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Strong IgG Antibody Responses to *Borrelia burgdorferi* Glycolipids in Patients with Lyme Arthritis, a Late Manifestation of the Infection

Kathryn L. Jones[a], **Robert J. Seward**[a,c], **Gil Ben-Menachem**[b], **Lisa J. Glickstein**[a], **Catherine E. Costello**[c], and **Allen C. Steere**[a,*]

[a]Division of Rheumatology, Allergy, and Immunology, Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

[b]National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

[c]Department of Biochemistry, Mass Spectrometry Resource, Boston University School of Medicine, Boston, MA 02118.

Abstract

In this study, the membrane lipids of *B. burgdorferi* were separated into 16 fractions; the components in each fraction were identified, and the immunogenicity of each fraction was determined by ELISA using sera from Lyme disease patients. Only the 2 glycolipids, acylated cholesteryl galactoside (ACG, *Bb*GL-I) and monogalactosyl diacylglycerol (MgalD, *Bb*GL-II), were immunogenic. Early in the infection, 24 of 84 patients (29%) who were convalescent from erythema migrans and 19 of the 35 patients (54%) with neuroborreliosis had weak IgG responses to purified MgalD, and a smaller percentage of patients had early responses to synthetic ACG. However, almost all of 75 patients with Lyme arthritis, a late disease manifestation, had strong IgG reactivity with both glycolipids. Thus, almost all patients with Lyme arthritis have strong IgG antibody responses to *B. burgdorferi* glycolipid antigens.

Keywords

Lyme disease; Bacterial infection; *Borrelia burgdorferi*; Antibodies; Glycolipid antigens

Introduction

Lyme disease, which is caused by the tick-borne spirochete, *Borrelia burgdorferi*, typically begins with an expanding skin lesion, erythema migrans (1). Within days to weeks, spirochetal dissemination often occurs, particularly to the nervous system, heart or joints. Soon after dissemination, early neurologic or cardiac involvement may develop. Months later, untreated

*Corresponding author. Massachusetts General Hospital, 55 Fruit Street, Charlestown Navy Yard 149/8301, Boston, MA 02114; telephone 617-726-1527, fax 617-726-1544 *E-mail address*: asteere@partners.org,..

patients often have intermittent or persistent arthritis in a few large joints, especially the knee, over a period of several years.

Antibody responses in Lyme disease develop gradually to a large array of spirochetal proteins (2-4). In both the human disease and in murine models of the infection, many of the immunogens are plasmid-encoded outer-surface lipoproteins (4,5). *B. burgdorferi*-infected mice have T-cell-independent B-cell responses to certain outer-surface lipoproteins (6). However, in patients with Lyme disease, the responses to most spirochetal lipoproteins exhibit IgG class-switching; the titers are highest late in the infection (2,7), and antibody reactivity declines slowly after spirochetal eradication with antibiotic therapy (7,8). These features are suggestive of T-helper-cell dependent B-cell responses.

In an early study in which *B. burgdorferi* was separated into 4 fractions by thin layer chromatography (TLC), patients with Lyme disease were found to have IgM or IgG antibody responses to lipid or glycolipid components of the spirochete, but the actual components of these fractions were not identified (9). In a later study, using TLC and mass spectrometry, *B. burgdorferi* was reported to have 2 phospholipids and a glycolipid component, α-galactosyl diacyglycerol (10), similar to a previously reported glycolipid in other *Borrelia* or *Treponema* (11). Ten patients each with stage II or III Lyme disease had antibody responses to this glycolipid, as determined by Western blotting, but the antibody isotypes were not identified. Antibody responses were not found in 5 patients with stage I disease, and the phospholipids were not immunogenic (10). The clinical manifestations of patients at each stage of the disease were not reported.

Subsequently, using nuclear magnetic resonance spectroscopy, two *B. burgdorferi* glycolipids were identified (12). One was a novel glycolipid, cholesteryl 6-*O*-acyl-*β*-D-galactopyranoside, called acylated cholesteryl galactoside (ACG or *Bb*GL-I); the other was the previously recognized α-galactosyl diacyglycerol, which was further characterized as 1,2-di-*O*-acyl-3-*O*-*α*-D-galactopyranosyl-*sn*-glycerol, called monogalactosyl diacylglycerol (MgalD or *Bb*GL-II). When mice were immunized with these glycolipids in complete Freund's adjuvant, each glycolipid induced low-level antibody responses, primarily of the IgM isotype, as determined by ELISA (12). In a similar study, the presence of these 2 *B. burgdorferi* glycolipids was confirmed, and it was noted that ACG also existed in a non-acylated form (13). Three of 4 patients with early Lyme disease had IgG responses to ACG (*Bb*GL-I), and 1 had IgG reactivity with MgalD (*Bb*GL-II). Seven of 8 patients with late disease had IgG responses to ACG (*Bb*GL-I), but none had reactivity with MgalD (*Bb*GL-II).

Taken together, the description of antibody responses to *B. burgdorferi* glycolipids in humans and mice were incomplete or contradictory. One study of Lyme disease patients noted reactivity with MgalD (10), and the other primarily with ACG (13). One noted IgG responses to ACG (13), and the other did not report antibody isotypes to MgalD (10). In mice immunized with these glycolipids, only weak responses were noted, primarily of the IgM isotype, but the responses were not determined after infection with *B. burgdorferi*. Thus, the significance of these anti-glycolipid antibody responses was not clear.

As a part of our ongoing survey of immune responses in Lyme disease, our goal here was to assess the immunogenicity of the membrane lipids of *B. burgdorferi* using serum samples from human patients with early or late manifestations of Lyme disease.

Materials and Methods

Patients

All patients met the criteria of the Centers for Disease Control and Prevention (CDC) for the diagnosis of Lyme disease (14). They had either culture-confirmed erythema migrans or a later manifestation of the illness accompanied by a positive antibody response to a sonicated preparation of *B. burgdorferi* by ELISA and Western blot, interpreted according to the CDC criteria (15). The Human Investigation Committees at Yale University School of Medicine (1976-1987), Tufts Medical Center (1987-2002), and Massachusetts General Hospital (2002-2007) approved the study.

Serum samples were selected from 194 antibiotic-treated patients with erythema migrans, early neuroborreliosis, or Lyme arthritis. Since glycolipid antigen preparations were limited and since more patients with erythema migrans have antibody reactivity during convalescence than during active infection (16,17), only convalescent-phase serum samples were tested from all 84 patients with culture-confirmed erythema migrans who participated in a field study of early Lyme disease in Wakefield, Rhode Island or East Lyme, Connecticut (1998-2001) in whom serum samples were still available (18). By the time of early, disseminated infection with organ system involvement, antibody responses to *B. burgdorferi* sonicates are positive during active infection (16,17). Thus, all available serum samples were tested from 35 patients with early neuroborreliosis, 20 with facial palsy alone and 15 with combinations of meningitis, cranial neuropathy, or radiculoneuropathy, who were seen from 1976-2007. Finally, the first serum sample was tested from 75 patients with active Lyme arthritis, 23 with antibiotic-responsive arthritis and 52 with antibiotic-refractory arthritis, who participated in a study called “Immunity in Lyme Arthritis” from 1987-2007 (19). This proportion of responsive and refractory cases is reflective of our role as a referral center.

For initial screening for the immunogenicity of *B. burgdorferi* membrane fractions, serum samples were pooled from 10 patients, 2 with erythema migrans, 3 with neuroborreliosis, and 5 with Lyme arthritis. To determine the natural history of the antibody response, 4 serial serum samples were tested from 10 non-antibiotic-treated patients seen in the late 1970s who were followed throughout early and late infection prior to the use of antibiotic therapy to treat this illness.

To assess the decline in antibody responses after antibiotic therapy, serum samples obtained during the acute or convalescent phases of the illness and again approximately 6 months after the completion of antibiotic therapy were available in 38 (16%) of the 194 patients, 9 with erythema migrans, 7 with acute neuroborreliosis, and 22 with Lyme arthritis. These samples were obtained by protocol and not because of disease activity after antibiotic therapy. A normal control group consisted of serum samples from 70 subjects; 60 from individuals with no history of Lyme disease who were seen for well patient visits in Wakefield, Rhode Island or East Lyme, Connecticut, and 10 from healthy individuals in our laboratory. To screen the membrane fractions, sera from the 10 laboratory workers were pooled.

Extraction and fractionation of *B. burgdorferi* membrane lipids

To isolate membrane lipids, *B. burgdorferi* N40 spirochetes were grown to a density of ~1×10^9 spirochetes per ml in 1 L of Barbour-Stoenner-Kelly II media (BSK-II; Sigma, St. Louis, MO). The culture was centrifuged at 10,409 × g for 20 minutes, resuspended twice with phosphate buffered saline (PBS, pH 7.4; Fisher Scientific, Pittsburgh, PA), and centrifuged again after each wash for an additional 20 minutes. The resulting spirochetal pellet (0.37 g, wet weight) was flash frozen and stored at -80°C.

Membrane lipids were isolated using a modified Bligh and Dyer extraction protocol (20). All solvents were HPLC grade (Honeywell Burdick & Jackson, Morristown, NJ). For analysis, the frozen *B. burgdorferi* pellet was thawed on ice and resuspended in 3 ml of water. Methanol:chloroform (2:1) (11.25 ml) was added to the cell suspension, and the mixture was vortexed briefly each minute for 15 minutes at room temperature. Because of concern that highly glycosolated lipids might be lost in the standard 2-phase (aqueous/organic) separation, additional chloroform and water were not added. The lipid extract was dried under a stream of nitrogen, and stored under nitrogen at -80°C.

The extract was fractionated using the protocol of Ben-Menachem et al. (12), with slight modifications. Silica gel 60 (230-400 mesh, EMD Chemicals, Gibbstown, NJ) was heated to 130°C for 2 hours to remove adsorbed water. A 3-cm diameter glass column was packed to a 3-cm height with silica gel in chloroform and washed with chloroform. The extract was resuspended in 10 ml of chloroform and filtered through glass wool onto the column. A step-elution technique was used to separate the membrane lipids using increasing concentrations of methanol in chloroform (100 ml each of 0, 2.5, 5, 10, 20, 50 and 100 % solutions). Three fractions (33.3 ml each) of each concentration of methanol were collected in glass tubes and dried under a nitrogen stream. Each fraction was resuspended in 1.5 ml of the same methanol:chloroform mixture in which it had been eluted. The tubes were flushed with nitrogen and stored at -80°C.

To obtain large quantities of MgalD (*Bb*GL-II), a second preparation was made with slight modifications. First, lipids in the spirochetal pellet (1.2 g wet weight) were extracted by the Bligh and Dyer protocol (20), as described above. However, for this preparation, more chloroform:water (1:1) (7.5 ml) was added to the extract; the mixture was vortexed and centrifuged at 2,988 × g for 5 minutes for separation into two phases. The lower lipid-containing phase was transferred to a clean glass tube, dried under a stream of nitrogen, and stored under nitrogen at -80°C. The procedure yielded 21.2 mg of membrane lipids. Second, to increase resolution, a longer column (10 cm rather than 3 cm) was used for separation. The 3 fractions that contained only MgalD were pooled and dried under a stream of nitrogen, and the combined fraction was resuspended in 5 ml of 5% methanol in chloroform.

Mass spectrometry analysis

One aliquot (2.5 ul) of each fraction was analyzed by matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometry using a 2,5-dihydroxybenzoic acid matrix with a Bruker Reflex IV MALDI-TOF mass spectrometer (Billerica, MA) operated in positive ion reflectron mode. Individual mass spectral peaks were assigned corresponding to the monoisotopic *m/z* of known lipid ionic forms of *B. burgdorferi* (11). The identities of these ions were confirmed by tandem mass spectrometry on an Applied Biosystems QSTAR Pulsar i- quadrupole orthogonal time-of-flight mass spectrometer (Foster City, CA) fitted with either a Proxeon nanospray source (San Mateo, CA) or an o-MALDI1 source.

Antibody responses to *B. burgdorferi* glycolipids

To screen the membrane fractions, the first fraction in which lipid material appeared (called fraction 1) and the subsequent 15 fractions (2-16) were analyzed by ELISA. The fractions were diluted 1:100 in 100% ethanol. Because only the fractions containing ACG (*Bb*GL-I) or MgalD (*Bb*GL-II) appeared to be immunogenic, antibody reactivity with these glycolipids was then determined in individual samples from large numbers of Lyme disease patients. Although chemically synthesized ACG (*Bb*GL-I) was available (21), a synthetic form of MgalD (*Bb*GL-II) was not, and therefore, this glycolipid was purified from cultured spirochetes, and its purity was verified by mass spectrometry.

In initial experiments in which polystyrene (PS) or polyvinyl chloride (PVC) plates were used, serum samples from normal control subjects gave high background absorbances (~0.5). Better results were obtained using polystyrene plates coated with polyvinylidine fluoride (PVDF) membranes. However, since such plates are opaque, the samples had to be transferred to PS plates for reading. In this way, background absorbances were reduced to negligible levels for IgG determinations (~0.1), but not for IgM determinations, which remained high (~0.5). IgM responses, which were tested in all patients, rarely exceeded this absorbance. Because IgM responses in patients could not usually be distinguished from those in control subjects, only IgG responses are reported here. Insufficient synthetic ACG or purified MgalD remained to determine IgA reactivity or IgG subtype responses.

Polystyrene 96-well microtiter plates with PVDF membranes (Millipore #MSIPN4550, Billerica, MA) were coated with either ACG (*Bb*GL-I) or MgalD (*Bb*GL-II) (100 μl per well) and incubated overnight, loosely covered to facilitate ethanol evaporation. All steps were done at room temperature. The optimal concentration of chemically synthesized ACG (*Bb*GL-I) in 100% chloroform was 2 μg/ml in ethanol, and the optimal dilution of the preparation containing MgalD (*Bb*GL-II) was 1:40 in ethanol. The next day, the plates were incubated with 5% milk-PBS (blocking buffer) (150 μl) for 2 hours and then washed 3 times with PBS alone. After dilution in blocking buffer (1:100), patient or control sera (100 μl) were added to each well and incubated for 1 hour with shaking. After 3 washes with PBS, goat anti-human IgG conjugated to horseradish peroxidase (Kierkegaard and Perry Labs, Gaithersberg, MD) (100 μl), diluted 1:10,000 in blocking buffer, was added to each well for 1 hour with shaking. After 3 more washes, the plates were incubated with a 1:1 mixture of the substrate, 3,3′,5,5′-tetramethylbenzidine, and 0.01% hydrogen peroxide (100 μl, Kierkegaard and Perry Labs). The reaction was stopped after 10 minutes with 1M phosphoric acid (100 μl). To measure the absorbance (450 nm), 100μl from each well were transferred to clear-bottomed 96-well plates (Corning, Lowell, MA).

Cut-off values for positive responses in individual patient samples were 3 standard deviations above the mean value of 22 normal control samples included on the same plate. For serial patient samples, cut-off values were 3 standard deviations above the mean values of 11 normal control samples included on the same plate.

Statistical analysis

IgG antibody responses to *Bb*GL-I or *Bb*GL-II were compared between patient groups by Mann-Whitney rank sum test. The percentage of patients with positive IgG responses to either glycolipid was compared by chi-square test. Among the patients in whom serum samples were available during the period of active arthritis and ~6 months after antibiotic therapy, the decline in IgG antibody responses was assessed by the Wilcoxin Signed-Rank test. All P-values are two-tailed. P-values ≤ 0.05 were considered statistically significant.

Results

Identification and immunogenicity of *B. burgdorferi* membrane lipids

Using a step gradient technique in which *B. burgdorferi* membrane lipids were separated into 16 fractions and analyzed by MALDI-TOF mass spectrometry, 2 glycolipids, ACG (*Bb*GL-I) and MgalD (*Bb*GL-II), non-acylated cholesteryl galactoside, and 2 phospholipids, phosphatidylglycerol and phosphatidylcholine, were identified. The verification of MgalD (*Bb*GL-II) is shown in Figure 1.

In a screening ELISA, the immunogenicity of each fraction was determined using pooled sera from 10 patients with Lyme disease or 10 normal control subjects. The IgG antibody responses

to fractions 3-6, which contained MgalD (*Bb*GL-II), were clearly greater in Lyme disease patients than in normal control subjects, and the responses to ACG (*Bb*GL-I) in fractions 1-3 appeared to be higher in patients than in control subjects (Figure 2). However, there was no reactivity with the 2 membrane phospholipids in either patients or control subjects. Although this screening assay showed antibody responses in normal control subjects against the material in fractions 1-3, which contained ACG (Figure 2), normal subjects did not react with synthetic ACG (Figure 3A). Thus, we focused on determining the immunogenicity of the 2 *B. burgdorferi* glycolipids in patients with early or late manifestations of Lyme disease using a synthetic preparation of ACG (*Bb*GL-I) and a purified preparation of MgalD (*Bb*GL-II).

IgG antibody responses to *B. burgdorferi* glycolipids

Early in the infection, only 8 of the 84 patients (10%) who were in the convalescent-phase of the illness, 3-4 weeks after the initiation of antibiotic treatment for erythema migrans, and only 10 of 35 patients (29%) with early neuroborreliosis who were seen 2-8 weeks after disease onset had weak IgG antibody responses to synthetic ACG (*Bb*GL-I) (Figure 3A). Late in the illness, months to several years after disease onset, 74 of the 75 patients (99%) with Lyme arthritis had strong IgG responses to this glycolipid (median absorbance = 1.0).

In comparison, 24 of the 84 patients (29%) who were convalescent from erythema migrans, 19 of the 35 patients (54%) with neuroborreliosis, and 71 of the 75 patients (95%) with Lyme arthritis had IgG antibody reactivity with purified MgalD (*Bb*GL-II) (Figure 3B). The median absorbance was lowest in patients who were convalescent from erythema migrans (0.38), intermediate in those with neuroborreliosis (0.55), and highest (0.95) in those with Lyme arthritis. Normal control subjects had minimal or no reactivity with either glycolipid. Thus, patients with early infection (erythema migrans or acute neuroborreliosis) had more frequent reactivity with MgalD (*Bb*GL-II) than with ACG (*Bb*GL-I), but later in the illness, almost all patients with Lyme arthritis had antibody responses to both glycolipids (Table 1). Reactivity with either glycolipid in the 52 patients with antibiotic-refractory arthritis was not significantly different than that in the 23 patients with antibiotic-responsive arthritis (data not shown). Thus, some patients with early infection had weak IgG antibody responses to MgalD and less often to ACG, but most patients with Lyme arthritis had strong IgG reactivity with both glycolipids.

Longitudinal evaluation of the antibody responses to *B. burgdorferi* glycolipids

To determine the natural history of antibody reactivity to *B. burgdorferi* glycolipids in individual patients followed throughout the infection, IgG antibody levels to the 2 glycolipids were determined in serial serum samples from 10 non-antibiotic-treated patients who were followed throughout their 2- to 5-year course of the illness in the late 1970s before the etiologic agent of Lyme disease was known. It was later shown that PCR testing for *B. burgdorferi* DNA remained positive throughout this several-year disease course (22), and therefore, the spirochete is thought to be present in the natural infection until eradication by the immune response at the time of arthritis resolution.

Among the 10 patients, only 1 had an antibody response to ACG (*Bb*GL-I) during early infection, whereas all 10 had reactivity with this glycolipid late in the illness when arthritis developed (Figure 4A). In comparison, 5 of the 10 patients had antibody responses to MgalD (*Bb*GL-II) during early infection, and all 10 had reactivity with this glycolipid when arthritis was present (Figure 4B). With both glycolipids, reactivity was higher during periods of arthritis than in early disease, and the strongest median responses occurred at the time of arthritis resolution, 2- to 5-years after disease onset. Thus, immune processing of *B. burgdorferi* glycolipids appears to continue throughout the infection.

IgG antibody responses to *B. burgdorferi* glycolipids after antibiotic therapy

To assess the decline in antibody responses to the 2 glycolipids after antibiotic therapy, initial samples obtained during the acute or convalescent phase of the illness and again approximately 6 months later were tested in 38 of the 194 patients (16%) in whom such samples were available. Nine of the patients had erythema migrans, 7 had neuroborreliosis, and 22 had Lyme arthritis. Since the decline in responses could only be determined in patients who had positive antibody responses in initial samples, the decrease in responses to ACG (*Bb*GL-I) could only be assessed in the 22 patients with Lyme arthritis (Figure 5A). In these patients, the median absorbance value during active arthritis (1.2) declined slightly ~6 months after antibiotic therapy (1.0), a difference of possible significance (P=0.06).

Because more patients with early infection had reactivity with MgalD (*Bb*GL-II), it was possible to measure the decline in antibody responses to this glycolipid in each patient group (Figure 5B). In the 9 patients with erythema migrans, the median absorbance during convalescence (0.46) declined below the cut-off value 6 months after antibiotic treatment (0.12). Of the 9 patients, 2 still had values above the cut-off value, but all 9 patients remained well after antibiotic treatment. In the 7 patients with neurologic involvement, the decrease in responses over a 6-month period after treatment was minimal. These changes in patients with erythema migrans or neuroborreliosis were not statistically significant. However, among the 22 patients with Lyme arthritis, the slight decline in the median value from 0.96 during active arthritis to 0.90 ~6 months after treatment was statistically significant (P = 0.008). With both glycolipids, the minimal decline in reactivity in the 14 patients with antibiotic-refractory arthritis was similar to that in the 8 patients with antibiotic-responsive arthritis (data not shown).

Discussion

In this study, 10% of the study patients who were convalescent from erythema migrans 3-4 weeks after the onset of illness, 29% with neuroborreliosis 2-8 weeks after disease onset had IgG antibody responses to MgalD (*Bb*GL-II), and a smaller percentage of patients had early responses to ACG (*Bb*GL-I). However, almost all patients with Lyme arthritis, which occurs months to years later, had strong IgG reactivity with both glycolipids. In untreated patients, these responses were often highest when the arthritis resolved 2-5 years after disease onset, whereas the titers in antibiotic-treated patients declined gradually after antibiotic therapy.

Similarly, greater frequency of responses and higher titers late in the infection and gradual decline in reactivity after antibiotic therapy are characteristic of the responses to most protein antigens of *B. burgdorferi*. For example, in a previous analysis of antibody responses to *B. burgdorferi* sonicates, which contained multiple spirochetal proteins and membrane components, 18% of the patients who were convalescent from erythema migrans, 85% with early neuroborreliosis or carditis, and 100% with Lyme arthritis had IgG antibody responses to this preparation (17). One of the most prominent antibody responses in Lyme arthritis patients is to *B. burgdorferi* decorin binding protein A (DbpA) (7). As was seen here with responses to MgalD, about half of patients with early, disseminated infection had low-level reactivity with DbpA, and almost all patients with Lyme arthritis had marked IgG responses to this protein. Moreover, in untreated patients, the responses to DbpA were often highest at the time of arthritis resolution, whereas reactivity in antibiotic-treated patients declined slowly after antibiotic therapy, except in patients with erythema migrans in whom low-level reactivity decreased somewhat faster (7,17,23). Thus, these general features of the antibody responses seemed similar to protein and glycolipid antigens of the spirochete.

Antibody responses to spirochetal lipoproteins are directed against protein epitopes, and not the lipid moiety (24). Therefore, what portions of *B. burgdorferi* glycolipids constitute antibody epitopes? A previous analysis suggested that the galactose moiety is an essential component

of the MgalD (*Bb*GL-II) epitope. Periodate treatment, which eliminated the galactose residue, abrogated antibody responses to α-MgalD (10). In addition, we observed antibody reactivity with α-MgalD, but not with commercially available β-MgalD that was isolated from spinach (Matreya, Pleasant Gap, PA) (data not shown). Thus, it would appear that anti-MgalD antibodies bind the alpha configuration of the galactose moiety. No information is yet available regarding the antibody-binding site for ACG. In previous experiments in which potential cross-reactivity between antibodies to ACG (*Bb*GL-I) and MgalD (*Bb*GL-II) was assessed using hyperimmune mouse sera in IgM and IgG immunodiffusion assays, no evidence of cross-reactivity was found (personal communication, Dr. Gil Ben-Menachem). Thus, these 2 borrelial glycolipids appear to have different antibody epitopes that induce separate antibody responses.

Because lipid antigens often elicit T-cell independent B-cell responses, we anticipated that glycolipid antigens of *B. burgdorferi* might induce transient responses early in the infection. Instead, the converse was found. The antibody responses to *B. burgdorferi* glycolipids included IgG class switching, persistent high titers late in the illness, and gradual decline in reactivity after spirochetal eradication, features which typify T-cell-dependent B-cell responses. It was recently reported that NK T cells may provide lipid antigen-specific cognate help for B cells (25). Moreover, it was recently learned that the MHC-like CD1d molecule on antigen presenting cells presents *B. burgdorferi* MgalD (*Bb*GL-II) to NK T cells in vitro (26). One possibility is that the B cell receptor binds the galactose moiety of MgalD, internalizes the glycolipid, and presents the lipid portion via the CD1 molecule to NK T cells. Another hypothesis is that each of these glycolipids is a part of a protein-glycolipid complex. In that case, the glycolipid may bind to the B cell receptor, internalize the complex, and present a peptide from the complex via traditional class II MHC molecules to CD4+ T helper cells. As yet, there is no evidence that *B. burgdorferi* glycolipids are covalently attached to spirochetal proteins. However, spirochetal fragments in which glycolipids and proteins are non-covalently complexed could contribute to T-cell-dependent anti-glycolipid antibody responses.

Might the antibody response to *B. burgdorferi* glycolipids be useful as a diagnostic test or in vaccination? In syphilis, which is caused by another spirochete, *Treponema pallidum*, reactivity with the lipid antigen cardiolipin, which is diphosphatidylglycerol (27), is measured by the VDRL test. The response to this lipid antigen declines ≥4-fold or disappears within 6 months after antibiotic therapy, and therefore, the success of antibiotic treatment can be confirmed with this test. Although *B. burgdorferi* has phosphatidylglycerol, rather than diphosphatidylglycerol, this closely related phospholipid was not immunogenic, and the antibody responses to *B. burgdorferi* glycolipids did not decline rapidly after treatment. Thus, *B. burgdorferi* infection does not appear to induce a response to either a lipid or glycolipid antigen that could be used as a surrogate marker for spirochetal eradication in Lyme disease patients.

The original vaccine for human Lyme disease consisted of recombinant, lipidated outer-surface protein A (OspA) (28,29). Three injections of this vaccine gave high antibody titers to OspA, but the titers declined rapidly (28). Previously, mice immunized with *B. burgdorferi* glycolipids had only weak IgM anti-glycolipid antibody responses, primarily to ACG (12). In contrast, in patients with Lyme arthritis, antibody responses to *B. burgdorferi* lipoproteins last for years after spirochetal killing (7), and re-infection has not been observed in these patients. As shown here, antibody reactivity with *B. burgdorferi* glycolipids also appears to be long lasting. Thus, immunization with *B. burgdorferi* glycolipids, perhaps in combination with particular spirochetal lipoproteins, may be beneficial for vaccination.

In summary, some patients with early Lyme disease had weak IgG antibody responses to MgalD (*Bb*GL-II) and less often to ACG (*Bb*GL-I), but most patients with Lyme arthritis had strong IgG reactivity with both glycolipids.

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References

1. Steere AC. Lyme disease. New Engl. J. Med 2001;345:115–125. [PubMed: 11450660]
2. Akin E, McHugh GL, Flavell RA, Fikrig E, Steere AC. The immunoglobulin (IgG) antibody response to OspA and OspB correlates with severe and prolonged Lyme arthritis and the IgG response to P35 correlates with mild and brief arthritis. Infect. Immun 1999;67:173–181. [PubMed: 9864212]
3. Dressler F, Whalen JA, Reinhardt BN, Steere AC. Western blotting in the serodiagnosis of Lyme disease. J. Infect. Dis 1993;167:392–400. [PubMed: 8380611]
4. Barbour AG, Jasinskas A, Kayala MK, Davies DH, Steere AC, Baldi P, Felgner PL. A genome-wide proteome array reveals a limited set of immunogens in natural infections of humans and white-footed mice with *Borrelia burgdorferi*. Infect. Immun 2008;76:3374–3389. [PubMed: 18474646]
5. Nowalk AJ, Gilmore RD Jr. Carroll JA. Serologic proteome analysis of *Borrelia burgdorferi* membrane-associated proteins. Infect. Immun 2006;74:3864–3873. [PubMed: 16790758]
6. Fikrig E, Barthold SW, Chen M, Chang CH, Flavell RA. Protective antibodies develop, and murine Lyme arthritis regresses, in the absence of MHC class II and CD4+ T cells. J. Immunol 1997;159:5682–5686. [PubMed: 9548512]
7. Kannian P, McHugh G, Johnson BJ, Bacon RM, Glickstein LJ, Steere AC. Antibody responses to *Borrelia burgdorferi* in patients with antibiotic-refractory, antibiotic-responsive, or non-antibiotic-treated Lyme arthritis. Arthritis Rheum 2007;56:4216–4225. [PubMed: 18050219]
8. Heikkilä T, Huppertz H-I, Seppälä I, Sillanpää H, Saxen H, Lahdenne P. Recombinant or peptide antigens in the serology of Lyme arthritis in children. J. Infect. Dis 2003;187:1888–1894. [PubMed: 12792865]
9. Wheeler CM, Monco J.C. Garcia, Benach JL, Golightly MG, Habicht GS, Steere AC. Nonprotein antigens of *Borrelia burgdorferi*. J. Infect. Dis 1993;167:665–674. [PubMed: 8440937]
10. Hossain H, Wellensiek HJ, Geyer R, Lochnit G. Structural analysis of glycolipids from *Borrelia burgdorferi*. Biochimie 2001;83:683–692. [PubMed: 11522398]
11. Livermore BP, Johnson RC. Lipids of the Spirochaetales: comparison of the lipids of several members of the genera Spirochaeta, Treponema, and Leptospira. J. Bacteriol 1974;120:1268–1273. [PubMed: 4436256]
12. Ben-Menachem GJ, Kubler-Kielb J, Coxon B, Yergey A, Schneerson R. A newly discovered cholesteryl galactoside from *Borrelia burgdorferi*. Pro. Nat. Acad. Sci. U.S.A 2003;100:7913–7918.
13. Schroder NW, Schombel U, Heine H, Gobel UB, Zahringer U, Schumann RR. Acylated cholesteryl galactoside as a novel immunogenic motif in *Borrelia burgdorferi* sensu stricto. J. Biol. Chem 2003;278:33645–33653. [PubMed: 12810705]
14. CDC. Case definitions for public health surveillance. MMWR Morb. Mort. Wkly. Rpt 1990;39:1–43.
15. CDC. Recommendations for test performance and interpretation from the Second International Conference on serologic diagnosis of Lyme disease. MMWR Morb. Mort. Wkly. Rpt 1995;44:590–591.

16. Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J. Clin. Microbiol 1996;34:1–9. [PubMed: 8748261]

17. Steere AC, McHugh G, Damle N, Sikand VK. Prospective study of serologic tests for Lyme disease. Clin. Infect. Dis 2008;47:188–195. [PubMed: 18532885]

18. Jones KL, Glickstein LJ, Damle N, Sikand VK, McHugh G, Steere AC. *Borrelia burgdorferi* genetic markers and disseminated disease in patients with early Lyme disease. J. Clin. Microbiol 2006;44:4407–4413. [PubMed: 17035489]

19. Steere AC, Angelis SM. Therapy for Lyme arthritis; strategies for the treatment of antibiotic-refractory arthritis. Arthritis Rheum 2006;54:3079–3085. [PubMed: 17009226]

20. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can. J. Med. Sci 1959;37:911–917.

21. Pozsgay V, Kubler-Kielb J. Synthesis of an experimental glycolipoprotein vaccine against Lyme disease. Carbohydrate Res 2007;342:621–626.

22. Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N. Engl. J. Med 1994;330:229–234. [PubMed: 8272083]

23. Peltomaa M, McHugh G, Steere AC. Persistence of antibody response to the VlsE sixth invariant region (IR_6) peptide of *Borrelia burgdorferi* after successful antibiotic treatment of Lyme disease. J. Infect. Dis 2003;187:1178–1186. [PubMed: 12695996]

24. Sears JE, Fikrig E, Nakagawa TY, Deponte K, Marcantonio N, Kantor FS, Flavell RA. Molecular mapping of OspA-mediated immunity against *Borrelia burgdorferi*, the agent of Lyme disease. J. Immunol 1991;147:1995–2000. [PubMed: 1716290]

25. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, Besra GS, Brenner MB. NK T cells provide lipid antigen-specific cognate help for B cells. Proc. Nat. Acad. Sci. U.S A 2008;105:8339–8344.

26. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, Zajonc DM, Ben-Menachem G, Ainge GD, Painter GF, Khurana A, Hoebe K, Behar SM, Beutler B, Wilson IA, Tsuji M, Sellati TJ, Wong CH, Kronenberg M. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat. Immunol 2006;7:978–986. [PubMed: 16921381]

27. De Bruijn JH. Chemical structure and serological activity of natural and synthetic cardiolipin and related compounds. Br. J. Venereal Dis 1966;42:125–128. [PubMed: 5330042]

28. Steere AC, Sikand VK, Meurice F, Parenti DL, Fikrig E, Schoen RT, Nowakowski J, Schmid CH, Laukamp S, Buscarino C, Krause DS. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface protein A with adjuvant. N. Engl. J. Med 1998;339:209–215. [PubMed: 9673298]

29. Sigal LH, Zahradnik JM, Lavin P, Patella ST, Bryant G, Haselby R, Hilton E, Kunkel M, Adler-Klein D, Doherty T, Evans J, Molloy PJ, Seidner AL, Sabetta JR, Simon HJ, Klempner MS, Mays J, Marks D, Malawista SE. A vaccine consisting of recombinant *Borrelia burgdorferi* outer-surface protein A to prevent Lyme disease. Recombinant outer-surface protein A Lyme disease vaccine study. N. Engl. J. Med 1998;339:216–222. [PubMed: 9673299]

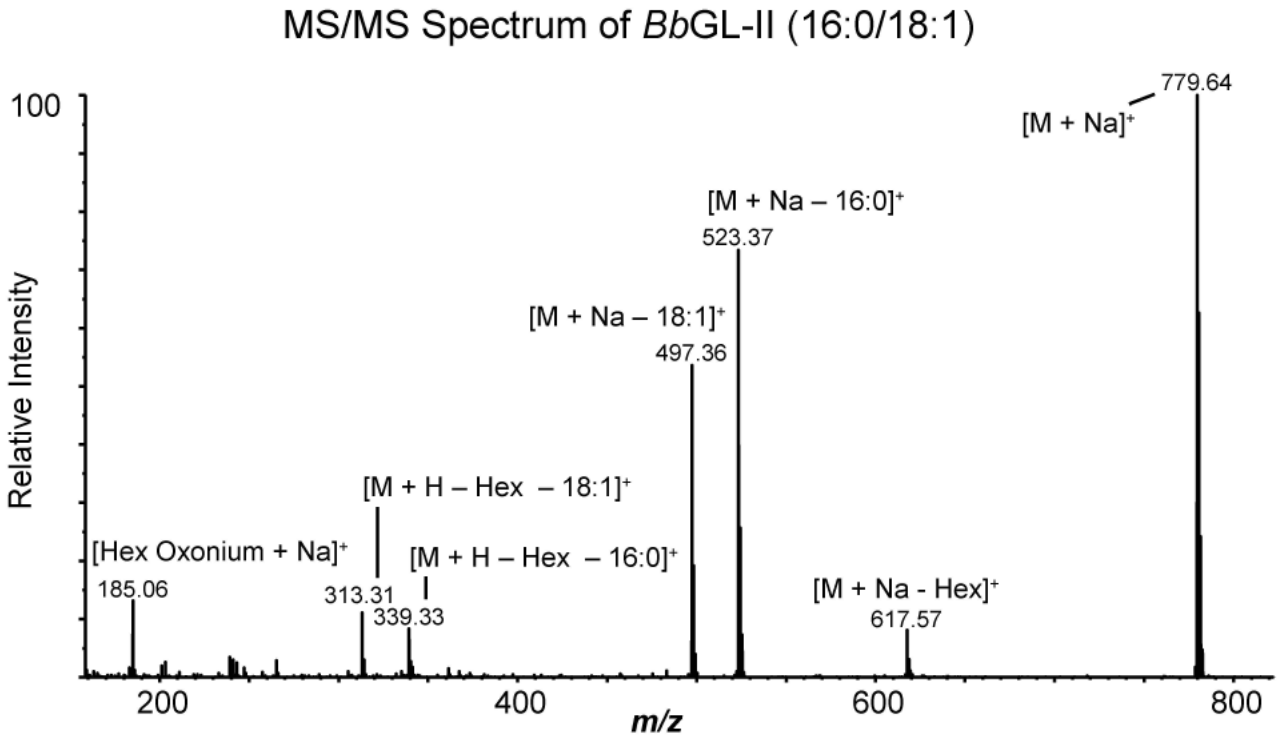

Figure 1.

Positive mode MALDI quadrupole orthogonal TOF product ion spectrum of MgalD (BbGl-II). The *Bb*GL-II-containing fraction was spotted (4 μl) on a stainless steel target with DHB matrix. The ion at m/z 779.64 (16:0/18:1, $[M + Na]^+$) was subjected to collisionally activated dissociation with argon collision gas. The figure shows the product ion spectrum of $[M +Na]^+$, 16:0/18:1 with characteristic neutral losses of fatty acyl chains (-16:0, -18:1) and hexose (-Hex), as well as the hexose oxonium ion.

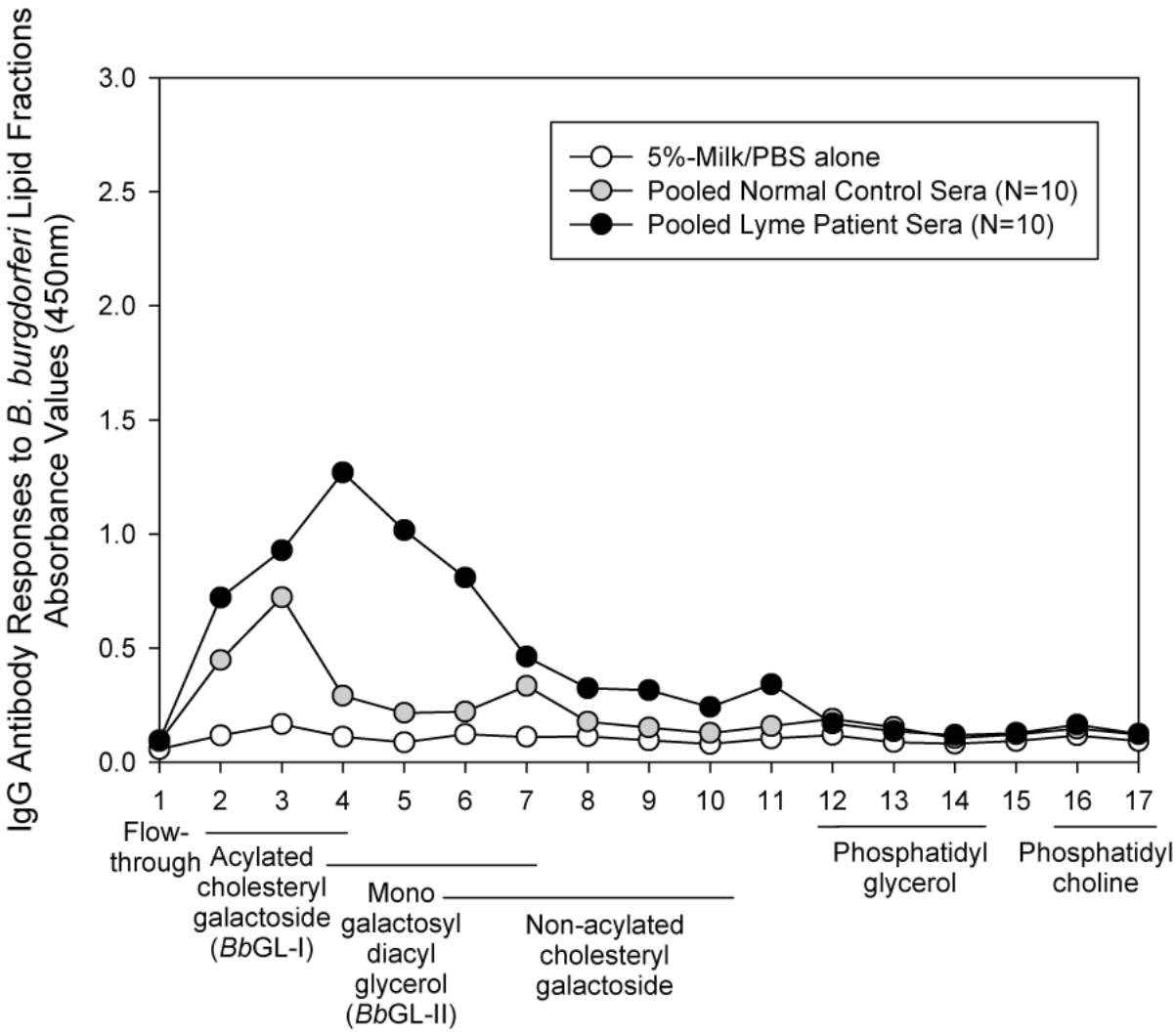

Figure 2.

After fractionation by normal phase chromatography, each fraction was tested by ELISA in a screening assay using pooled serum samples from patients with erythema migrans, early, acute neuroborreliosis, or Lyme arthritis sera (N=10) and pooled samples from normal control subjects (N=10).

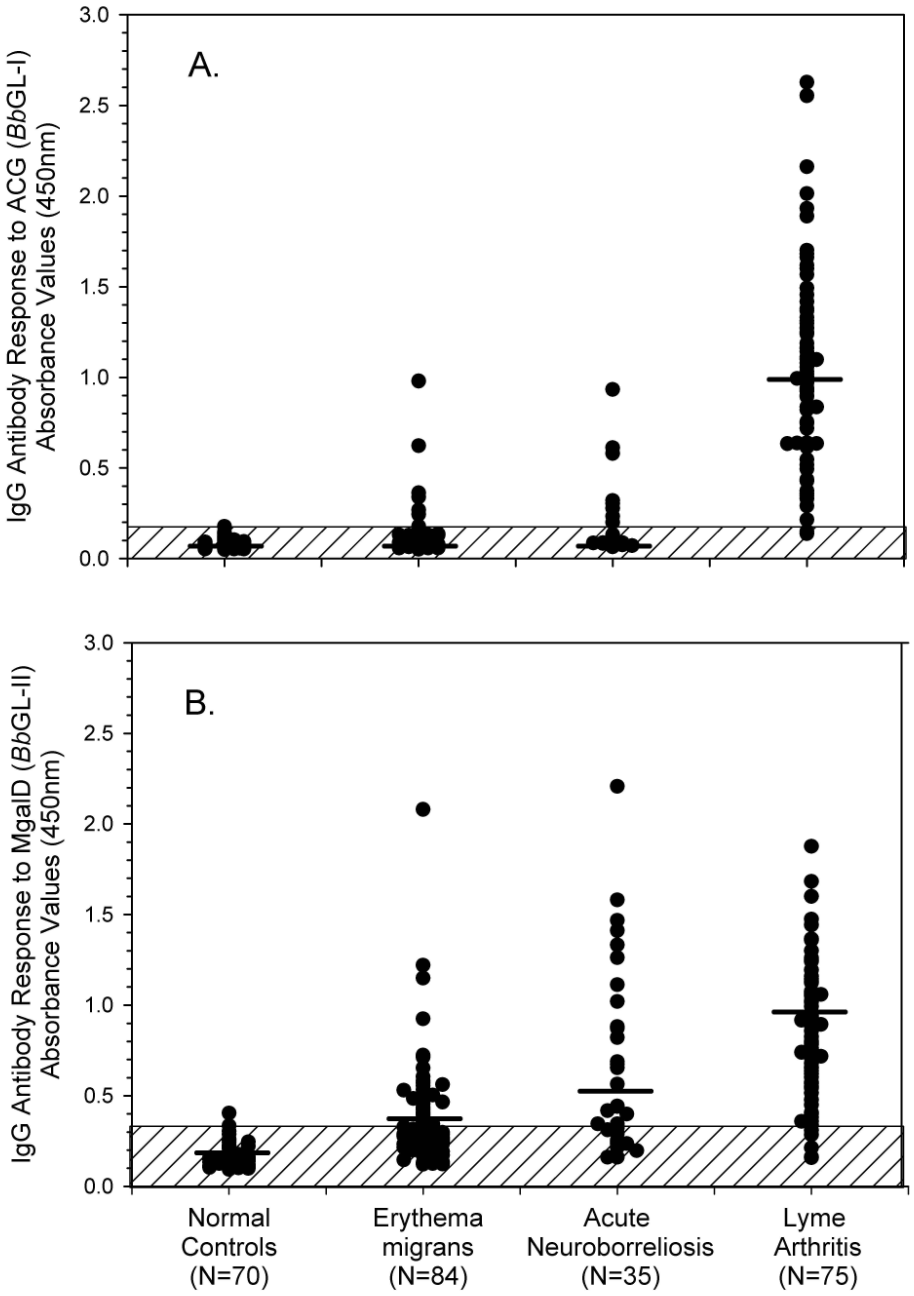

Figure 3.

IgG antibody responses to synthetic ACG (*Bb*GL-I) (panel A) and purified MgalD (*Bb*GL-II) (panel B) in sera from patients with Lyme disease or normal control subjects. The horizontal bars indicate median absorbance values. Cut-off values for positive responses in individual patient samples were ≥3 standard deviations above the mean value of 22 normal control samples included on the same plate. A few patients with erythema migrans had reactivity with ACG, but the differences between these patients and normal control subjects were not statistically significant. Compared with patients with erythema migrans, more of those with early, acute neuroborreliosis had responses to ACG (P=0.05), and compared with the other groups, more patients with Lyme arthritis had responses to this glycolipid (in each instance, P=0.001).

Compared with normal control subjects, patients with each manifestation of Lyme disease had higher responses to MgalD (in each instance, P=0.001). Patients with neuroborreliosis had significantly higher reactivity than those with erythema migrans (P=0.05), and those with arthritis had higher responses than the other groups (P≤0.01). ACG = acylated cholesteryl galactoside, MgalD = monogalactosyl diacylglycerol.

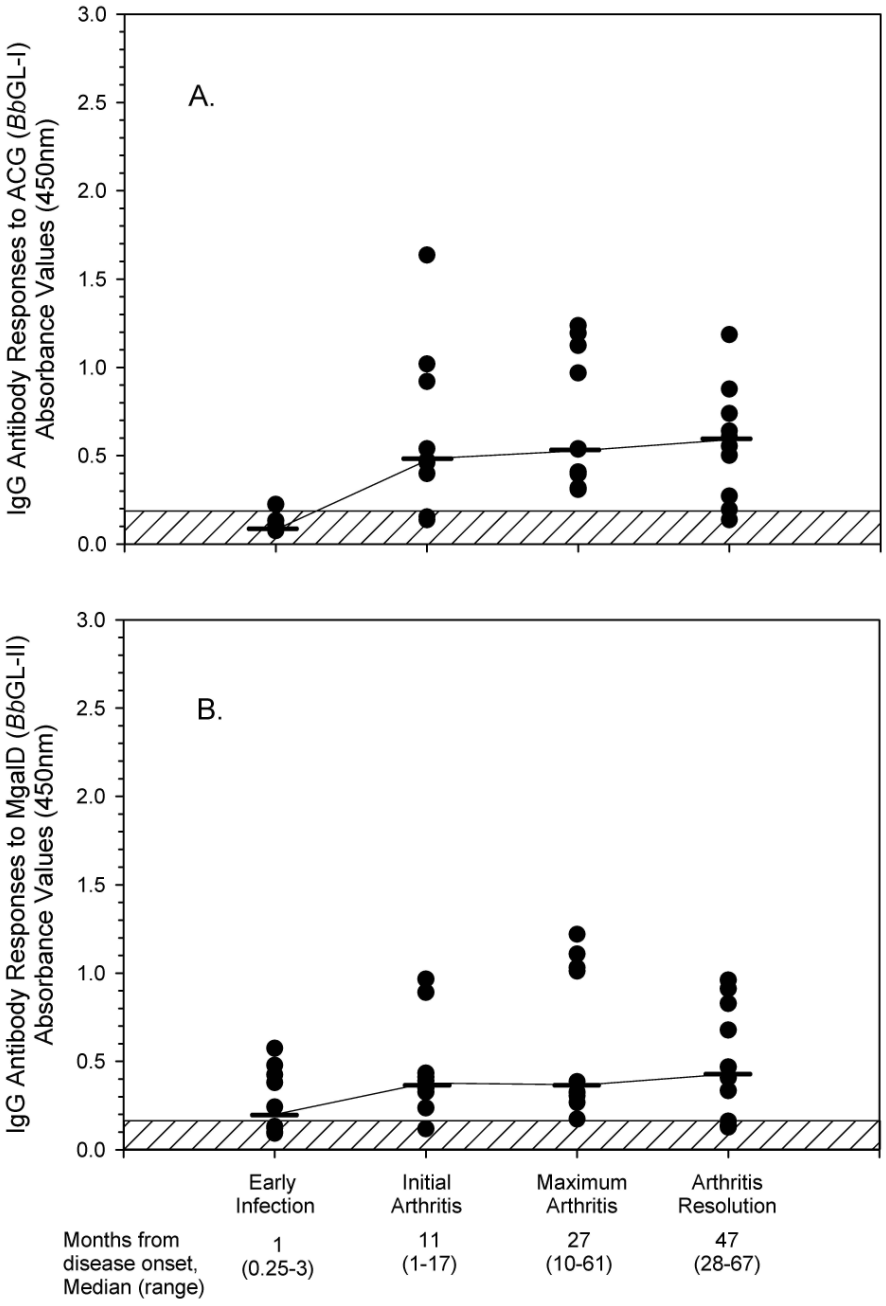

Figure 4.

Longitudinal IgG antibody responses to synthetic ACG (*Bb*GL-I) (panel A) and purified MgalD (*Bb*GL-II) (panel B) in serial serum samples from untreated patients followed throughout the course of Lyme disease. Four samples were tested from each patient; one was obtained early in the infection, a second during initial periods of arthritis, a third during maximal periods of arthritis, and a fourth near the time of resolution of arthritis. The horizontal bars indicate the median absorbance at each time point. Cut-off values for positive responses in individual patient samples were ≥3 standard deviations above the mean value of 22 normal control samples included on the same plate. ACG = acylated cholesteryl galactoside, MgalD = monogalactosyl diacylglycerol.

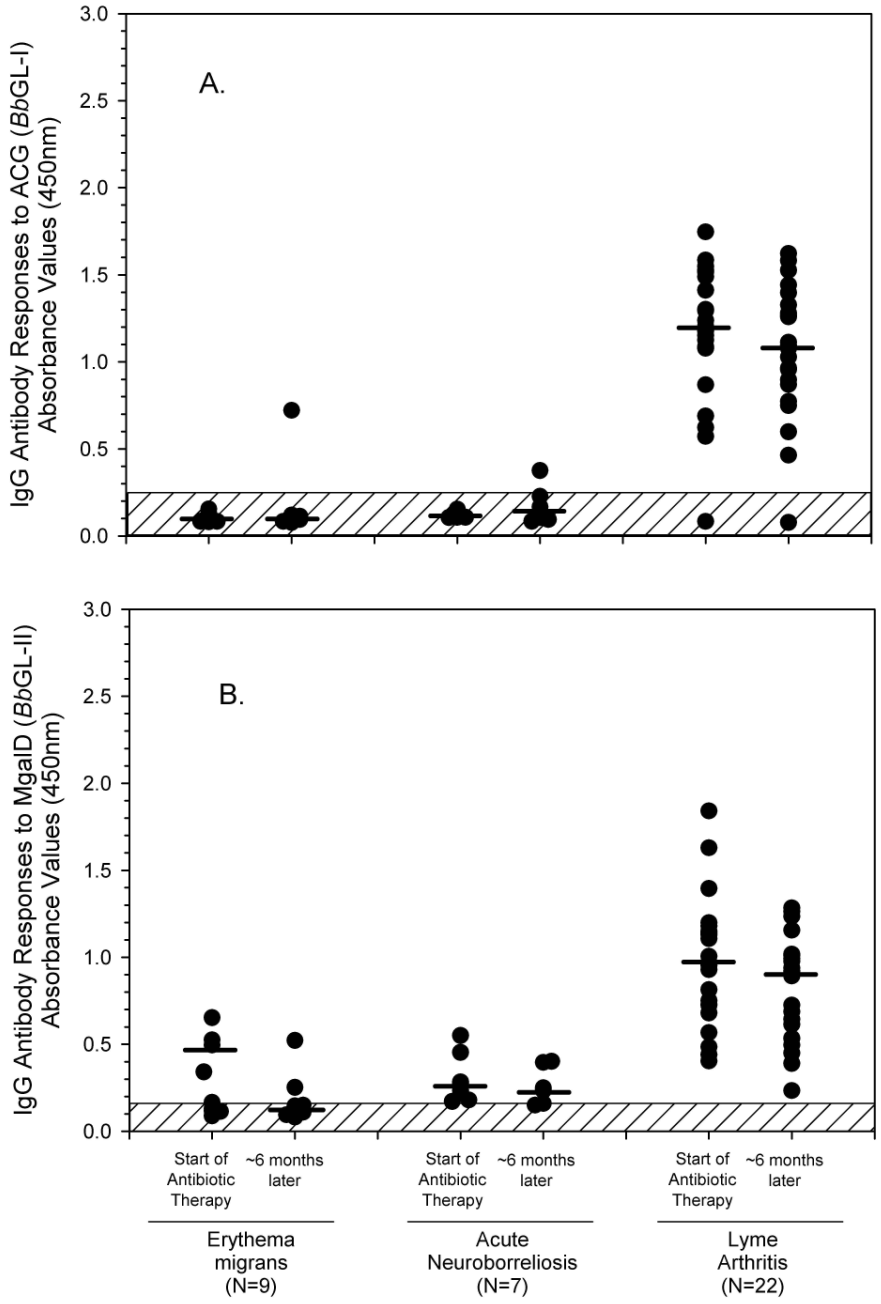

Figure 5.

Decline in IgG antibody responses to synthetic ACG (*Bb*GL-I) (panel A) and purified MgalD (*Bb*GL-II) (panel B) in patients with erythema migrans, neuroborreliosis, or Lyme arthritis after antibiotic treatment. Absorbance values were compared in initial samples obtained during the acute-phase or convalescent-phase of the illness and again approximately 6 months after the completion of antibiotic therapy. The horizontal bars indicate median absorbance values, which were calculated only for patients who had positive values in initial samples. Cut-off values for positive responses in individual patient samples were ≥3 standard deviations above the mean value of 22 normal control samples included on the same plate. The median values at the 2 time points were not significantly different in patients with erythema migrans or

neuroborreliosis. However, among the patients with arthritis, the difference of in values was a possible significance for ACG (P = 0.06) and was significantly different for MgalD (P = 0.008). ACG = acylated cholesteryl galactoside, MgalD = monogalactosyl diacylglycerol.

Table 1

Percent of patients with a positive IgG antibody response to the *B. burgdorferi* glycolipids.

	Percent Positive		
	ACG[a] (*Bb*GL-I)	MgalD[a] (*Bb*GL-II)	P Value[b]
Patients with Lyme disease			
Erythema migrans (N = 84)	10	29	≤0.001
Early neuroborreliosis (N = 35)	29	54	0.05
Lyme arthritis (N = 75)	96	99	NS
Normal control subjects (N = 70)	2	0	NS

[a] ACG = acylated cholesteryl galactoside, and MgalD = monogalactosyl diacylglycerol.

[b] Statistical significance of comparisons tested by chi square test.