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Experimental Immunization with *Borrelia burgdorferi* Induces Development of Antibodies to Gangliosides

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Patients with neuroborreliosis produce antibodies, mostly of the immunoglobulin M (IgM) class, to gangliosides, particularly to those with Gal(β1-3)GalNac terminal sequences. Lewis rats were immunized with a nonpathogenic strain of *Borrelia burgdorferi* and with a chloroform-methanol extract (nonprotein) of this organism (CM) to determine whether antibodies to *B. burgdorferi* also recognized gangliosides. Rats were also immunized with asialo-GM1 to determine whether the elicited antibodies recognized antigens in *B. burgdorferi*. Rats immunized with *B. burgdorferi* produced low levels of IgM antibodies that cross-reacted with asialo-GM1 and GM1. Rats immunized with CM had marked IgM reactivity to asialo-GM1 and GM1. Immunization with asialo-GM1 resulted in antibodies that cross-reacted with *B. burgdorferi* antigens. Although antibodies to *B. burgdorferi* were of both the IgM and IgG classes, those to CM and to asialo-GM1 and GM1 were predominantly in the IgM fraction. Reactivity of the IgM antibodies decreased after adsorption with the heterologous and the homologous antigens, indicating bidirectional cross-reactivity between CM, asialo-GM1, and GM1 and that immunization with one produces antibodies to the other. There was no in vivo deposition of Ig in peripheral nerves, nor was there nerve pathology as a result of immunizations, but IgM antibodies to asialo-GM1 and CM recognized homologous antigens in the nodes of Ranvier of peripheral nerves from nonimmunized rats. This immunization model suggests that antibodies to gangliosides in Lyme disease have a microbial origin and are potentially relevant in pathogenesis.

Lyme disease has neurological manifestations which fall under the broad designation of neuroborreliosis. This disease is a complex spirochetosis caused by *Borrelia burgdorferi* (6, 7, 42) and can include dysfunction of both the central and peripheral nervous systems. The various manifestations can be of early as well as late onset and have a broad clinical spectrum (14, 41).

The pathogenesis of neuroborreliosis is not completely understood. Overt neurological manifestations have not been reported in rodent models of Lyme disease, although the organisms are known to penetrate the central nervous system (17, 32). Experimental studies have shown that *B. burgdorferi* can adhere to cells of neural origin (16, 18, 46), initiating a process which can lead to cell injury (19). This organism also induces C6 glioma cells to secrete interleukin 6 (21), a cytokine also found in the cerebrospinal fluid of patients (52). There is evidence for the development of autoreactive antibodies to myelin and myelin components (15, 29, 44). A series of studies have described cross-reactive polyclonal and monoclonal antibodies which recognize flagellar antigenic determinants as well as epitopes on neural cells (1, 12, 40).

Prior evidence has shown that patients with neuroborreliosis and syphilis develop antibodies to gangliosides. In studies with European patients there was a marked immunoglobulin M (IgM) reactivity to GM1 in the cerebrospinal fluid of neuroborreliosis patients (51), although serum reactivity appeared to be composed of both IgM and IgG antibodies (50). In an earlier study, we examined reactivity of a subset of neuroborreliosis patients to gangliosides with the Gal(β1-3)GalNac terminal sequence (gangliosides GM1, GD1b, and asialo-GM1 [gangliotetraosyl ceramide]). IgM antibodies to gangliosides were significantly more frequent in patients with neuroborreliosis than in patients with other manifestations of Lyme disease (20). Monoclonal IgM and polyclonal antibodies to gangliosides with variable specificities have been associated with neurological disorders, particularly with lower motor neuron syndromes (33, 34, 37, 47, 54) but with other conditions as well (11, 26, 35).

Gangliosides are amphipathic acidic glycosphingolipids which are composed of a ceramide molecule and various combinations of one or more sialic acids, amino sugars, and monohexoses. The ceramide molecule is, in turn, composed of a sphingosine backbone and fatty acids. Many types of gangliosides are expressed on the surface of cells, where they perform various functions, including being binding sites of bacterial toxins (cholera and diphtheria), having a role in signal transduction and immunomodulation, and acting as markers of neoplasia (22, 39).

The role of antibodies to gangliosides, if any, in the pathogenesis of Lyme disease is unclear. Their origin is not known either. These antibodies could develop as a result of cross-reactivity to spirochetal antigens in a manner analogous to that of antibodies which develop to gangliosides following *Campylobacter* infection (10, 30, 31, 49, 55, 56). Although the glycolipid antigens of *B. burgdorferi* are not as well characterized as those of the lipopolysaccharide (LPS) of enteric bacteria, such molecules have been detected and are known to have biological and antigenic activities (4, 9, 36, 45, 53). Spirochetes are unable to synthesize their own lipid macromolecules, simply incorporating available long-chain fatty acids (5); this may represent a means of generating strain diversity. Nonetheless, partial characterization of nonprotein antigens of *B. burgdorferi* has been achieved (5, 9).

As is the case with many disorders in which antibodies to gangliosides occur, their pathophysiological importance is not entirely understood. These antibodies may initiate the disease

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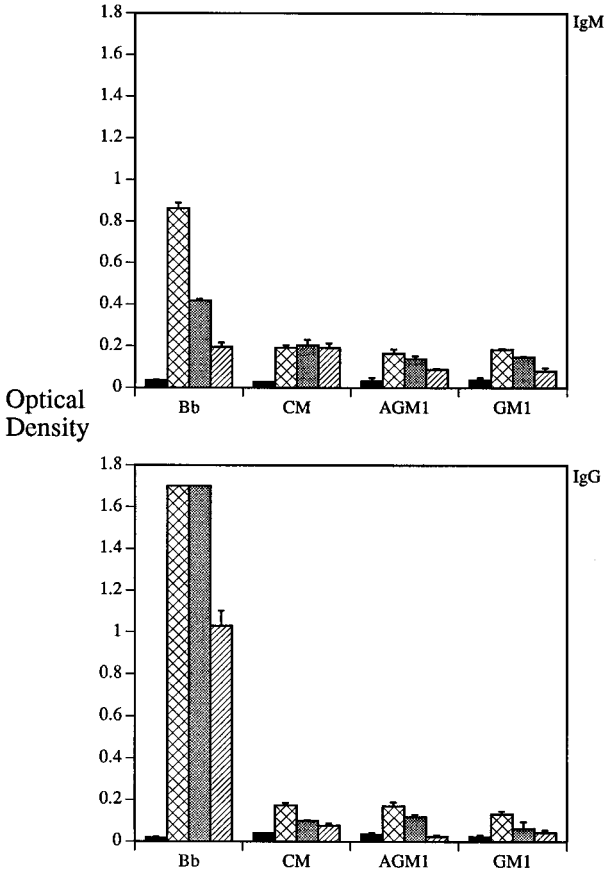

FIG. 1. Serologic responsiveness of rats immunized with live *B. burgdorferi* to *B. burgdorferi* antigen (Bb), CM, asialo-GM1 (AGM1), and GM1. Symbols: ■, day 0 baseline value (preimmunization), ▩, 28 days postimmunization; ▤, 42 days postimmunization; ▨, 56 days postimmunization.

process or be the product of associated responses to the disease. Thus, on the basis of both the precedent that Lyme disease patients have antibodies to gangliosides and the *Campylobacter jejuni* anti-GM1 response, we considered the possibility that antibodies to gangliosides in experimental animals arise as a consequence of immunization, not infection, with live nonpathogenic *B. burgdorferi* and *B. burgdorferi* nonprotein antigens. Conversely, we also considered whether antibodies to this organism or its components could develop bidirectionally as a result of immunization with endogenous glycosphingolipids.

MATERIALS AND METHODS

Bacteria and animals. The B31 strain of *B. burgdorferi* grown in serum-free medium was used for all experiments (7). This strain was chosen for immunizations because, as a high-passage strain, it is not infectious for laboratory animals. Male Lewis rats (200 to 300 g) were used for these experiments.

Experimental design. Groups of six rats each were immunized subcutaneously on days 0, 7, 14, and 25 for a total of four inoculations each as follows. Group 1 received 5×10^7 *B. burgdorferi* spirochetes in Hanks' balanced salt solution. Group 2 received 1 mg of chloroform-methanol extract of *B. burgdorferi* (CM) (see below) emulsified in 100 μl of incomplete Freund's adjuvant (IFA). Group 3 received 1 mg of asialo-GM1 (gangliotetraosyl ceramide; Matreya Inc., Pleasant Gap, Pa.) emulsified in 100 μl of IFA. Group 4 received 100 μl of IFA, and group 5 served as an untreated control.

Immediately prior to the first inoculation on day 0, the rats were bled from the tail veins to provide samples for baseline serology. On days 28, 42, and 56, two to four rats from each group were sacrificed by CO_2 inhalation and bled by cardiac puncture and both sciatic nerves were removed.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was carried out essentially as described by Thomas et al. (48) in polyvinyl chloride plates (Falcon/Becton Dickinson Co., Oxnard, Calif.). Plates were coated with sonicated *B. burgdorferi* antigen at a concentration of 5 μg of protein per ml in high-pH carbonate buffer (2.145 g of $Na_2HCO_3 \cdot 10H_2O$ and 1.456 g of $NaHCO_3$ in 500 ml of distilled water at pH 9.6). CM, asialo-GM1, and ganglioside GM1 (Matreya) were dissolved in methanol also at a concentration of 5 μg/ml. For some experiments, sphingosine and ceramide (Sigma, St. Louis, Mo.) were dissolved in chloroform-methanol (1:1, vol/vol) and the polyvinyl chloride plates were also coated with gangliosides GD1a, GT1B, and GM2 (Matreya, Inc.) dissolved in methanol at the same concentrations. Each well received 100 μl of each antigen for a final concentration of 0.5 μg per well. The plates were incubated at 4°C overnight so that the methanol would evaporate. Following three washes with phosphate-buffered saline (PBS; pH 7.4), the plates were blocked with 200 μl of PBS supplemented with 2% bovine serum albumin (BSA) for 4 h at 4°C; this was followed by three PBS washes, after which the plates were dried and stored in aluminum foil at −20°C until used.

Serum diluted at 1:100 in PBS supplemented with 1% BSA and 0.25% Tween 20 was incubated at 4°C overnight and then washed three times with the same diluent. Horseradish peroxidase-conjugated anti-rat IgM (μ chain specific) and anti-rat IgG (γ chain specific) (Organon-Teknika, Durham, N.C.) in diluent were added and incubated for 2 h at 37°C; this was followed by three washes and addition of 100 μl of substrate (0.05 M Na_2HPO_4, 0.024 M sodium citrate, 0.04% *O*-phenylenediamine; pH 5.0) and 0.0003% hydrogen peroxide. Optical density was determined in an ELISA spectrophotometer (model MR100; Dynatech Laboratories, Alexandria, Va.) at a wavelength of 490 μm. Commercially purchased rabbit antisera to asialo-GM1 and GM1 (Matreya, Inc.) and horseradish peroxidase-conjugated anti-rabbit Ig (polyvalent) were used as controls in each plate to ascertain that asialo-GM1 and GM1 were bound to the wells. Control wells were blocked with PBS–2% BSA but did not contain any antigens. The baseline sera (day 0) and the sera collected after sacrificing each rat were tested as paired specimens together in the same plate to minimize variation from test to test. Possible endogenous reactivity for borrelial antigens, asialo-GM1, and

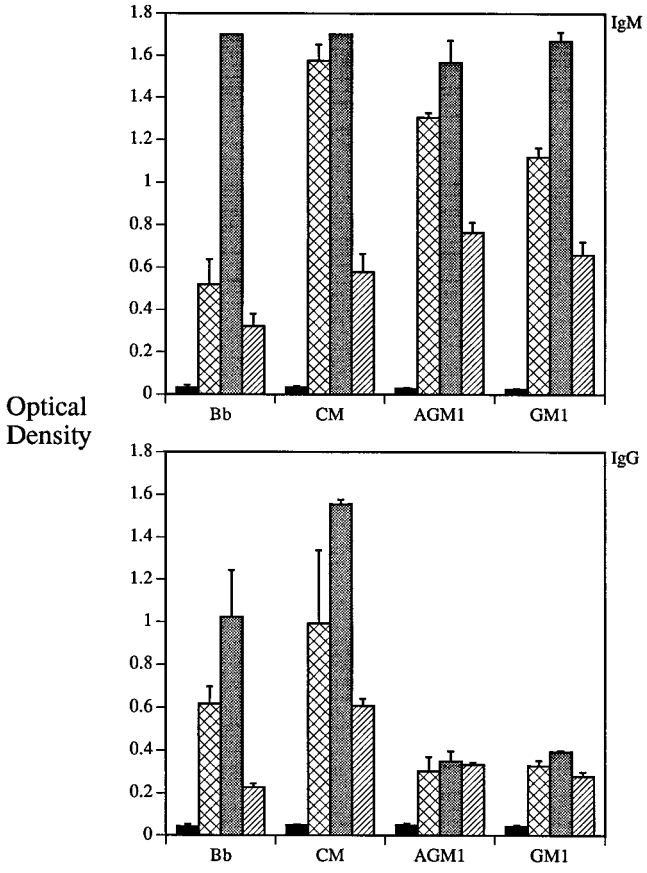

FIG. 2. Serologic responsiveness of rats immunized with CM to *B. burgdorferi* antigen (Bb), CM, asialo-GM1 (AGM1), and GM1. Symbols: ■, day 0 baseline value (preimmunization), ▩, 28 days postimmunization; ▤, 42 days postimmunization; ▨, 56 days postimmunization.

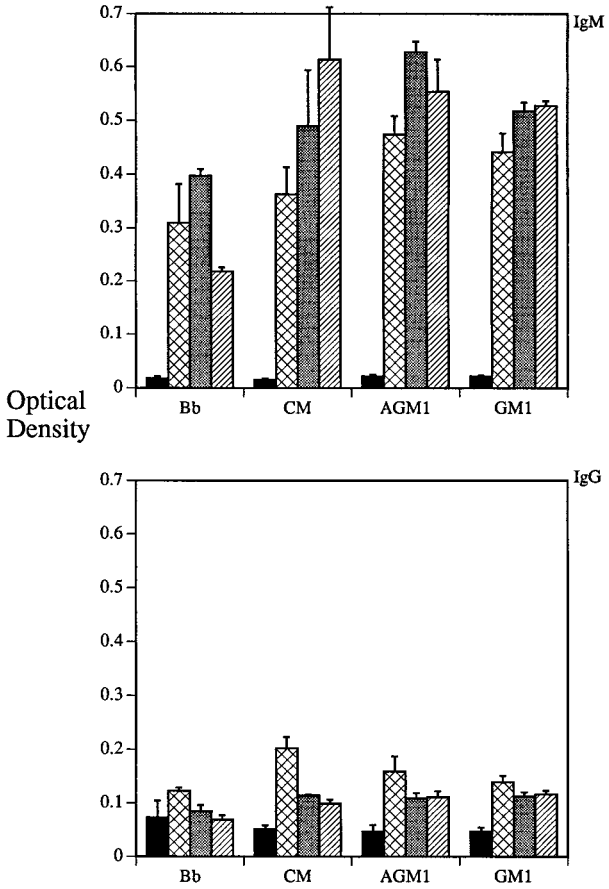

FIG. 3. Serologic responsiveness of rats immunized with asialo-GM1 to *B. burgdorferi* antigen (Bb), CM, asialo-GM1 (AGM1), and GM1. Symbols: ■, day 0 baseline value (preimmunization), ▨, 28 days postimmunization; ▤, 42 days postimmunization; ▨, 56 days postimmunization.

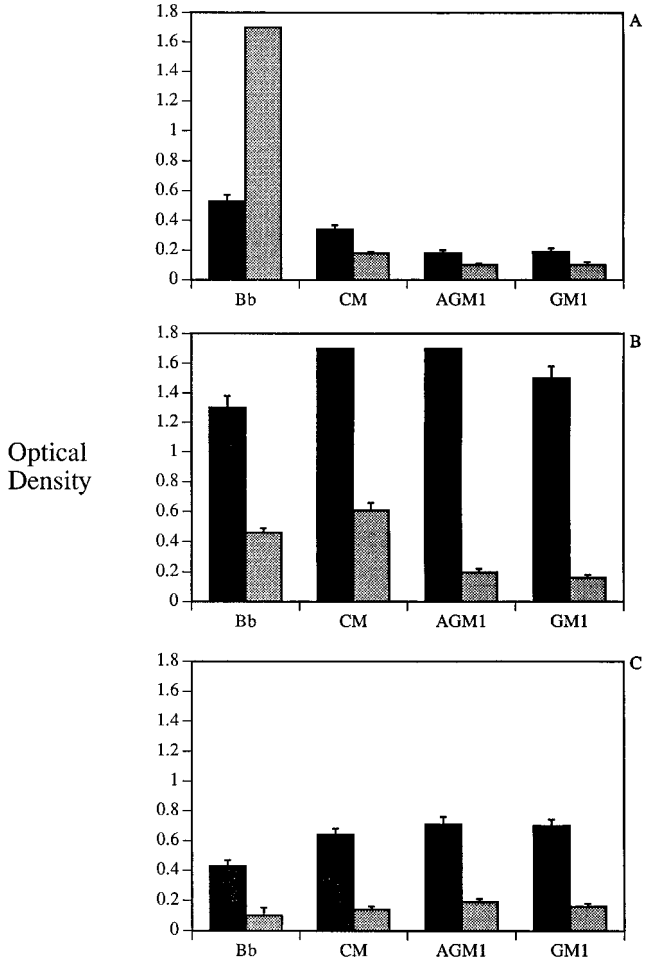

FIG. 4. Ig class-specific response of rats immunized with live *B. burgdorferi* (A) CM (B), and asialo-GM1 (C) to *B. burgdorferi* antigen (Bb), CM, asialo-GM1 (AGM1), and GM1. ■, IgM fraction of rat serum; ▤, IgG fraction of rat serum.

GM1 by the conjugates used in the assays was ruled out a priori by carrying out an ELISA without primary antibody (rat serum).

Preparation of borrelial antigens. CM was prepared as described elsewhere (13) with the modifications introduced by Wheeler et al. (53). Briefly, *B. burgdorferi* was grown in serum-free BSK medium to yield 2 g (wet weight), and after three washes in PBS, the pellet was dried under a stream of nitrogen gas and extracted in a 2:1 (vol/vol) chloroform-methanol mixture (13). The organic material was again dried under nitrogen and dissolved in 6 parts of 2:1 chloroform-methanol and washed with 1 part of double-distilled water. Solvents were evaporated to collect the extracts with a Brinkman Rotavapor R to permit direct weighing. From an initial 2 g of spirochetes, approximately 20 mg of extract was usually obtained. Aliquots of CM were evaporated and resuspended in 500 μl of 1% Triton in water for detection of contaminating polypeptides by a bicinchoninic acid assay (Pierce, Rockford, IL). Only extracts which did not contain contaminating polypeptides by the bicinchoninic acid assay were used. For immunizations, the desired quantities of CM were emulsified in IFA.

Purification of Igs and adsorption. Serum from individual rats (and in some cases pooled sera from two to three rats sacrificed on the same day) was treated with saturated ammonium sulfate and centrifuged at 10,000 × *g* for 30 min, and the precipitate was resuspended in PBS and dialyzed. Further purification of Igs was obtained by affinity chromatography using protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) for IgG and a matrix of the IgG fraction of anti-rat IgM bound to protein G-Sepharose for IgM. The affinity products were dialyzed against distilled water, vacuum concentrated to 1 to 2 mg of IgM per ml and 8 to 10 mg of IgG per ml in PBS, and stored at −70°C until used. The purity of the fraction was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue using purified rat IgM and IgG as controls (Accurate Diagnostics, Westbury, N.Y.), and the concentrations were determined by ELISA using alkaline phosphatase-conjugated anti-rat IgM and IgG.

Asialo-GM1 and GM1 affinity chromatography was done as described previously (25). Briefly, 1 mg of GM1 and 1 mg of asialo-GM1 were bound separately to 1 ml (packed volume) of prewashed octyl-Sepharose CL 4B (Pharmacia Fine Chemicals) in a solution of methanol-water (1:1, vol/vol) and 0.1 M KCl for 1 h at room temperature. The suspension was heated to 70°C, poured into a 10-ml liquid chromatography column, and washed extensively with PBS. The asialo-GM1- and GM1-bound octyl-Sepharose CL 4B was incubated overnight with 1.5 mg of rat IgM per ml of PBS at 4°C in a rocking platform. The unadsorbed fraction was eluted with PBS until the optical density measured at 280 nm was zero. This procedure left the IgM antibodies adsorbed to the GM1 and asialo-GM1 in the column. The eluate was dialyzed, and the concentration of the IgM was measured by Bradford assay; this was followed by reconstitution in PBS-BSA to a concentration of 0.1 mg/ml so that 100 μl of IgM suspension would be equivalent to a 1:100 dilution of rat serum (assuming rat serum to have a normal concentration of 1 mg of IgM per ml) and subsequent use for the serologic assays. Octyl-Sepharose CL 4B served as a control for nonspecific adsorption.

Adsorption with whole-cell *B. burgdorferi* was done by incubating 1 ml (1.5 mg/ml) of IgM with 10^7 organisms at 4°C for 4 h in a rocking platform and then centrifuging the mixture at 7,000 × *g* for 10 min to separate the spirochetes. The spirochete pellet was incubated with fluorescein isothiocyanate-conjugated anti-rat IgM to visualize antibody binding to the organisms. Adsorption with CM was done by incubating 1.5 mg of rat IgM per ml on polyvinyl chloride plates coated with 10 μg of CM per well. This was done by an overnight incubation at 4°C. The plates were developed by ELISA as above to determine the success of the adsorption using horseradish peroxidase-conjugated anti-rat IgM. The IgM adsorbed with whole-cell *B. burgdorferi* and CM was reconstituted to a concentration of 0.1 mg/ml each as indicated above for the adsorption with asialo-GM1 and GM1.

Nerve examination. Sciatic nerves were removed after exsanguination of the rats and split into 1-cm longitudinal pieces. Some nerve segments were immediately embedded in OCT compound (Miles Inc., Elkhart, Ind.) for both longitudinal and cross sections and frozen in 2-methylbutane-isopentane and liquid nitrogen. The frozen blocks were sectioned (4-μm thickness) and stored at

TABLE 1. Results of adsorptions of the pooled IgM fraction from rats sacrificed on day 42 after immunization with CM

Substance with which IgM was adsorbed	Mean optical density ± SD (% decrease from control)[a] in sera tested against:			
	B. burgdorferi	CM	Asialo-GM1	GM1
None (unadsorbed control)	1.30 ± 0.08	1.7	1.7	1.50 ± 0.09
B. burgdorferi	0.41 ± 0.10 (69)***	0.63 ± 0.09 (63)***	0.58 ± 0.07 (66)***	0.51 ± 0.09 (62)***
CM	0.96 ± 0.10 (27)*	0.41 ± 0.09 (76)***	0.39 ± 0.06 (77)***	0.42 ± 0.08 (72)***
Asialo-GM1	0.94 ± 0.11 (28)**	0.39 ± 0.08 (67)***	0.31 ± 0.05 (82)***	0.33 ± 0.06 (78)***
Octyl-Sepharose CL 4B	1.10 ± 0.09 (16)	1.51 ± 0.07 (11)	1.40 ± 0.06 (18)	1.23 ± 0.06 (18)

[a] Values are from triplicate wells. Levels of statistical significance by one-way ANOVA, compared with unadsorbed controls: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

−70°C until used for immunofluorescence studies. Primary antibodies were incubated at the predetermined optimal dilutions for 1 h at 37°C in a moist chamber. The sections were washed in PBS, and fluorescein isothiocyanate- and rhodamine-conjugated anti-rat IgM or IgG secondary antibodies (conjugates) also at predetermined optimal dilutions were incubated for 1 h at 37°C and subjected to a final PBS wash. Slides were mounted in Slow Fade (Molecular Probes, Inc., Eugene, Oreg.) and examined by light or confocal microscopy using a Nikon inverted Diaphot with an Odyssey laser confocal system (Noran, Middleton, Wis.). Image capture was done with Image I software from Universal Imaging, Westchester, Pa.

Sciatic nerve segments were also fixed in 10% buffered formalin, embedded in paraffin, sectioned at a width of 4 μm, deparaffinized, and stained with hematoxylin and eosin and with Luxol fast blue–periodic acid-Schiff stain.

Statistics. Student's *t* tests and one-way analysis of variance (ANOVA) were done with StatView (Abacus Concepts, Berkeley, Calif.).

RESULTS

Antibodies to *B. burgdorferi* react with asialo-GM1 and GM1. The responses of rats immunized with live *B. burgdorferi* showed a substantial increase in both IgM and IgG antibodies against the homologous antigen (Fig. 1, Bb). IgM and IgG antibodies to CM, asialo-GM1, and GM1 developed by day 28 (Fig. 1), but reactivity to these antigens was much less than the reactivity noted in the sera of rats immunized with *B. burgdorferi* (Fig. 1).

Immunization with CM resulted in the development of IgM antibodies which recognized all four antigens (Fig. 2). These findings indicate that immunization with CM results in antibody recognition of *B. burgdorferi* antigen since the CM is a constituent of the live organisms. The IgM responses of rats immunized with CM to asialo-GM1 and GM1 suggest a cross-reactive phenomenon. IgG reactivity also developed against *B. burgdorferi* antigen and the homologous CM but was less pronounced against asialo-GM1 and GM1 (Fig. 2).

Antibodies to asialo-GM1 react with components of *B. burgdorferi*. Immunization with asialo-GM1 resulted in the development of IgM antibodies to all four antigens (Fig. 3) which reacted at approximately the same levels to CM, asialo-GM1, and GM1 (Fig. 3). These results are consistent with those obtained for the IgM antibodies of rats immunized with CM (Fig. 2) and are taken as evidence of cross-reactivity. All sera used in this study were tested in triplicate against control wells blocked with 2% BSA (see Materials and Methods), with no detectable reactivity. BSA is known to bind fatty acids and could be a source of assay contaminants since the growth medium for spirochetes does not contain serum (another possible source of contaminants) but does contain BSA. Thus, the possibility that this bidirectional cross-reactivity was due to culture medium or assay contaminants has been minimized.

Antibodies which recognized ganglioside GM1 and asialo-GM1 also recognize components of *B. burgdorferi*. There is some IgG reactivity present in the sera of rats immunized with asialo-GM1 to *B. burgdorferi*, CM, asialo-GM1, and GM1. Control rats (immunized with IFA and untreated) had serologic values similar to the preimmunization levels in all the rats in both IgM and IgG ELISAs (data not shown).

Cross-reactive antibodies are mostly of the IgM class. Given the marked IgM rise in immunized rats and the possibility of bidirectional cross-reactivity between *B. burgdorferi* antigens and asialo-GM1 and GM1, the Ig from the sera collected on day 42 postimmunization (which in most of the rats had the highest optical density values for all antigens) was precipitated and further separated into purified IgM and IgG fractions by affinity chromatography. Thus, rat IgM and IgG were tested against all four antigens by using an IgM concentration of 0.1 mg/ml and an IgG concentration of 1 mg/ml. Rats immunized with live *B. burgdorferi* had a marked IgG response to *B. burgdorferi* antigen, but a greater response to CM, asialo-GM1, and GM1 was found in the IgM fractions (Fig. 4A). On the other hand, the IgM fraction of rats immunized with CM contained the bulk of the reactivity to all four antigens (Fig. 4B). Slight IgG reactivity was present as well. The same pattern of reactivity was noted in the IgM fraction of rats immunized with asialo-GM1 (Fig. 4C). The results obtained with purified IgM and IgG (Fig. 4) are consistent with those obtained with serum (Fig. 1 to 3), and so immunization of rats with nonpathogenic (but clearly immunogenic) live *B. burgdorferi* may result in an expected pattern of IgM reactivity to whole-cell *B. burgdorferi* antigens followed by an isotype switch to IgG. Interestingly, the reactivity to CM, asialo-GM1, and GM1 remained predominantly in the IgM fractions. In contrast, rats immunized with CM developed a strong IgM response to asialo-GM1 and GM1. Some IgG reactivity was detectable (Fig. 2, 3, and 4B and C), but there was no expansion in the reactivity of this Ig class. From these results, it can be concluded that IgM antibodies which develop following immunization with live *B. burgdorferi* and CM recognize asialo-GM1 and GM1. The converse, that IgM antibodies generated after immunization with asialo-GM1 recognize spirochetal antigens as well as GM1, is also true.

Cross-reactivity can be demonstrated by adsorption with heterologous antigens. Purified IgM suspensions from rats immunized with CM and asialo-GM1 were adsorbed with whole-cell live *B. burgdorferi*, CM, and asialo-GM1 and with octyl-Sepharose CL 4B as a control and retested against all four antigens. Significantly decreased reactivities after the various adsorptions of IgM from rats immunized with CM (Table 1) or with asialo-GM1 (Table 2) further demonstrated the cross-reactive nature of these antibodies. The adsorptions with the four antigens of the sera from rats immunized with asialo-GM1 did not result in significantly decreased reactivity to *B. burgdorferi* antigen (Table 2). This may indicate that the cross-reactive antigens are not readily accessible in the intact organisms, but adsorption with the CM extract resulted in significant reductions in reactivity to both asialo-GM1 and CM, under-

TABLE 2. Results of adsorptions of the pooled IgM fraction from rats sacrificed on day 42 after immunization with AGM1

Substance with which IgM was adsorbed	Mean optical density ± SD (% decrease from control)[a] in sera tested against:			
	B. burgdorferi	CM	Asialo-GM1	GM1
None (unadsorbed control)	0.43 ± 0.04	0.64 ± 0.04	0.71 ± 0.05	0.71 ± 0.04
B. burgdorferi	0.33 ± 0.04 (23)*	0.51 ± 0.07 (20)*	0.58 ± 0.07 (18)*	0.53 ± 0.05 (25)**
CM	0.37 ± 0.06 (14)	0.24 ± 0.05 (63)***	0.21 ± 0.03 (70)***	0.21 ± 0.03 (70)***
Asialo-GM1	0.39 ± 0.05 (10)	0.21 ± 0.03 (67)***	0.18 ± 0.03 (75)***	0.19 ± 0.03 (73)***
GM1	0.39 ± 0.04 (10)	0.22 ± 0.04 (66)***	0.18 ± 0.02 (75)***	0.17 ± 0.03 (76)***
Octyl-Sepharose CL 4B	0.54 ± 0.03	0.43 ± 0.04 (33)**	0.59 ± 0.03 (17)*	0.59 ± 0.04 (17)*

[a] Results are from triplicate wells. Levels of statistical significance by one-way ANOVA, compared with unadsorbed controls: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

scoring that the cross-reactive antigen(s) is in the CM fraction (Table 2).

Fine specificity of the IgM fractions. Purified IgM from rats immunized with *B. burgdorferi*, CM, and asialo-GM1 did not show any reactivity to sphingosine and ceramide ($P > 0.05$, compared with BSA controls), indicating that the reactivity seen in these sera requires a carbohydrate component not found in the lipid backbones of GM1 and asialo-GM1 (Table 3). In separate experiments, purified IgM from rats immunized with live organisms, CM, and asialo-GM1 were tested for reactivity to gangliosides GD1a, GT1b, and GM2 (Table 3). Purified IgM from rats immunized with live *B. burgdorferi* did not react to any of these glycosphingolipids ($P > 0.05$, compared with BSA controls). IgM from rats immunized with CM showed reactivity to gangliosides GD1a and GT1b (mean optical densities of 0.32 ± 0.04 and 0.39 ± 0.05, respectively; $P < 0.05$, compared with BSA controls). Values obtained for GD1a and GT1b were, however, significantly less ($P < 0.01$) than those obtained for GM1 and asialo-GM1 (1.31 ± 0.06 and 1.44 ± 0.05, respectively). IgM from rats immunized with asialo-GM1 had slight reactivity to gangliosides GD1a, GT1b, and GM2 ($P < 0.05$, compared with controls).

Neuropathology. Direct immunofluorescence of sciatic nerves removed from all the immunized rats did not disclose any evidence of Ig deposition. Purified IgM (at 0.1 mg/ml) from rats immunized with CM and from rats immunized with asialo-GM1 bound to the nodes of Ranvier in sections of sciatic nerves from untreated rats (Fig. 5B). Each axon is surrounded by myelin formed by a series of adjacent Schwann cells. The nodes of Ranvier are gaps in the myelin sheath representing spaces between adjacent Schwann cells. The nodes of Ranvier are known to contain glycosphingolipids and glycoproteins which share the Gal(β1-3)GalNac terminus (2, 8, 38, 43, 48). Reactivity was measured to a dilution of 0.05 mg/ml (twofold dilution of original 0.1 mg/ml) and disappeared thereafter. Untreated rat IgM did not bind to the nodes of Ranvier at the same concentrations. As a control, sciatic nerve sections were stained with rhodamine-conjugated peanut agglutinin, a lectin with terminal Gal(β1-3)GalNac specificity (Vector Laboratories, Burlingame, Calif.) (Fig. 5A). Hematoxylin-and-eosin- and Luxol fast blue–periodic acid-Schiff-stained sections of nerves revealed no significant pathological findings.

DISCUSSION

Antibodies to gangliosides can be demonstrated in patients with neuroborreliosis (20, 50, 51). The present study sought to determine whether such antibodies could be induced experimentally by immunization of laboratory animals with a nonpathogenic strain of *B. burgdorferi* and nonprotein components of this organism. Our reason for using a nonpathogenic but immunogenic strain stemmed from the hypothesis that immunization with *B. burgdorferi* antigens could result in the development of antibodies to gangliosides. Several lines of evidence suggest that this is the case. The first is that antibodies elicited after immunization with live *B. burgdorferi* recognize asialo-GM1 and GM1 at approximately the same levels as do antibodies elicited after immunization with their own nonprotein fraction (CM). Second, immunization with CM induced high levels of IgM antibodies to asialo-GM1 and GM1. Likewise, antibodies elicited by immunization with asialo-GM1 cross-reacted with antigenic determinants present in *B. burgdorferi* (Fig. 1 to 3). A third line of evidence is provided by the demonstration of reactivity after adsorption with homologous and heterologous antigens (Tables 1 and 2).

Asialo-GM1 was chosen as the immunizing agent because patients with neuroborreliosis had the highest levels of antibodies to this neutral glycosphingolipid (20). In addition, the lack of sialic acid in asialo-GM1 decreased the possibility that antibody binding is due primarily to the presence of negative charges. The experimental results presented here parallel our earlier findings of human IgM antibodies to glycosphingolipids with the Gal(β1-3)GalNac terminal sequence. The contribution of this terminal sequence is suggested by the parallel reactivity to asialo-GM1 and GM1 in ELISA, the lack of reactivity to sphingosine and ceramide (which have no sugars), and the lack of or decrease in reactivity to other gangliosides which do not have this terminal carbohydrate sequence (Table 3).

TABLE 3. Reactivity (optical density) of purified IgM from rats sacrificed on day 42 after immunization with to sphingosine, ceramide, and three gangliosides

Immunizing agent	Optical density ± SD[a] in sera tested against:					
	BSA	Sphingosine	Ceramide	GD1a	GT1b	GM2
B. burgdorferi	0.07 ± 0.01	0.05 ± 0.01	0.09 ± 0.03	0.06 ± 0.02	0.04 ± 0.01	0.06 ± 0.02
CM	0.03 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.32 ± 0.04*	0.39 ± 0.05*	0.04 ± 0.02
Asialo-GM1	0.03 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.16 ± 0.03**	0.18 ± 0.04**	0.11 ± 0.04**

[a] Levels of statistical significance by one-way ANOVA, compared with BSA controls: *, $P < 0.01$; **, $P < 0.05$.

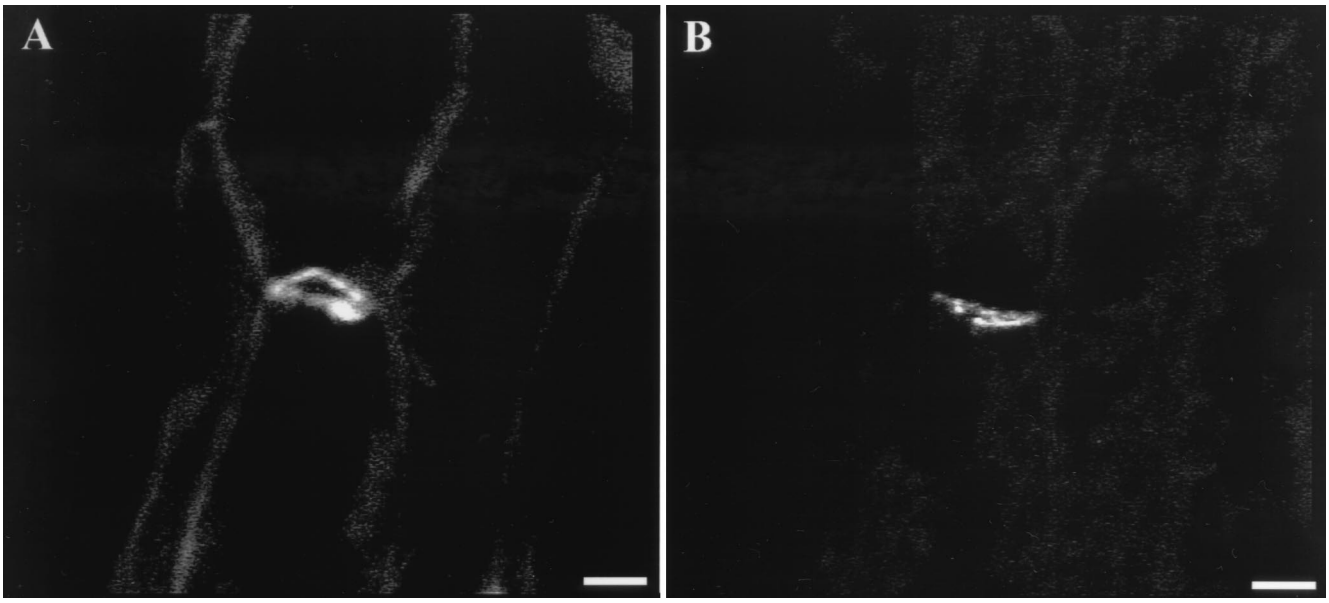

FIG. 5. Frozen untreated rat sciatic nerve (4-μm thickness) stained with rhodamine-conjugated peanut agglutinin (10 μg/ml) (A) and with 50 μg of purified IgM from rats immunized with borrelial CM extract per ml and with fluorescein isothiocyanate-conjugated anti-rat IgM (B). Identical results were obtained with IgM purified from rats immunized with asialo-GM1 at the same concentration. Confocal images were collected at 0.25 μm per slice to a depth of 4 μm and were then merged for the composite final images. Bars = 5 μm.

These experimental results are consistent with the hypothesis of a microbial origin for some of these potentially autoreactive antibodies. This situation is analogous to the induction of antibodies to GM1 after infection with *C. jejuni* (10, 30, 31, 49, 54–56). In this particular model, the origin of the cross-reactivity has been attributed to the LPS of this bacterium which carries sugar sequences similar or identical to those in ganglioside GM1 (3, 27, 55). In the case of *B. burgdorferi*, the nonprotein elements are less well characterized as to their composition and molecular structure, and borrelial glycolipid may be different from the LPS of enteric bacteria (45). Carrying this analogy further, it is noteworthy that a large number of patients who develop Guillain-Barré syndrome (30, 31, 49, 54) and other neuropathies (10, 56) had a history of a prior infection with *C. jejuni* and had antibodies to GM1. While Guillain-Barré syndrome is not a prominent finding in Lyme neuroborreliosis (14, 41), other neurological deficits more frequent in this disease could also be associated with the presence of these antibodies.

The predominance of IgM antibodies in rats immunized with CM in the recognition of asialo-GM1 and GM1, as well as in rats immunized with asialo-GM1, suggests a T-cell-independent mechanism for development of these antibodies, such as that described for other antigens of *B. burgdorferi* (28). This would be consistent with the T-cell-independent genesis of antibodies to LPS and to carbohydrates in general. Future studies could be designed to determine whether this is the case here.

There were no in vivo deposits of Ig at the nodes of Ranvier of the immunized rats. This is unlike the results obtained in rabbits immunized with GM1, in which an experimental neuropathy was produced (48); neurophysiological findings were consistent with myelin dysfunction, although pathologically only a mild axonal degeneration was found with in vivo Ig deposits at the nodes of Ranvier. The antibodies elicited in the immunized rats should have recognized the glycosphingolipids and similar carbohydrate components in the glycoproteins present in the nodes of Ranvier (2, 8, 38, 39, 43, 48), and at high IgM concentrations, we were able to document deposition on nerves of nonimmunized rats. Timing factors as well as in vivo antibody concentration could have played a role in the lack of deposition in the immunized rats. Another possibility is that the immunization regimens did not alter the permeability of the blood-nerve barrier. Nonetheless, the finding that the sera of rats immunized with CM reacted with the glycoconjugates or glycoproteins of the nodes of Ranvier indicates that these antibodies can recognize antigens presented directly on tissue.

The peripheral neuropathy of neuroborreliosis is primarily axonal (24), while demyelinating neuropathies are rare, and Ig deposition in nerves has not been demonstrated in patients. Thus, these disease characteristics are concordant with our lack of in vivo Ig deposition in the nodal areas of the nerves.

Since both monoclonal and polyclonal antibodies to gangliosides have been associated primarily with motor neuropathies (33, 34, 37), Guillain-Barré syndrome (26, 35) and other diseases (11), there exists the possibility for an association of these antibodies with neurological dysfunction in Lyme disease as well. An epidemiological study found an increased frequency of antibodies to *B. burgdorferi* in patients with amyotrophic lateral sclerosis from an area where Lyme disease is endemic (23). Since there may be cross-reactivity between gangliosides and *B. burgdorferi*, the possibility for false-positive serologies in diseases unrelated to neuroborreliosis exists. In addition to these analogous roles for antibodies to gangliosides in Lyme disease, there are other potential avenues for further documentation of other roles for these antibodies in pathogenesis. CM is antigenic (9, 53) and has proinflammatory properties via activation of macrophages (36). Thus, the inflammatory and antibody responses elicited by the cross-reactive antigens could also be responsible for injury at the cellular and tissue levels.

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REFERENCES

1. **Aberer, E., A. C. Brunner, G. Suchanek, A. G. Barbour, G. Stanek, and H. Lausmann.** 1989. Molecular mimicry and Lyme borreliosis: a shared antigenic determinant between *Borrelia burgdorferi* and human tissue. Ann. Neurol. **26:**732–737.
2. **Apostolski, S., S. A. Sadiq, A. P. Hays, M. Corbo, L. Suturkova-Milosevic, P. Chaliff, K. Stefansson, R. G. LeBaron, E. Ruoslahti, A. P. Hays, and N. Latov.** 1994. Identification of Gal(β1-3)GalNac bearing glycoproteins at the nodes of Ranvier in peripheral nerve. J. Neurosci. Res. **38:**134–141.
3. **Aspinall, G. O., S. Fujimoto, A. G. McDonald, H. Pang, L. A. Kurjanczyk, and J. L. Penner.** 1994. Lipopolysaccharides from *Campylobacter jejuni* associated with Guillain-Barré syndrome patients mimic human gangliosides in structure. Infect. Immun. **62:**2122–2125.
4. **Beck, G., G. S. Habicht, J. L. Benach, and J. L. Coleman.** 1985. Chemical and biological characterization of a lipopolysaccharide extracted from the Lyme disease spirochete (*Borrelia burgdorferi*). J. Infect. Dis. **152:**108–117.
5. **Belisle, J. T., M. E. Brandt, J. D. Radolf, and M. V. Norgard.** 1994. Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. J. Bacteriol. **176:**2151–2157.
6. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308:**740–742.
7. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grundwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216:**1317–1319.
8. **Corbo, M., A. Quattrini, N. Latov, and A. P. Hays.** 1993. Localization of GM1 and Gal(β1-3) GalNAc antigenic determinants in peripheral nerve. Neurology **43:**809–814.
9. **Eiffert, H., H. Lofter, K. Jarecki-Khan, and R. Thomssen.** 1991. Identification of an immunoreactive non-proteinaceous component in *Borrelia burgdorferi*. Med. Microbiol. Immunol. **180:**229–237.
10. **Enders, U., H. Karch, K. V. Toyka, M. Michels, J. Zielasek, M. Pette, J. Heesemann, and H. P. Hartung.** 1993. The spectrum of immune responses to *Campylobacter jejuni* and glycoconjugates in Guillain-Barré syndrome and in other neuroimmunological disorders. Ann. Neurol. **34:**136–144.
11. **Endo, T., D. D. Scott, S. S. Stewart, S. K. Kundu, and D. M. Marcus.** 1984. Antibodies to glycosphingolipids in patients with multiple sclerosis and SLE. J. Immunol. **132:**1793–1797.
12. **Fikrig, E., R. Berland, M. Chen, S. Williams, L. H. Sigal, and R. A. Flavell.** 1993. Serologic response to the *Borrelia burgdorferi* flagellin demonstrates an epitope common to a neuroblastoma cell line. Proc. Natl. Acad. Sci. USA **90:**183–187.
13. **Folch, J., M. Lees, and G. H. Sloane Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
14. **Garcia-Monco, J. C., and J. L. Benach.** 1995. Lyme neuroborreliosis. Ann. Neurol. **37:**691–702.
15. **Garcia-Monco, J. C., J. L. Coleman, and J. L. Benach.** 1988. Antibodies to myelin basic protein in Lyme disease patients. J. Infect. Dis. **158:**667–669.
16. **Garcia-Monco, J. C., B. Fernandez Villar, and J. L. Benach.** 1989. Adherence of the Lyme disease spirochete to glial cells and cells of glial origin. J. Infect. Dis. **160:**497–506.
17. **Garcia-Monco, J. C., B. Fernandez Villar, J. Calvo, and J. L. Benach.** 1990. *Borrelia burgdorferi* in the central nervous system: experimental and clinical evidence for early invasion. J. Infect. Dis. **161:**1187–1193.
18. **Garcia-Monco, J. C., B. Fernandez Villar, R. C. Rogers, A. Szczepanski, C. M. Wheeler, and J. L. Benach.** 1992. *Borrelia burgdorferi* and other related spirochetes bind to galactocerebroside. Neurology **42:**1341–1348.
19. **Garcia-Monco, J. C., B. Fernandez Villar, A. Szczepanski, and J. L. Benach.** 1991. Cytotoxicity of *Borrelia burgdorferi* for cultured rat glial cells. J. Infect. Dis. **163:**1362–1366.
20. **Garcia-Monco, J. C., C. M. Wheeler, J. L. Benach, R. A. Furie, S. A. Lukehart, G. Stanek, and A. C. Steere.** 1993. Reactivity of neuroborreliosis patients (Lyme disease) to cardiolipin and gangliosides. J. Neurol. Sci. **117:** 206–214.
21. **Habicht, G. S., L. I. Katona, and J. L. Benach.** 1991. Cytokines and the pathogenesis of neuroborreliosis: *Borrelia burgdorferi* induces glioma cells to secrete interleukin 6. J. Infect. Dis. **164:**568–574.
22. **Hakamori, S.** 1990. Bifunctional role of glycosphingolipids. J. Biol. Chem. **265:**18713–18716.
23. **Halperin, J. J., G. P. Kaplan, S. Brazinsky, T. F. Tsai, T. Cheng, A. Ironside, P. Wu, J. Delfiner, M. G. Golightly, R. H. Brown, R. J. Dattwyler, and B. J. Luft.** 1990. Immunologic reactivity against *Borrelia burgdorferi* in patients with motor neuron disease. Arch. Neurol. **47:**586–594.
24. **Halperin, J. J., B. J. Luft, D. J. Volkman, and R. J. Dattwyler.** 1990. Lyme neuroborreliosis: peripheral nervous system manifestations. Brain **113:**1207–1221.
25. **Hirabayashi, Y., T. Suzuki, Y. Suzuki, T. Taki, M. Matsumoto, H. Higashi, and S. Kato.** 1983. A new method for purification of anti-glycosphingolipid antibody. Avian anti-hematoside (NeuGc) antibody. J. Biochem. **94:**327–330.
26. **Ilyas, A. A., H. J. Willison, R. H. Quarles, F. B. Jungalwala, D. R. Cornblath, B. R. Trapp, D. E. Griffin, J. W. Griffin, and G. M. McKhann.** 1988. Serum antibodies to gangliosides in Guillain-Barré syndrome. Ann. Neurol. **23:**440–447.
27. **Kuroki, S., T. Saida, M. Nukina, T. Haruta, M. Yoshioka, Y. Kobayashi, and H. Nakanishi.** 1993. *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome belong mostly to Penner serogroup 19 and contain β-N-acetylglucosamine residues. Ann. Neurol. **33:**243–247.
28. **Ma, Y., and J. J. Weis.** 1993. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic activity and cytokine-stimulatory properties. Infect. Immun. **61:**3843–3853.
29. **Martin, R., J. Ortlauf, V. Sticht-Groh, V. Bagdahn, S. F. Goldman, and H. G. Mertens.** 1988. *Borrelia burgdorferi*-specific and autoreactive T cell lines from cerebrospinal fluid in Lyme radiculomyelitis. Ann. Neurol. **24:**509–516.
30. **Mishu, B., and M. J. Blaser.** 1993. Role of infection due to *Campylobacter jejuni* in the initiation of Guillain-Barré syndrome. Clin. Infect. Dis. **17:**104–108.
31. **Mishu, B., A. A. Ilyas, C. L. Koski, F. Vriesendorp, S. D. Cook, F. A. Mithen, and M. J. Blaser.** 1993. Serologic evidence of previous *Campylobacter jejuni* infection in patients with the Guillain-Barré syndrome. Ann. Intern. Med. **118:**947–953.
32. **Pachner, A. R., and A. Itano.** 1990. *Borrelia burgdorferi* infection of the brain: characterization of the organism and response to antibiotics and immune sera in the mouse model. Neurology **40:**1535–1540.
33. **Pestronk, A., R. N. Adams, L. Clawson, D. Cornblath, R. W. Kuncl, D. Griffin, and D. B. Drachman.** 1988. Serum antibodies to GM1 ganglioside in amyotrophic lateral sclerosis. Neurology **38:**1457–1461.
34. **Pestronk, A., V. Chaudry, E. L. Feldman, J. W. Griffin, D. R. Cornblath, E. H. Denys, M. Glasberg, R. W. Kuncl, R. K. Olney, and W. C. Yee.** 1990. Lower motor neuron syndromes defined by patterns of weakness, nerve conduction abnormalities and high titer of antiglycolipid antibodies. Ann. Neurol. **27:**316–326.
35. **Quarles, R. H., A. A. Ilyas, and H. J. Willison.** 1990. Antibodies to gangliosides and myelin proteins in Guillain-Barré syndrome. Ann. Neurol. **27**(Suppl.)**:**S48–S52.
36. **Radolf, J. D., L. L. Arndt, D. R. Akins, L. L. Curetty, M. E. Levi, Y. Shen, L. S. Davis, and M. V. Norgard.** 1995. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocyte/macrophages. J. Immunol. **154:**2866–2877.
37. **Salazar-Grueso, E. F., M. J. Routbort, J. Martin, G. Dawson, and R. P. Roos.** 1990. Polyclonal IgM anti-GM1 ganglioside in patients with motor neuron disease and variants. Ann. Neurol. **27:**558–563.
38. **Santoro, M., F. P. Thomas, M. E. Fink, D. J. Lange, A. Uncini, N. A. Wadia, N. Latov, and A. P. Hays.** 1990. IgM deposits at nodes of Ranvier in a patient with amyotrophic lateral sclerosis, anti-GM1 antibodies and multifocal conduction block. Ann. Neurol. **28:**373–377.
39. **Schwerer, B., H. Lassmann, K. Kitz, and H. Berkheimer.** 1986. Ganglioside GM1, a molecular target for immunological and toxic attacks: similarity of neuropathological lesions induced by ganglioside antiserum and cholera toxin. Acta Neuropathol. **72:**55–61.
40. **Sigal, L. H., and A. H. Tatum.** 1988. Lyme disease patients' serum contains IgM antibodies to *Borrelia burgdorferi* that crossreact with neuronal antigens. Neurology **38:**1439–1442.
41. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
42. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308:**733–740.
43. **Streit, W. J., B. A. Schulte, S. S. Spicer, and J. D. Balentine.** 1985. Histochemical localization of galactose containing glycoconjugate at peripheral nodes of Ranvier in the rat. J. Histochem. Cytochem. **33:**33–39.
44. **Suchanek, G. W., W. Kristoferitsch, G. Stanek, and H. Bernheimer.** 1986. Antimyelin antibodies in cerebrospinal fluid and serum of patients with meningopolyneuritis Garin-Bujadoux-Bannwarth and other neurological disorders. Zentralbl. Bakteriol. Hyg. **A263:**160–168.
45. **Takayama, K., R. J. L. Rothenberg, and A. G. Barbour.** 1987. Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect. Immun. **55:**2311–2313.
46. **Thomas, D. D., D. Cadavid, and A. G. Barbour.** 1994. Differential association of Borrelia species with cultured neural cells. J. Infect. Dis. **169:**445–448.
47. **Thomas, F. P., A. M. Lee, S. N. Romas, and N. Latov.** 1989. Monoclonal IgM's with anti-Gal(β1-3)GalNac activity in lower motor neuron disease: identification of glycoprotein antigens in neural tissues and crossreactivity with serum immunoglobulins. J. Neuroimmunol. **23:**167–174.
48. **Thomas, F. P., W. Trojaborg, C. Nagy, M. Santoro, S. A. Sadiq, N. Latov, and A. P. Hays.** 1991. Experimental autoimmune neuropathy with anti-GM1 antibodies and immunoglobulin deposits at the nodes of Ranvier. Acta Neuropathol. **82:**378–383.

49. **Vriesendorp, F. J., B. Mishu, M. J. Blaser, and C. L. Koski.** 1993. Serum antibodies to GM1, GD1b, peripheral nerve myelin, and *Campylobacter jejuni* in patients with Guillain-Barré syndrome and controls: correlation and prognosis. Ann. Neurol. **34:**130–135.
50. **Weller, M., A. Stevens, N. Sommer, J. Dichgans, B. Kappler, and H. Wietholter.** 1992. Ganglioside antibodies: a lack of diagnostic specificity and clinical utility? J. Neurol. **239:**455–459.
51. **Weller, M., A. Stevens, N. Sommer, and H. Wietholter.** 1992. Are CSF or serum ganglioside antibodies related to peripheral nerve demyelination in neuroborreliosis, Guillain-Barre syndrome, or chronic inflammatory demyelinating polyradiculoneuropathy? Eur. Arch. Psychiatry Clin. Neurosci. **242:** 122–126.
52. **Weller, M., A. Stevens, N. Sommer, H. Wietholter, and J. Dichgans.** 1991. Cerebrospinal fluid interleukins, immunoglobulins, and fibronectin in neuroborreliosis. Arch. Neurol. **48:**837–841.
53. **Wheeler, C. M., J. C. Garcia-Monco, J. L. Benach, M. G. Golightly, G. S. Habicht, and A. C. Steere.** 1993. Nonprotein antigens of *Borrelia burgdorferi*. J. Infect. Dis. **167:**665–674.
54. **Wirguin, I., L. Suturkova-Milosevic, P. Della-Latta, T. Fisher, R. H. Brown, Jr., and N. Latov.** 1994. Monoclonal IgM antibodies to GM1 and asialo-GM1 in chronic neuropathies cross-react with *Campylobacter jejuni* lipopolysaccharides. Ann. Neurol. **35:**698–703.
55. **Yuki, N., T. Taki, F. Inagaki, T. Kasama, M. Takahashi, K. Saito, S. Handa, and T. Miyatake.** 1993. A bacterium lipopolysaccharide that elicits Guillain-Barré syndrome has a GM1 ganglioside-like structure. J. Exp. Med. **178:** 1771–1775.
56. **Yuki, N., H. Yoshino, S. Sato, and T. Miyatake.** 1990. Acute axonal polyneuropathy associated with anti-GM1 antibodies following *Campylobacter enteritis*. Neurology **40:**1900–1902.