Antibody Response to IR₆, a Conserved Immunodominant Region of the VlsE Lipoprotein, Wanes Rapidly after Antibiotic Treatment of Borrelia burgdorferi Infection in Experimental Animals and in Humans

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Invariable region (IR)₆, an immunodominant conserved region of VlsE, the antigenic variation protein of Borrelia burgdorferi, is currently used for the serologic diagnosis of Lyme disease in humans and canines. A longitudinal assessment of anti-IR₆ antibody levels in B. burgdorferi-infected rhesus monkeys revealed that this level diminished sharply after antibiotic treatment (within 25 weeks). In contrast, antibody levels to P39 and to whole-cell antigen extracts of B. burgdorferi either remained unchanged or diminished less. A longitudinal analysis in dogs yielded similar results. In humans, the anti-IR₆ antibody titer diminished by a factor of 25 since national surveillance began in 1982. At the same time, tangible progress has been made in the prevention and diagnosis of this emerging infectious disease. A commercial vaccine is available, and efforts to improve diagnostic serologic analysis have yielded algorithms to aid in the standardization of serologic methods. Research is in progress at the National Institutes of Health (NIH) to try to discern whether the so-called posttreatment Lyme disease syndrome (PTLDS; i.e., the occurrence of persistent signs and symptoms of disease, despite the administration of what is currently considered to be adequate antibiotic therapy) is due to ongoing active borrelial infection, to a postinfectious syndrome, to irreversible sequelae of earlier tissue injury, or to a condition altogether unrelated to Lyme disease.

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Study patients gave informed consent for punch biopsies.

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dorferi could be used as an indicator of infection status. Longitudinal analyses of serum titers, measured both by fluorescence and by ELISA, were performed. The consensus from these studies is either that IgG (or IgM) antibody titers do not change significantly after treatment or, when they do, the changes do not correlate with presence or absence of cure. In these ELISAs, whole-cell extracts of B. burgdorferi were used as antigen [8, 9].

Recently, a sensitive and specific serologic test for Lyme disease based on the detection of antibody to a conserved immunodominant region of VlsE was developed [4, 10–12]. VlsE, the antigenic variation lipoprotein of B. burgdorferi, has 2 invariable domains at the amino and carboxyl termini, respectively, and a central variable domain [13]. The variable domain contains 6 variable regions, VR1–VR6, and 6 invariable regions, IR1–IR6. The latter remain unchanged during antigenic variation [13] and are conserved among strains and genospecies of B. burgdorferi sensu lato [10]. IR6 is the most conserved of the IRs and is immunodominant both in monkeys and in humans infected with B. burgdorferi [10]. The serologic test developed is based on an ELISA that uses a peptide (C6) as antigen. This peptide reproduces the sequence of IR6 of the IP90 strain of the European genospecies B. garinii [4].

During a longitudinal assessment of the antibody response to C6 in rhesus monkeys infected with B. burgdorferi, we noticed that the anti-C6 antibody level diminished sharply after the animals were treated with antibiotics. In untreated animals, in contrast, this level remained essentially unchanged several years after infection [4]. This prompted us to compare the rate of decrease of anti-C6 antibody levels with that of antibodies to P39 (BmpA) [14] and to whole-cell antigen extracts of B. burgdorferi. We further compared these rates in B. burgdorferi–infected antibiotic-treated dogs and humans. Here we describe the results of these studies.

Materials and Methods

Antibiotic treatment, serum collection, and serologic analysis in monkeys and dogs. Seven 2–4-year-old rhesus macaques (Macaca mulatta) of both sexes were inoculated with the JD1 strain of B. burgdorferi sensu stricto by tick bite, as described elsewhere [15]. At postinoculation (PI) week 12, the animals were treated orally with doxycycline for 60 days (2 mg/kg twice a day). Peak and trough levels of serum doxycycline were determined, to ensure that the MIC had been reached. The doxycycline concentration at the peak was 7 times the MIC (0.3 mg/L); at the trough it was at or below the MIC. All of the animals were culture positive before treatment, as determined by cultivation of skin biopsy samples within the first 4 weeks after infection. Tissue cultivation was done as described elsewhere [16]. Serum samples were collected weekly for 40 weeks.

We used 3 serologic assays with these serum specimens: C6 ELISA, standard Lyme disease diagnostic ELISA, and P39 ELISA. The C6 ELISA was done as described elsewhere [4]. The standard ELISA, using whole-cell extract of B. burgdorferi as antigen, is available commercially (MarDx) and was performed according to the manufacturer’s instructions. Antigen for the P39 ELISA was a purified recombinant fusion protein that consisted of a fragment of BmpA, lacking the first 12 aa of the mature form of this protein, fused to the maltose-binding protein (MBP) of Escherichia coli. The BmpA gene was isolated from B. burgdorferi strain JD1 [17].

The optimum antigen and antibody conjugate concentrations were determined by checkerboard analysis. Immunopassay flat-bottom plates (Costar) were coated with 1 μg/mL of BmpA-MBP or with MBP alone in a 0.1 M carbonate buffer, pH 9.6, and were incubated for 3 h at 37°C in a humidified incubator. Unbound antigen and unbound reagents from each of the ensuing incubations were removed by 3 washes with PBS containing 0.05% Tween 20 (PBS-T). Subsequent incubations were for 1 h at 37°C with (1) 0.2 mL/well of a 3% (vol/vol) blocking solution of liquid gelatin (Norland Laboratories) in PBS-T; (2) 0.05 mL/well of monkey serum at a dilution of 1:200; or (3) 0.1 mL/well of a 1:14,000 dilution of horseradish peroxidase–labeled anti–human IgG antibody (γ-chain specific; catalog no. 074-1002; Kirkegaard & Perry). Plates were developed for 10 min by adding 0.1 mL/well of a solution that contained tetramethylbenzidine chromogen and hydrogen peroxide, as prepared by the manufacturer (catalog no. 50-76-00; Kirkegaard & Perry). The reaction was stopped with 0.1 mL/well of 0.1 N phosphoric acid, and the optical density (OD) was read at 450 nm in a Tecan Spectra II ELISA reader (SLT Lab Instruments). Net OD values were obtained by subtracting OD values obtained with MBP as antigen from OD values obtained with BmpA-MBP.

Serum samples from 4 additional rhesus macaques were serially assessed with the C6 ELISA. These animals had been inoculated with B. burgdorferi B31 spirochetes for another study [13] and were never treated with antibiotics.

We tested serial serum samples from 16 dogs (specific pathogen–free 6-week-old beagles of both sexes) that were infected with B. burgdorferi by exposure to feral ticks. Infection was confirmed in all of the dogs by culture and by polymerase chain reaction (PCR), as described elsewhere [18]. Starting on day 120 of the experiment, 3 groups of 4 dogs each were treated with doxycycline, ceftriaxone, or azithromycin, respectively, for 30 consecutive days. The remaining 4 animals were not treated and served as controls. Blood samples were collected every 2 weeks and were tested for anti–B. burgdorferi antibodies by a kinetic ELISA with whole-cell B. burgdorferi extract as antigen. Details of these procedures have been described elsewhere [18]. For the present study, serum samples from ceftriaxone–treated dogs and from control group animals were assessed for anti-C6 antibody with the C6 ELISA, as described elsewhere [12].

Serum samples from patients with early Lyme disease. We analyzed serum samples from Lyme disease patients participating in two clinical studies: the Lyme disease culture study of New York Medical College [19] and the “azithromycin trial” [20]. Patients in the first group were eligible for participation if they had ≥1 erythema migrans (EM) rashes (as defined in CDC surveillance criteria [21]) and had undergone a 2-mm skin biopsy. Patients with a positive culture of skin and/or blood samples were evaluated at baseline, at 7–10 and 21–28 days, at 3, 6, and 12 months, and annually thereafter. At these times, patients were interviewed and examined and a blood sample was taken. All of the patients were treated with antibiotics as per recommendations of the Infectious Diseases
Society of America [5]. They were considered to be cured if they were free of the signs and symptoms shown at presentation and had no additional clinical evidence of infection.

The serum samples from the azithromycin trial had been serially collected from 7 culture-confirmed patients with early Lyme disease during the first year after their treatment at the Marshfield clinic, Marshfield, Wisconsin [20]. The azithromycin trial was a double-blinded, randomized, controlled trial in which the efficacies of the antibiotics azithromycin and amoxicillin in the treatment of EM were compared. Serum specimens were collected serially at varying times for 26 weeks to >1 year after presentation [20]. In addition, a group of 4 serum samples from patients with early Lyme disease who presented with EM were obtained from the A. I. duPont Hospital for Children, Wilmington, Delaware. Serum specimens were obtained at presentation and ~6 months later. Patients were treated with cefuroxime axetil for 20 days, and all were free of signs and/or symptoms by week 12 after presentation.

Serum samples from patients with late Lyme disease and PTLDS. Patients with late Lyme disease included arthritis patients (n = 11) from the duPont Hospital for Children who had been treated either with amoxicillin or with doxycycline for 4 weeks and were free of signs and symptoms at the time of the last blood sample. The time between blood samplings varied between 4 and 76 weeks. The first sample was taken at presentation. In addition, serum samples from 1 patient with chronic treatment-resistant Lyme arthritis and from 2 PTLDS patients were serially obtained at the research hospital of the National Institute of Allergy and Infectious Diseases (NIAID; Bethesda, MD).

Serologic analysis of human serum specimens. C6 ELISA with human serum samples was performed as described elsewhere [4]. Anti-C6 antibody titers were determined by 2-fold serial dilutions. The end point was defined as the first dilution at which the OD value was below the cutoff line. The latter was set at the mean OD value of 10 serum specimens from patients living in an area where Lyme disease is not endemic plus 3 times the SD of that mean. Standard ELISA values and titers were determined by using B. burgdorferi whole-cell antigen extracts, as described elsewhere [22].

Results

Course of C6 antibody response in monkeys. The longitudinal analysis of serum antibody levels in monkeys yielded a striking result. Although antibody levels determined with the 3 assays (C6 ELISA, whole-cell antigen [standard] ELISA, and P39 ELISA [figure 1A, 1B, and 1C, respectively]) increased gradually in all animals during the initial course of infection, they declined at markedly different rates after antibiotic treatment. Between weeks 12 and 21, when the animals were treated with antibiotics, antibody levels to both C6 and whole-cell antigens declined, except in animal AA68, in which the standard ELISA OD remained essentially unchanged (figure 1B, arrow). As mentioned, the decline in the OD values was more pronounced with the C6 ELISA than with the standard ELISA. Anti-P39 antibody levels were not determined in this period, except for animal V134 (figure 1C, arrow). Surprisingly, by PI week 34 (13 weeks after treatment termination), the anti-C6 antibody levels had reached the background level for this assay (figure 1A). In contrast, the standard ELISA OD had not yet reached its corresponding cutoff line by PI week 40. In addition, for 5 of the 7 animals in the study, the antibody levels, as measured by the standard ELISA, had reached a plateau that was 1.5–4 times the cutoff value (figure 1B). The response to P39 followed a similar pattern. With the exception of the 2 animals that did not respond to this antigen, the OD value of the P39 ELISA had reached a plateau of 5–10 times the cutoff value for this assay by PI week 31 (figure 1C). In fact, the anti-P39 antibody levels appeared to be essentially unaffected by the doxycycline treatment.

Levels of anti-C6 antibody are fairly stable in the absence of antibiotic treatment. We previously determined that the anti-C6 antibody response in chronically infected monkeys remains high and at a level essentially unchanged for >3 years, the longest period studied [4]; however, in monkeys, during a short period at the beginning of the infection process, the anti-C6 antibody level transiently diminished. This dip in the C6 ELISA OD was observed in virtually every infected animal between PI weeks 5 and 12 and was unrelated to antibiotic treatment. Figure 2 shows the serial C6 ELISA OD values in 4 rhesus monkeys infected (via tick bite) with B. burgdorferi B31. The OD values dip by PI weeks 5–12. The dip also is visible in the experiment shown in figure 1A in animals V822, V760, V134, and AA68. In the remaining 3 animals (AL93, AK53, and AA67), the dip may have overlapped the doxycycline treatment.

Course of anti-C6 antibody response in dogs. In a previous investigation, Straubinger et al. [18] surveyed the serial antibody response to whole-cell antigen extracts of B. burgdorferi in the same dogs that were used in the present study. Their survey and ours included time points before and after ceftriaxone treatment. Such antibody levels, which were determined by kinetic ELISA, had reached a plateau at ~60% of the highest level reached in the study period by PI week 47 (i.e., 6 months after the termination of treatment) [18]. In contrast, the anti-C6 antibody level had already reached a plateau at this time, essentially at background level (figure 3). Hence, as with monkeys, the anti-C6 antibody response waned faster and more radically after antibiotic treatment than the antibody response to whole-cell antigens.

Before antibiotic treatment, spirochetes could be cultured from each skin sample of all the dogs during the first 120 days of infection. In contrast, after treatment, only control dogs yielded skin biopsy samples that were culture positive, and only rarely were skin samples PCR positive in treated dogs [18]. At postmortem examination, no treated dog yielded tissue samples that were culture positive, whereas untreated dogs yielded multiple tissue samples that were culture positive for B. burgdorferi [18]. Thus, the antibiotic either greatly reduced or totally eliminated the infectious burden of treated dogs, in comparison with untreated dogs. Concomitantly, the anti-C6 antibody decreased almost to background levels in treated dogs but remained at
Figure 1. Longitudinal assessment of anti-C_{6} (A), anti-whole-cell antigen extract (B), and anti-P39 (C) antibody response in rhesus monkeys infected with *Borrelia burgdorferi* JD1 and treated with doxycycline. Vertical lines, times of initiation and termination of treatment. B and C. Arrows indicate standard ELISA curve for animal AA68 and P39 antibody curve for animal V134, respectively (see text). Cutoff line was set at mean optical density (OD) value of preinfection serum samples plus 3 × SD of the mean.

The highest levels in control animals throughout the study period (figure 3).

**Course of anti-C_{6} antibody response in humans.** We next compared the decline in anti-C_{6} antibody titer with the decline in antibody titer to whole-cell extracts in 4 patients with early Lyme disease and in 11 patients with late Lyme disease seen at the duPont Hospital for Children, who responded to treatment. In all but 1 case, the anti-C_{6} antibody titer fell faster than the anti-whole-cell antigen titer. Interestingly, the anti-C_{6} antibody titer decreased by a factor of $\geq 4$ in all of the patients...
Figure 2. Serial anti-C₆ antibody response in 4 rhesus macaques (L457, J831, L131, and M021) infected with *Borrelia burgdorferi* B31. OD, optical density.

when assessed at >20 weeks after presentation. The geometric mean decline in anti-C₆ antibody titer was 9.1 (range, 4–32) when the second serum sample was obtained ≥21 weeks after presentation and 4.0 (range, 1–8) when obtained at ≤20 weeks. Similar results were obtained with serum samples obtained at presentation and at years 1, 2, 4, or 5 after treatment from 9 patients with early Lyme disease seen at the New York Medical College. These patients were free of signs and symptoms at the later visit. Regardless of the time elapsed, at the time of the last visit, the anti-C₆ antibody titer had fallen by a factor of ≥4 (median, 9.3; range, 4–64). When serum specimens from the duPont Hospital patients who were tested for anti-C₆ antibody were tested by standard (whole-cell antigen) ELISA, the geometric mean decline in titer for samples collected ≥21 weeks after presentation was 1.6 (range, 1–4) and for specimens collected before that time likewise was 1.6 (range, 1–8). These results, and those with experimental animals, suggest that the rate of decline in anti-C₆ antibody titer, unlike that of antibody to whole-cell antigen, could serve as an indirect indicator of spirochetal burden (i.e., a test to assess response to Lyme disease therapy or to assess whether a *B. burgdorferi* infection had been eliminated).

*Initial assessment of C₆ ELISA as a test of response to therapy or of cure.* To examine the C₆ ELISA as a test of response to therapy or cure, we used serum samples from patients who had unmistakable signs and symptoms of treatment-resistant Lyme disease. We first assessed patients with early culture-confirmed Lyme disease who had participated in the azithromycin trial [20] and whose serum specimens were serially collected during the first posttreatment year. All but 1 of the 7 patients

Figure 3. Anti-C₆ antibody response in dogs infected with *Borrelia burgdorferi* and treated with ceftriaxone (ceft; dotted lines) or not treated (solid lines). Vertical lines, times of initiation and termination of treatment. Cutoff was set at mean optical density (OD) value of preinfection serum samples plus 3 × SD of the mean.
were successfully treated. Patient MC2, in whom initial treatment failed, received iv ceftriaxone 1 year after treatment with azithromycin. This patient had intense migratory joint pain that started at 7 months and a small right knee effusion at 11 months that was positive for *B. burgdorferi* by PCR [23]. Except for patient MC2, whose anti-C₆ antibody level remained essentially unchanged for 1 year, the antibody levels of all patients either reached the cutoff line or likely would have done so shortly after the last time point we assessed (figure 4). As before, during a 6-month posttreatment period, titers diminished by a factor of ≧4 for all of the patients who were free of symptoms, except for patient MC17, whose initial serum sample had a low OD value (figure 4). The geometric mean decline in titer for cured patients was 8.6 (range, 3–128). In contrast, the serum titer of patient MC2, who was treatment resistant, increased 4-fold instead of diminishing.

Three additional serum samples from patients with either chronic treatment-resistant Lyme arthritis or PTLDS who were treated at the NIAID research hospital were assessed. Patient 1 (with PTLDS) was a 36-year-old man who had an EM rash in April 1996 and fever, fatigue, myalgias, and arthralgias. In July 1996, he developed right knee arthritis, followed by right ankle and left elbow arthritis. Both standard ELISA and Western blot were positive. He was treated with iv ceftriaxone for 1 month after the July visit. One month after finishing the therapy, the symptoms returned (myalgias, arthralgias, fatigue, difficulty concentrating, short-term memory loss, anxiety, and irritability). He was treated with amoxicillin but did not improve. The relative invariance of the anti-C₆ antibody titer in patient 1, despite the repeated treatment, correlates with the persistence of symptoms. Blood samples were collected at roughly 6-month intervals (table 1).

Patient 4 (with treatment-resistant Lyme arthritis), a 76-year-old woman, was seen early in 1997 with severe depression. Brain magnetic resonance imaging showed multiple white matter lesions. Lyme ELISA and IgG Western blot analyses were positive. Symptoms of depression had started ~1990. He had a history of right facial palsy in 1985, followed by multiple episodes of recurrent bilateral knee arthritis from 1987 to 1989. He was treated iv for 3 weeks with ceftriaxone (completed 13 August 1994). One year after treatment, his symptoms had not resolved, and his anti-C₆ antibody titer was unchanged (table 1). Thus, in the 3 patients with treatment-resistant Lyme disease or PTLDS, there was a strict correlation between presence of symptoms and change of anti-C₆ antibody titers by a factor of <4.

**Discussion**

We have shown that the antibody response to IR₆, as measured by the C₆ ELISA, wanes rapidly after antibiotic treatment of a *B. burgdorferi* infection in experimental animals and humans. Both anti-C₆ antibody levels, as measured by the ELISA OD, and, of more importance, antibody titers decreased more quickly than antibody to *B. burgdorferi* whole-cell extracts. This happened regardless of host species and was not the trivial consequence of comparing antibody level elicited against mul-

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**Figure 4.** Anti-C₆ antibody level as function of time after treatment in antibiotic-treated patients from the Marshfield clinic (Marshfield, WI). Cutoff was defined as mean optical density (OD) value of serum samples from 10 patients from a hospital in an area where Lyme disease is not endemic plus 3 × SD of the mean.
The development of such a test for Lyme disease has been elusive, perhaps because the focus has been antibody to whole-cell antigen extracts [8, 9, 25]. Many of these antigens, including P39, are probably efficient elicitors of B cell memory. Periodic restimulation of B memory cells with antigens is thought to be required for the long-term maintenance of both an antibody response and the memory B cell pool [26, 27]. Thus, availability of antigens in the form of immune complexes is a prerequisite for the maintenance of long-term serum antibody responses. These antigens are stored in folliculodendritic cells (FDCs), which are highly adapted to serve as depot for antigen-antibody complexes that drive the B cell differentiation process [28]. FDCs are thought to sequester antigens in an immunogenic form for up to several years, making antigens available for the restimulation of memory cells and the maintenance of serum antibody levels [26, 28].

*B. burgdorferi* antigens that can stimulate a sustained serum antibody response long after spirochetes have been killed must be molecules that are both abundant and stable in the host milieu and possibly are released (or shed) by live (or dead) spirochetes so as to easily form immune complexes. Above all, they should not be subjected to rapid turnover by the spirochete. This last attribute is the one we believe is key, for antigens that are rapidly turned over by living spirochetes shortly after their synthesis are probably nonabundant and unstable and/or disappear rapidly after spirochetal death. Production of antibodies to such antigens would not be maintained after spirochetal death.

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### Table 1. Serum samples from patients with late treatment-resistant Lyme arthritis or posttreatment Lyme disease syndrome.

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<thead>
<tr>
<th>Patient</th>
<th>Sampling date</th>
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<tr>
<td>Patient 1</td>
<td>1/28/97</td>
<td>12,800&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Previously treated with ceftriaxone and amoxicillin; no antibiotics at evaluation</td>
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<td>12,800</td>
<td>Bilateral knee arthritis remained; PCR of synovial fluid weakly positive</td>
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<td>Persistent arthritis; negative synovial PCR</td>
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<td>8/20/97</td>
<td>25,600&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 Weeks of ceftriaxone treatment completed 1 week before evaluation</td>
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<td>9/11/97</td>
<td>12,800–25,600</td>
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**NOTE.** C<sub>6</sub> titer ratio (initial:final) was 2 for patients 1 and 4 and 1 for patient 7. iv, intravenous; MRI, magnetic resonance imaging; PCR, polymerase chain reaction.

<sup>a</sup> Initial.

<sup>b</sup> Final.

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multiple antigens with that elicited against a single one, as evidenced by the persistent response to P39 observed in antibiotic-treated monkeys. In fact, the anti-P39 response was remarkably stable, when compared with the anti-C<sub>6</sub> response.

In dogs and monkeys with untreated *B. burgdorferi* infections, the response to C<sub>6</sub> remains stable. In serum specimens from treatment-resistant Lyme disease patients, the C<sub>6</sub> antibody titer also remained fairly constant. We submit, therefore, that the anti-C<sub>6</sub> antibody response should be further investigated as a possible test to assess response to therapy or cure from a *B. burgdorferi* infection. Such a test could be used not only to ascertain whether treatment of early Lyme disease is successful, thereby preventing the transition to the late, more intractable form of the disease, but also to distinguish among the possible etiologies of PTLS. A test for cure of spirochetal infection has long been available for syphilis. Antibiotic treatment efficacy in patients with syphilis is assessed by using the quantitative forms of the VDRL test or the rapid plasma reagin test. Both tests are based on the quantification of serum antibody that reacts with a mixture of cardiolipin, cholesterol, and lecithin [24]. Quantitative reactions are reported in terms of the highest (last) dilution at which the specimen is fully reactive. Treatment is considered successful if titer declines ≥4-fold [24].
might be a T cell–independent (TI) antigen. By using mice lacking both αδ+ and γδ+ T cells, McKisc and Barthold [30] recently identified several B. burgdorferi antigens that can elicit an antibody response during infection in such animals [30]. Proteins of molecular masses of 21, 32, 34, 39, 58, and 66 kDa were among the TI antigens identified [30]. The mitogenic effect of bacterial lipoproteins on B cells is well known [31–34]. It is thus conceivable that an organism such as B. burgdorferi, in which 11% of the genome encodes potential lipoproteins [35], can generate an array of TI (and T cell–dependent) antibody responses. In preliminary experiments, we verified that normal mice infected with B. burgdorferi and subsequently treated with ceftriaxone experience a decline in anti-C6 antibody levels similar to those observed in monkeys and dogs. In contrast, antibody levels to P39 remained essentially unchanged (M.T.P., M.B.J., and J.E.P., unpublished data). If normal immunocompetent mice can mount primarily TI antibody responses to the IR6, as detected by the C6 peptide ELISA, it is possible that such responses would be short-lived after antibiotic treatment, since some TI antigens are poor elicitors of B cell memory responses [36, 37]. We are now testing this alternative hypothesis.

In conclusion, the swift waning of the anti-C6 antibody response in the wake of antibiotic treatment, as seen both in experimental animals and in humans, suggests that the quantification of C6 antibody titer as a function of time may serve as a test to assess whether a B. burgdorferi infection has been eliminated. We are in the process of further evaluating the validity of this contention in an expanded retrospective study by using serum samples from patients with both early and late Lyme disease. If this study yields a satisfactory result, it will be followed by a prospective evaluation.

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References