34 cells because of the lack of GM1 in this cell line.

With respect to the specific portion of the extracellular domains of Nav channels recognized by the anti-Kdo2-Lipid A antibody, immunofluorescence staining of NSC-34 cells showed that chicken serum C1.4 stained the cell surface (Fig. 5B). Anti-Nav1.4 pAb did not react with the outside-membrane domains of Nav1.4 channel without Triton X-100 treatment (Fig. 5A(4)), but did react with the inside-membrane domain of this channel after Triton X-100 treatment (Fig. 5A(5)). This result is in line with the observation that anti-Nav1.4 pAb does not elicit the inhibition of the resulting Na⁺ current (Fig. 4B).

The Nav channel is a large multimeric complex, composed of an α subunit and smaller β subunits (Yu and Catterall, 2003). The Na⁺ ion-conducting pore is located entirely within the α subunit, which represents the essential element of the channel functions, such as channel opening, ion selectivity, and inactivation. Nine α subunits (Nav1.1 to Nav1.9) have been functionally characterized and shown to localize in four extracellular domains (domains I–IV, Fig. 9) whose primary sequences are highly conserved with similarities in the 3D structure (Diss et al., 1998). The α subunit of Nav channels is also reported to be a highly N-glycosylated

protein (Schmidt and Catterall, 1987; Johnson et al., 2004). Recent studies have demonstrated that N-glycosylation is present on the extracellular domain I (Fig. 9) that serves to modulate the gating of the Nav1.4 channel. Reduced N-glycosylation results in insufficient gating (Bennett et al., 1997). NSC-34 is a neuroblastoma-spinal cord hybrid cell line displaying more multipolar motor neuron-like phenotypes than NSC-19 does (Cashman et al., 1992; Matsumoto et al., 1995). Benatar et al. (1999) failed to show any effect of anti-GM1 on Nav channels of NSC-19 cells by whole-cell patch-clamping. Previously, we have reported an effect of an anticiliary neurotropic factor receptor (CNTFR) that induces cell death of NSC-34 cells, which can be rescued by GM2, a marker ganglioside for motor neurons (Usuki et al., 1999, 2001). In the present study, NSC-34 cells were examined for Nav channel functions because expression of Nav 1.4 was demonstrated by RT-PCR, and the blockade of the resulting current could be detected by μ -Conx. This toxin is a selective blocker for Na^+ current of Nav1.4 in skeletal muscle cells (Ukomadu et al., 1992). Our finding on the effect of μ -Conx on NSC-34 cells suggests that functional Nav1.4 channels are also expressed in this motor neuron cell line. For this reason, we presumed that Nav1.4 might be an important target for anti-Kdo2-Lipid A antibody in the VLA2/18 sera. Anti-Kdo2-Lipid A was found in the rabbit polyclonal antibody, although it was produced against the 19-mer peptide of Nav1.4 protein. The 19-mer peptide was a specific sequence of Nav1.4, but Nav1.4 pAb did not show the resulting inhibition of Nav1.4 currents. The 19-mer peptide was chosen because this peptide sequence is unique for the Nav1.4 channel protein and not present in any other Nav channel proteins. More importantly it shows structural similarity with Kdo2-Lipid A on the basis of expectation of 3D structural overlapping (Fig. 8). As we expected from the immuno-fluorescence staining study that the 19-mer peptide sequence is located inside the cell and not readily accessible to anti-Nav1.4 pAb, it is not surprising that the antibody did not exert any inhibitory effect on the Na^+ current in this cell line (Fig. 4B). The question arose as to what the binding epitope was for anti-Kdo2-Lipid A to bind to cause its depressive effect on Na^+ current in NSC-34 cells. Our assumption was that the effect must reside on a ubiquitous epitope in the extracellular domains of Nav channels. One attractive possibility was that the N-glycan serves in the epitopes recognized by anti-Kdo2-Lipid A. The α subunit of Nav channels is a highly N-glycosylated protein. Recent studies have demonstrated that N-glycosylation is required to modulate the gating of Nav channels; reduced glycosylation results in insufficient gating (Schmidt and Catterall, 1987; Johnson et al., 2004). Concerning the glycosylation status of the channel protein, although N-glycosylation is required for maintenance of functional voltage-gated Na^+ channels, our data indicate that the N-linked carbohydrate portion does not participate in the resulting current inhibition by anti-Kdo2-Lipid A. The N-linked carbohydrate portion is instead dependent on an epitope on the protein part of the channel. However, this possibility was ruled out on the basis of the finding that tunicamycin treatment did not change the resulting Na^+ current together with anti-Kdo2-Lipid A (Fig. 6A). Thus, it is suggested that there may be a multiplicity of peptide epitopes on the extracellular domains of Nav channels with structural similarity to Kdo2-Lipid A and other peptides in addition to the 19-mer peptide.

These peptide epitopes of Nav may be a multitarget for anti-Kdo2-Lipid A antibody. This assumption was borne out; we predicted that the dipeptide (Leu³-Lys⁴) of the 19-mer peptide was relevant to the homology of the v-Lipid A on the basis of molecular modeling (Fig. 8B). Analysis of the shape and available surface of extracellular domains I to IV by molecular overlapping suggests that the dipeptides Ser-Trp, Ser-Tyr, and Ser-Glu with the first Ser residue in domains I to III, respectively, can contribute to the homology of v-Lipid A. The homologous peptide of domain IV is the dipeptide Lys-Lys with the first residue as Lys (Tables III and IV). With respect to the interaction of anti-Kdo2-Lipid A and the Nav channel, our findings suggest that the dipeptide epitope on each of the outer domains may be involved. The caveat is that there is no homology of the dipeptides in domains I and III of Nav1.6, domain IV of Nav1.8, and domain II of Nav1.9. A region near the N-acyl bond of a reducing terminal glucosamine of v-Lipid A is homologous to the first Ser or Lys residue of the dipeptide epitope. In addition,

the second amino acid of the dipeptides appears to be occupied by an amino acid with an aromatic residue or a long side chain, such as Tyr, Trp, Glu, Arg, or His. Thus, it may be safe to conclude that the dipeptide, with the first Ser or Lys residue present in the α subunit of the Nav channel, may be responsible for cross-interaction with anti-Kdo2-Lipid A through a molecular mimicry mechanism. This represents one of the examples of cross-reactivity between peptide and carbohydrate antigens. Further studies are directed to the demonstration of the specific binding site by using possible multiple peptide antigens including dipeptides and the anti-Kdo2-Lipid A antibody.

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Fig. 1. TLC-immunooverlay of LOS with chicken and human sera. Samples of 100 ng of GM1 and LOS (strain HS19 of *C. jejuni*) were developed on HPTLC plates using n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v) as the developing solvent. The plates were examined by TLC-immunooverlay using 1:100 dilution of antisera in PBS buffer. **A:** Chicken sera from the infection experiment (A1.1, HS6; B1.1, HS21; C1.1, VLA2/18), **B:** chicken sera from the immunization experiment with ganglioside GM1, GM2, or GM3 (D1.1, anti-GM1 antibody; E1.1, anti-GM2 antibody; F1.1, anti-GM3 antibody). **C:** Human sera from the laboratory worker with campylobacteriosis (H1.1 day 1, day 1 after isolation of the strain VLA2/18; H1.1 day 22, day 22 after isolation of the strain VLA2/18).

Fig. 2.

Effect of antibody immunoabsorption on voltage-gated Na^+ channel in NSC-34 cells. **A:** Peak Nav channel currents in the control solution (○) and D 1.1 (●) were plotted against the test-pulse potentials. Each point in the current Na voltage curve represents the mean $\pm$ SD ($n = 9$). **B:** Histograms of relative Na^+ currents in NSC-34 cells after addition of immunoabsorbed chicken serum. **1:** Control, a healthy chicken serum; C1.4, (anti-GM1⁻, anti-GM3⁻, anti-Kdo2-Lipid A⁺); C1.5, (anti-GM1⁻, GM3⁻, and Kdo2-Lipid A⁻). **2:** Control, a healthy chick serum; D1.2 (anti-GM1⁻, anti-Kdo2-Lipid A⁺); D1.3 (anti-GM1⁻, anti-Lipid A⁻); E1.2 (anti-GM2⁻, anti-Kdo2-Lipid A⁺); E1.3 (anti-GM2⁻, anti-Kdo2-Lipid A⁻); F1.2 (anti-GM3⁻, anti-Kdo2-Lipid A⁺); F1.3 (anti-GM3⁻, anti-Kdo2-Lipid A⁻). **Left closed columns:** D1.2, E1.2, and F1.2 are anti-ganglioside antibodies⁻ and anti-Kdo2-Lipid A⁺. **Right open columns:** D1.3, E1.3, and F1.3 are anti-ganglioside antibodies and anti-Kdo2-Lipid A⁻. **C:** Three Na^+ current traces recorded in control external solution and after addition of 10 μL of the chicken serum and anti-GD3 mAb; (1) D1.1, (2) E1.1, or (3) C1.1, and (4) or (5); R24 or D0143, and after washout. The lower diagrams of (1) to (5) are time course of inhibition of Na^+ current by the same serum or anti-GD3 as corresponding to the Na^+ current traces above.

Fig. 3.

RT-PCR and Western blot analysis. **A:** RT-PCR analysis for Nav 1.1 to 1.9. N18TG2, NSC-34, and NSC-19 cells were cultured in DMEM with 10% fetal bovine serum for 3 days. Total RNA was extracted, and the expression of mRNA of α subunit of Nav 1.1 to 1.9 was examined by RT-PCR, separated by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining. GAPDH was used as internal control for loading and efficiency of RT-PCR (lane b). The size markers of 100 bp to 1,000 kb were used as a reference (lane a). **B:** Expression of α subunit protein of Nav1.6 and Nav1.7 channels by N18TG2 and NSC-34 cells in culture. The total proteins (lane 2) were obtained after solubilization of the cells. The membrane proteins (lane 3) were prepared as described under Materials and Methods. The α subunit protein of Nav1.6 or Nav1.7 was detected by immunoblot with anti-Nav1.6 or Nav1.7 rabbit pAb. The membrane proteins containing the α subunit protein of Nav1.6 and Nav1.7 were also prepared from normal rat brain (lane 1) as a positive control. Nav1.6 and Nav1.7 are indicated by arrows.

Fig. 4.

Characterization of anti-Nav1.4 pAb. **A:** Samples of 100 ng of Kdo2-Lipid A, LPA1 (Lipid A, monophosphoryl from *E. coli* F583, Rd mutant), or LPA2 (Lipid A, diphosphoryl from *E. coli* F583, Rd mutant) were developed on HPTLC plates using n-propanol:H₂O:25% NH₃ (6:3:1, v/v/v) as the developing solvent and the plates were examined by TLC-immunooverlay using 1:100 dilution of anti-Nav1.4pAb in PBS buffer, the rabbit pAb against the 19-mer peptide (KELKDNHILNHVGLTDGPR) of α subunit of Nav1.4 channel. **B:** Three Na⁺ current traces recorded in control external solution (preapplication) and after addition of 10 μ L anti-Nav1.4pAb diluted by 1:200 or 1:66.7. Time course of inhibition of Na⁺ current by the same solutions as above and 1:40 diluted solution of anti-Nav1.4 pAb are shown in the right diagram.

Fig. 5.

Localization of binding sites in NSC-34 cells by anti-Kdo2-Lipid A antibody **A:** GM2 was localized on the outside membrane by double (1) and single (6) staining. The 19-mer peptide of Nav1.4 was localized on the inside membrane domains in the Nav1.4 α subunit (2). The overlay does not show colocalization of GM2 and Nav1.4 channel (3). Before immunostaining with anti-Nav1.4 pAb, cells were subjected to with (+) or without (–) 0.2% Triton X-100 treatment (4,5). **B:** The C1.4 chicken serum (containing anti-Kdo2-Lipid A antibody activity and none of the anti-ganglioside antibodies) immunostained the outside-membrane of NSC-34 cells (1). A Lipid A-like epitope was shown on the outside of membrane of NSC-34 cells by the phase contrast (2) and overlay (3). **C:** The C1.5 chicken serum (does not contain antibody activities against Kdo2-Lipid A and gangliosides) showed no staining for the Lipid A-like epitope in NSC-34 cells (1) or for phase contrast (2) and overlay (3).

Fig. 6.

Effect of tunicamycin on whole-cell membrane Na^+ current and on protein expression of Nav1.4 α subunit protein. **A:** Columns representing the Na^+ relative current in the presence (+) or absence (–) of 0.1 $\mu\text{g/mL}$ tunicamycin, followed by the addition of chicken serum: C1.5 (anti-GM1 $^-$, anti-GM3 $^-$, anti-Kdo2-Lipid A $^-$) or C1.4, (anti-GM1 $^-$, GM3 $^-$, anti-Kdo2-Lipid A $^+$). The values of columns are shown as percentage of the first column, the resulting Na^+ current (tunicamycin, anti-Kdo2-Lipid A $^-$). The values are average of two experiments. **B:** NSC-34 cells were cultured in the presence (+) or absence (–) of tunicamycin (0.1 $\mu\text{g/mL}$), and the total proteins (lane 2) were obtained after solubilization of the cells. The membrane proteins (lane 3) were prepared by the procedure as described in Materials and Methods. The Nav1.4 α subunit protein of Nav1.4 was detected by immunoblotting with anti-Nav1.4 pAb. The membrane proteins were also prepared from the normal rat brain (lane 1). The Nav1.4 α subunit protein is indicated by arrows.

Fig. 7.

Characterization of Nav1.4-specific current by μ -Conx. **A:** Na⁺ current traces recorded in control external solution (preapplication) and after addition of C1.3 serum (1:200 dilution) subsequent to 2 μ M of μ -Conx (1) and C1.4 serum (1:200 dilution) subsequent to 5 μ M of μ -Conx (4). Time course of inhibition of Na⁺ current by the same diluted solutions as above (2,5). The relative Na⁺ currents are shown in the right diagram (3,6). The values are average of two experiments. The bars are difference. **B:** Time course of Na⁺ current by the following treatments; 1 μ M TTX at 5 min; washout at 7 min; 1 μ M TTX at 12 min; C1.4 (1:200 dilution) at 15 min; washout at 20 min; 5 μ M μ -Conx at 25 min.

Fig. 8.

Molecular overlapping between v-Lipid A and 19-mer peptide in α subunit of Nav1.4 channel.

A: The 19-mer peptide of the α subunit in Nav1.4 channel (in Fig. 9) is drawn as the gray ribbon structure, of which the side chains of amino acid residue of dipeptide (Leu³-Lys⁴) is discriminated as the CPK representation (Leu, green; Lys, red). The phosphate group of v-Lipid A is shown in yellow. **B:** v-Lipid A is drawn as the CPK representation with different-colored atoms (oxygen, orange; phosphorus, red; carbon, gray; nitrogen, blue; hydrogen, white). **C:** The chemical structure of v-Lipid A is given as having the substituted groups, R1, R2, R3, R4, and R5 as indicated in Table II.

Fig. 9.

Schematic representation of the Nav1.4 channel α -subunit on the plasma membrane. The Nav1.4 channel α subunit molecule is illustrated to approximate scale, with channel topology adopted from current voltage-gated Na channel models (Yu and Catterall, 2003). Domains I through IV contain 6 transmembrane segments (S1–S6). The extracellular linker between S5 and S6 forms outer loops. The 19-mer peptide is specific for Nav1.4 and located in the intracellular domain. D1, D2, D3, and D4 are locations of dipeptide (Table III) as expected by molecular overlapping between domains I to IV and the v-Lipid A.

Fig. 10.

Domain structure and overlapping dipeptide. **A:** Domains I to IV of the Nav1.4 channel α subunit are drawn as the CPK representation. The dipeptides in Table III are highlighted in red-colored atoms. **B:** The v-Lipid A is drawn in the gray-colored atoms (oxygen, carbon, and nitrogen) and the yellow-colored atoms (phosphorus) without hydrogen as the CPK representation. The dipeptide (Ser-Tryp, Ser-Tyr, Ser-Glu, or Lys-Lys) is molecularly overlapped with the v-lipid A. The amino acid components are distinguished by different-colored atoms, as follows: Ser, green; Try, purple; Tyr, orange; Glu, blue; Lys, red.

TABLE I
Relationship Between Antibody Titer values and Immunoabsorption*

Serum ^a	Immunoabsorption ^b	Titer ^c				
		GM1	GM2	GD1a	GM3	GD1b
<i>Human</i>						
H1.1 (day 22)	—	3200	—	—	800	1600
H1.2 (day 22)	+GM1	—	—	—	400	1600
H1.3 (day 22)	+GM1,+Kdo2-Lipid A	—	—	—	—	—
<i>Chicken</i>						
A1.1 (HS 6)	—	400	—	—	800	800
A1.2 (HS 6)	+Kdo2-Lipid A	—	—	—	400	—
B1.1 (HS 21)	—	—	6400	—	800	800
B1.2 (HS 21)	+Kdo2-Lipid A	—	3200	—	400	—
C1.1 (VLA 2/18)	—	6400	—	—	3200	3200
C1.2 (VLA 2/18)	+GM1	—	—	—	1600	1600
C1.3 (VLA 2/18)	+GM3	3200	—	—	3200	1600
C1.4 (VLA 2/18)	+GM1,+GM3	—	—	—	—	800
C1.5 (VLA 2/18)	+GM1,+GM3,+Kdo2-Lipid A	—	—	—	—	—
D1.1 (GM1)	—	6400	—	—	—	1600
D1.2 (GM1)	+GM1	—	—	—	—	400
D1.3 (GM1)	+GM1,+Kdo2-Lipid A	—	—	—	—	—
E1.1 (GM2)	—	—	3200	—	—	800
E1.2 (GM2)	+GM2	—	—	—	—	400
E1.3 (GM2)	+GM2,+Kdo2-Lipid A	—	—	—	—	—
F1.1 (GM3)	—	—	—	—	3200	1600
F1.2 (GM3)	+GM3	—	—	—	—	800
F1.3 (GM3)	+GM3,+Kdo2-Lipid A	—	—	—	—	—

* Human serum (H1.1 to H1.3) and chick serum (A1.1 to A1.2, B1.1 to B1.2, C1.1 to C1.5, D1.1 to D1.3, E1.1 to E1.3, F1.1 to F1.3) were tested for ganglioside and lipid A antibody titration by ELISA and led to immunoabsorbed serum of human (H1.2 to H1.3) and chicken (A1.2 to B1.2, C1.2 to C1.5, D1.2 to D1.3, E1.2 to E1.3, F1.2 to F1.3) with immunoabsorption treatment by GM1, GM3, or Kdo2-Lipid A.

^aDays after isolation of strain VLA2/18, the infecting *C. jejuni* strain, or the ganglioside used for chicken immunization.

^bImmunoabsorption indicates pretreatment with ganglioside (+GM1, +GM2, or+GM3) or Kdo2-Lipid A (Kdo2-Lipid A).

^cTiters indicates dilutions of antisera giving an absorbance of ELISA of less than 0.1.

TABLE II

Structure of Lipid A

Kdo2-Liquid A	v-Liquid A
$R_1 = \begin{array}{c} \text{H} \\ \\ -\text{C}-\text{O}-\text{C}-\text{C}_{13}\text{H}_{27} \\ \\ \text{C}_{11}\text{H}_{23} \end{array}$	$-\text{C}_2\text{H}_4-\text{O}-\text{CH}_2\text{OH}$
$R_2 = \begin{array}{c} \text{H} \\ \\ -\text{C}-\text{O}-\text{C}-\text{C}_{11}\text{H}_{23} \\ \\ \text{C}_{11}\text{H}_{23} \end{array}$	$-\text{C}_2\text{H}_4-\text{O}-\text{CH}_2\text{OH}$
$R_3 = \begin{array}{c} \text{OH} \\ \\ -\text{CH}_2-\text{CH}-\text{C}_{11}\text{H}_{23} \end{array}$	$-\text{CH}_2\text{OH}$
$R_4 = \begin{array}{c} \text{OH} \\ \\ -\text{CH}_2-\text{CH}-\text{C}_{11}\text{H}_{23} \end{array}$	$-\text{CH}_2\text{OH}$
$R_5 = -\text{Kdo}-\text{Kdo}$	$-\text{H}$

TABLE III

Similar Structure to v-Lipid A in the Nav1.4 Extracellular Domains*

Domain of Nav1.4 α submit	Secondary structure prediction of amino acid residue sequence of extracellular loop following S5 segment
I	<p>**</p> <p>HGHIRQKCVRWPPPHDTHTTWYGNDTWYSHDTWYGNDTWYINDTWNSQESWAGNSTF CCCCCCEECCCCCCCCCECCCCCECCCCCCCCCCCCCCCCCCCCCCCCCCCC DWEAYINDEGNFYFLEGSNDIICGNSSDAGHCPEGYECIKAGRNPNYGYTS CCCHHHHCCCCCEEECCCCCECCCCCCCCCCCCCEEECCCCCCCCCCCC</p>
II	<p>**</p> <p>GKSYKECVCKIASDCNIPRWH CCCCCCCCCCCCCCCCCC</p>
III	<p>**</p> <p>FYYCYNTTTSERFDISVVNNKSESESIMYTGOWRWMNVKVN CEEEECCCCCEEEEECCCCCHHHHHHCCCEEECCCCC</p>
IV	<p>**</p> <p>KKESGIDDFN CCCCCCCCCC</p>

* H, alpha-helix; E, extended strand; C, coil; SW, Ser-Trp; SY, Ser-Tyr; SE, Ser-Glu; KK, Lys-Lys.

TABLE IV

Similat Strucutre to v-Lipid A in the Nav Extracellular Domains

	Domain I	Domain II	Domain III	Domain IV
Mouse Nav 1.1	Ser-Ile	Ser-Tyr	Ser-Asp	Lys-Arg
1.2	Ser-Phe	Ser-Tyr	Ser-Glu	Lys-Arg
1.3	Ser-Tyr	Ser-Tyr	Ser-Asp	Lys-Lys
1.4	Ser-Trp	Ser-Tyr	Ser-Glu	Lys-Lys
1.5	Ser-Val	Ser-Glu	Ser-Glu	Lys-Lys
1.6	—	Ser-Tyr	—	Lys-His
1.7	Ser-Glu	Ser-Tyr	Ser-Asp	Lys-Lys
1.8	Ser-Ser	Ser-Glu	Ser-Asp	—
1.9	Ser-Glu	—	Ser-Glu	Lys-Arg