Rapid Decline of OspC Borreliacidal Antibodies following Treatment of Patients with Early Lyme Disease

Dean A. Jobe, Todd J. Kowalski, Marissa Bloemke, S. D. Lovrich, and Steven M. Callister

Microbiology Research Laboratory and Section of Infectious Diseases, Gunderson Lutheran Medical Center and Department of Microbiology, University of Wisconsin—La Crosse, La Crosse, Wisconsin

Received 11 February 2011/Returned for modification 11 March 2011/Accepted 8 April 2011

We determined whether the levels of OspC borreliacidal antibodies declined following treatment of early Lyme disease and whether the OspC7 peptide enzyme-linked immunosorbent assay (ELISA) could be used as an alternative test for detecting the response. Serum samples were collected from 37 subjects at the onset of illness and 2 and 6 months after treatment with doxycycline. The ELISA detected IgM and IgG OspC7 antibodies within 2 months in 18 (49%) and 5 (14%) sera, respectively. Moreover, the sera from 12 subjects who tested positive by the ELISA also showed borreliacidal activity which was completely abrogated when the antibodies to OspC7 were removed. The borreliacidal activity decreased greater than 4-fold in each seropositive patient within 6 months after treatment, and the findings were accurately predicted by the IgM ELISA. The results confirmed that the ELISA was an effective alternative for detection of OspC borreliacidal antibodies produced during early Lyme disease in humans and also provided strong evidence that a significant decline in the response coincides with successful treatment of the illness.

Diagnostic testing for Lyme disease has improved significantly since the first tests were developed in the early 1980s, and the result has been increased accuracy, especially in areas where the illness is not endemic or in patients with atypical signs and symptoms. Moreover, the widespread availability of improved tests offers the distinct possibility that the cumbersome two-tiered system will become obsolete (1, 19, 20, 26). However, tests that can also be used to accurately predict successful elimination of the spirochetes after treatment have remained elusive. Such a test is especially important because other illnesses cause similar symptoms and because the spirochetes can persist despite aggressive treatment (2, 13, 31). In addition, repeated infections with *Borrelia burgdorferi* are possible (33). This can result in considerable confusion as patients and clinicians seek assurance that the treatment resolved the infection (6, 12).

To date, only tests that detect antibody responses against the *B. burgdorferi* antigenic variation protein (VlsE) or a peptide (C6) based on the sixth invariant region of VlsE have shown promise for predicting eradication of the spirochetes, but widespread utility remains controversial. For example, Marangoni et al. (22) showed that VlsE-positive patients with early Lyme disease were seronegative within 2 to 6 months after treatment. In addition, Philipp et al. (30) showed that C6 antibody titers declined at least 4-fold in 80% of treated early Lyme disease patients within 12 months. In contrast, however, Peltomaa et al. (29) showed that the C6 antibody levels failed to decrease significantly within time intervals up to 15 years in 86% of patients whose later stages of Lyme disease were treated successfully.

Another important antibody response produced during early Lyme disease is the production of OspC borreliacidal antibodies that kill the spirochetes by activation of complement (21, 32). In addition, a semiautomated flow cytometric test that detects the response provides sensitive and highly specific confirmation of the illness (4, 5). Unfortunately, the test is not widely used, primarily because the flow cytometry and the use of viable spirochetes add considerable technical complexity. More recently, however, researchers (16, 21) showed that the OspC borreliacidal antibodies produced during a natural human infection recognized a single epitope located within the 7 amino acids (AESPKKP) nearest the carboxy-terminus of the protein. In addition, Lovrich et al. (21) showed that the borreliacidal antibodies comprised the vast majority of the OspC-specific antibody response produced during early human illness. Moreover, Jobe et al. (15) showed that an enzyme-linked immunosorbent assay (ELISA) based on the corresponding peptide (designated OspC7) was an effective alternative for detecting the antibodies that also eliminated the technical difficulties of the flow cytometric procedure.

Interestingly, vaccines that provide immunity from Lyme disease by inducing borreliacidal OspA and OspC antibodies must be administered annually because the antibodies wane to undetectable levels (18, 28, 34). In addition, we only rarely detect borreliacidal antibodies in sera from healthy individuals or patients with other illnesses, even though our region is highly endemic for *B. burgdorferi* (14), and a significant number of donors have been treated previously for Lyme disease (unpublished observation). Moreover, IgM OspC borreliacidal antibodies, which predominate during several stages of Lyme disease (15, 16, 21, 23), only rarely switch to an IgG response. In fact, Mathiesen et al. (23, 24) speculated that the extensive cross-linking of OspC within the spirochetal membrane hinders interaction with T cells, and the T-cell-independent processing limits class switching. Based on these findings, we hypothesized that tests that detect OspC borreliacidal antibodies...
may also be useful for predicting successful elimination of the spirochetes after treatment. We therefore characterized the OspC borreliacidal antibody response after patients with early localized or early disseminated Lyme disease were treated with antibiotics. In addition, we compared the results to those obtained using the OspC7 ELISA.

The protocols were reviewed and approved by the Institutional Review Board (IRB) of the Gundersen Lutheran Medical Center. Pregnant patients or subjects with active connective tissue disease (e.g., systemic lupus erythematosi,s, rheumatoid arthritis), history of Lyme disease, or current immunosuppressing medications (e.g., prednisone) were excluded. The study population consisted of 37 adult patients who presented to the Gundersen Lutheran Medical Center urgent care department with exposure to ticks within 30 days before developing a single (early localized disease; \( n = 27 \)) or multiple (early disseminated disease; \( n = 10 \)) erythema migrans (EM). Twenty-nine subjects had EM lesions with characteristics (\( \geq 4 \) cm diameter with central clearing) sufficient to fulfill the Centers for Disease Control and Prevention surveillance criteria (7), and the remaining 8 patients had atypical lesions that ranged from petechiae to flat ovals without central clearing. The patients with atypical lesions were included because they also had appropriate additional clinical findings (e.g., a tick bite) and atypical lesions can occur (25). The most common constitutional complaints were fatigue (70%), myalgia (62%), headache (57%), stiff neck (47%), and arthralgia (43%).

Each subject received doxycycline (100 mg twice daily) for 10 to 21 days, and information from medical records and follow-up interviews was used to confirm resolution of the clinical abnormalities. Thirty-one (84%) subjects reported complete resolution (clinical cure) of the illness, and 6 (16%) reported persistent symptoms that included fatigue (\( n = 5 \)), stiff neck (\( n = 2 \)), and headache (\( n = 1 \)). However, no subjects reported ongoing symptoms with sufficient severity to suggest post-Lyme disease syndrome (35), and one subject who complained of a stiff neck and headache also had a previous diagnosis of osteoarthritis. The findings therefore provided strong evidence that the antibiotic treatment had successfully eliminated the spirochetes despite the persistence of mild symptoms in a few patients. As further support, Nowakowski et al. (27) showed that about 10% of patients with early Lyme disease reported persistent or intermittent nonspecific symptoms for months to years after the spirochetes were no longer present. In addition, Kowalski et al. (17) confirmed that treatment with doxycycline for as little as 7 days was rarely (<1%) unsuccessful.

Blood samples were collected by venipuncture in red-top Vacutainer tubes during the initial visit and approximately 2 and 6 months after treatment, and each was coded so that collection times and patient information were blinded. The serum was separated by centrifugation and stored in individual cryogenic vials (Corning Incorporated, Corning, NY) at \(-20^\circ C\) until tested.

The borreliacidal antibody test was performed as described previously (4). Briefly, 100-\( \mu \)l amounts of Barbour-Stoenner-Kelly (BSK) medium that contained \( 5 \times 10^5 \) log-phase \( B. \) burgdorferi 50772, which lacks the plasmid containing \( ospA \) and \( ospB \) and expresses OspC (32), was combined with 100-\( \mu \)l volumes of serum serially diluted in BSK and 5 \( \mu \)l guinea pig serum (\( \geq 200 \) 50% hemolytic complement units per ml). The suspensions were then incubated overnight at 35°C. Following incubation, 100 \( \mu \)l of each sample was combined with phosphate-buffered saline (PBS; pH 7.2) that contained acridine orange and then analyzed by flow cytometry. Samples yielding a \( \geq 13\% \) increase in fluorescence intensity compared to a normal serum control were considered positive (4, 5). In addition, positive assays were examined by dark-field microscopy to confirm the presence of characteristic blebbled and nonmotile spirochetes, and a positive control was included with each run to minimize interassay variability.

Antibodies that bound OspC or the OspC7 peptide were also detected by ELISA as described previously (15, 16). Briefly, a recombinant OspC (rOspC) or biotinylated OspC7 peptide was attached to individual wells of microtiter plates (Immulon 2 HB; Thermo Labsystems, Franklin, MA) via streptavidin (Pierce, Rockland, IL) and 100-\( \mu \)l amounts of each serial dilution of patient serum and 100-\( \mu \)l amounts of human IgM or IgG conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, IL) were added sequentially. Absorbances at 490 nm (SpectraMax 250 Molecular Devices, Sunnyvale, CA) were then determined after incubation with 0-phenylenediamine substrate (Sigma, St. Louis, MO), and samples that yielded significant levels of IgM (optical density [OD] of \( \geq 0.89 \)) or IgG (\( \geq 0.39 \)) compared to those of a normal serum control were considered positive. The cutoff levels were determined by calculating two standard deviations above the mean IgM and IgG reactivities of normal and potential cross-reactive sera (15), and the normal serum control had reactivity near the mean values. In addition, a well-characterized (4) early Lyme disease serum was included as a positive control and runs where the last dilution with significant reactivity was not identical to the previous run were repeated.

Twelve (32%) of the 37 initial (acute) sera contained IgM OspC7 antibodies, but only 2 (5%) were from the patients with a single EM (Table 1). The remaining 10 (27%) positive patients had multiple EM, and 3 (8%) also produced IgG antibodies. In addition, 6 single-EM patients produced IgM antibodies within 2 months after treatment and two sera also contained IgG antibodies. Moreover, the antibody titers decreased to undetectable levels 6 months after treatment in two IgM-positive sera and one IgG-positive serum from the pa-

| TABLE 1. Detection of OspC7 antibodies and borreliacidal activity in acute and convalescent-phase serum samples |
|-----------------------------------------------|
| Presence of EM | Time posttreatment | No. (%) of sera positive for indicated antibody detected by OspC7 ELISA | No. (%) of sera with borreliacidal activity |
|-----------------|-------------------|-------------------------------------------------|------------------------------------------|
| Single EM lesion | Acute | &times;100 | 2 (7) | 0 | 1 (4) |
| (n = 27)       | 2 mo | &times;30 | 8 (30) | 2 (7) | 3 (11) |
|                | 6 mo | &times;22 | 6 (22) | 1 (4) | 2 (7) |
| Multiple EM lesions | Acute | &times;100 | 10 (100) | 3 (30) | 9 (90) |
| (n = 10)       | 2 mo | &times;100 | 10 (100) | 3 (30) | 4 (40) |
|                | 6 mo | &times;50 | 5 (50) | 2 (20) | 1 (10) |
patients with single EM lesions and five IgM-positive sera and one IgG-positive serum from the patients with multiple EM. The findings from the borreliacidal antibody test were similar to the results from the OspC7 ELISA. Specifically, borreliacidal activity was detected in 10 (27%) acute-phase sera, and two (5%) additional sera were positive within 2 months after treatment. In addition, the borreliacidal activity was detected in only the sera that contained the highest levels (OD values ≥ 1.11) of IgM OspC7 antibodies. Additionally, the borreliacidal activity had decreased to undetectable levels within 6 months after treatment in all but three previously positive patients.

The close correlation provided strong evidence that the OspC7 peptide ELISA and borreliacidal antibody test were detecting the same antibody response. As a further confirmation, we then removed the OspC7 antibodies from three positive acute-phase sera by passage over columns that contained OspC7 as described previously (16, 21) and examined the effect on borreliacidal activity due to removing the OspC7 antibodies by adsorption. Without fail, removing the OspC7 antibodies abrogated the borreliacidal activity completely (Table 2). The findings therefore extended previous results (15) by confirming that the OspC7 ELISA was an effective alternative for detecting OspC borreliacidal antibodies.

In addition, we examined the effect of eliminating the OspC7 antibodies on the reactivity detected by an rOspC ELISA. Removal of the OspC7 borreliacidal antibodies completely eliminated the reactivity (Table 2). This finding also corroborates that of Lovrich et al. (21), who demonstrated that the immunodominant OspC antibody response during human Lyme disease was borreliacidal antibodies specific for OspC7. More significantly, the finding suggests that a human vaccine based on the epitope may provide comprehensive protection because the region of OspC defined by the peptide is conserved among the pathogenic genospecies.

This possibility contradicts findings of other investigators who have identified multiple epitopes within hypervariable regions of OspC that also induce complement-fixing antibodies (3, 8–11), indicating that a multivalent vaccine will be necessary for comprehensive protection. In fact, Earnhart and Marconi (11) showed that broad protection required at least 8 OspC borreliacidal antibody epitopes. To our knowledge, however, the epitopes were characterized by evaluating the immune responses in mice, and researchers have shown that there are distinct differences in the OspC borreliacidal antibodies produced by mice or humans. For example, Lovrich et al. (21) showed that mice do not produce borreliacidal OspC antibodies after challenge with host-adapted B. burgdorferi. In addition, Earnhart and Marconi (11) reported that mice failed to produce significant levels of borreliacidal antibodies after vaccination with a peptide that contained the borreliacidal antibody epitope contained within OspC7. The findings, therefore, cast doubt on the validity of mouse models to accurately predict the ability of vaccination with OspC-related proteins or peptides to provide human protection.

The low sensitivities of the borreliacidal antibody and OspC7 ELISAs in the subjects with single EM lesions should also be noted, because the finding contradicts earlier reports. For example, Jobe et al. (15) detected IgM OspC7 antibodies in 69 (80%) of 86 sera from patients with single EM lesions. In addition, Callister et al. (4) found borreliacidal antibodies in 16 (73%) of 22 sera from patients with early Lyme disease. Additional studies to explain this discrepancy are needed, but a possible explanation is that the earlier studies used sera from patients who were infected for a longer time period before

| Outcome                        | No. (%) of sera with borreliacidal activity | No. (%) of sera with indicated OspC7 antibody |
|-------------------------------|--------------------------------------------|---------------------------------------------|
|                               | Single EM (n = 3) | Multiple EM (n = 9) | Total (n = 12) | Single EM (n = 8) | Multiple EM (n = 10) | Total (n = 18) | Single EM (n = 2) | Multiple EM (n = 3) | Total (n = 5) |
| Declining follow-up titer     | 1 (33)          | 1 (11)             | 2 (15)       | 1 (12)         | 3 (30)             | 4 (22)         | 1 (50)          | 2 (67)             | 3 (60)          |
| Negative follow-up titer      | 2 (67)          | 8 (89)             | 10 (85)      | 2 (25)         | 7 (70)             | 9 (50)         | 1 (50)          | 1 (33)             | 2 (40)          |
| Total                         | 3 (100)         | 9 (100)            | 12 (100)     | 3 (38)         | 10 (100)           | 13 (72)        | 2 (100)         | 3 (100)            | 5 (100)         |

a Follow-up titer declined ≥4-fold with respect to baseline or convalescent-phase titer.
b Five subjects with a single EM seroconverted after 2 months and each titer then declined only 2-fold after 6 months.

TABLE 2. Elimination of borreliacidal activity and OspC antibodies by removing OspC7 antibodies

| Serum sample | OspC7 IgM ELISA* | Borreliacidal activity† | OspC IgM ELISA |
|--------------|------------------|-------------------------|----------------|
|              | Untreated | OspC7 adsorbed | Untreated | OspC7 adsorbed | Untreated | OspC7 adsorbed |
| 1            | 51,200    | <200          | 25,600   | <200          | 25,600   | <200          |
| 2            | 12,800    | <200          | 6,400    | <200          | 12,800   | <200          |
| 3            | 12,800    | <200          | 6,400    | <200          | 6,400    | <200          |

* Reciprocal of last dilution that yielded a ≥0.89 above that of a normal serum control.
† Reciprocal of last dilution that yielded a ≥13% shift in fluorescence compared to that of a normal serum control.

TABLE 3. Borreliacidal activity and OspC7 antibody responses to treatment
seeking medical advice. In support, OspC7-specific borreliacidal antibodies were detected in 2-month convalescent-phase sera of 8 additional single-lesion subjects. Another possibility is that some patients were not infected with *B. burgdorferi*, especially since we used only clinical findings to confirm the illness. Therefore, it will be necessary to confirm the illness by culture or PCR analysis in future studies to more accurately evaluate sensitivity.

Despite the low sensitivity, however, the preliminary findings still provide strong support for our hypothesis. Specifically, the borreliacidal activity decreased greater than 4-fold (*n* = 2) or disappeared entirely (*n* = 10) within 6 months after treatment in each seropositive subject (Table 3). In addition, the IgM (n = 13) and IgG (n = 5) OspC7 antibody titers decreased greater than 4-fold in each patient who had seroconverted at the time of diagnosis (acute-phase serum) and 2-fold in the patients who developed a detectable IgM antibody response after 2 months. Based on these findings, more comprehensive studies to determine the full utility of the OspC borreliacidal antibody test or OspC7 ELISA as a “test of cure” are warranted.

**REFERENCES**

1. Bacon, R. M., et al. 2003. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. J. Infect. Dis. 187:1187–1199.

2. Barthold, S. W., et al. 2010. Ineffectiveness of tigecycline against persistent *Borrelia burgdorferi*. Antimicrob. Agents Chemother. 54:643–651.

3. Buckles, E. L., C. G. Earnhart, and R. T. Marconi. 2006. Analysis of antibody response in humans to the type A OspC loop 5 domain and assessment of the potential utility of the loop 5 epitope in Lyme disease vaccine development. Clin. Vaccine Immunol. 13:1162–1165.

4. Callister, S. M. 2002. Ability of borreliacidal antibody test to confirm Lyme disease in clinical practice. Clin. Diagn. Lab. Immunol. 9:908–912.

5. Callister, S. M., D. A. Jobe, R. F. Schell, C. S. Pavia, and S. D. Lovrich. 1996. Sensitivity and specificity of the borreliacidal-antibody test during early Lyme disease: a gold standard? Clin. Diagn. Lab. Immunol. 3:339–402.

6. Cameron, D. J. 2010. Proof that chronic Lyme disease exists. Interdiscip. Perspect. Infect. Dis. doi:10.1155/2010/876450.

7. Centers for Disease Control. 1997. Case definitions for infectious disease conditions under public health surveillance. MMWR Recomm. Rep. 46(RR-10):1–57.

8. Earnhart, C. G., E. L. Buckles, J. S. Dummer, and R. T. Marconi. 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect. Immun. 73:7869–7877.

9. Earnhart, C. G., E. L. Buckles, and R. T. Marconi. 2007. Development of an OspC-based tetraivalent, recombinant, chimeric vaccine that elicits bactericidal antibody against diverse Lyme disease spirochete strains. Vaccine 25:466–480.

10. Earnhart, C. G., and R. T. Marconi. 2007. OspC phylogenetic analyses support the feasibility of a broadly protective polyvalent chimeric Lyme disease vaccine. Clin. Vaccine Immunol. 14:628–634.

11. Earnhart, C. G., and R. T. Marconi. 2007. An ovattent Lyme disease vaccine induces antibodies that recognize all incorporated OspC type-specific sequences. Hum. Vaccin. 3:281–289.

12. Fedor, H. M., Jr., et al. 2007. A critical appraisal of “chronic Lyme disease.” N. Engl. J. Med. 14:1242–1430.

13. Hodzic, E., S. Fang, K. Holden, K. J. Freet, and S. W. Barthold. 2008. Persistence of *Borrelia burgdorferi* following antibiotic treatment of mice. Antimicrob. Agents Chemother. 52:1728–1736.

14. Jackson, C. A., S. D. Lovrich, W. A. Agger, and S. M. Callister. 2002. Reassessment of a Midwestern Lyme disease focus for *Borrelia burgdorferi* and the human granulocytic ehrlichiosis agent. J. Clin. Microbiol. 40:2070–2073.

15. Jobe, D. A., et al. 2008. Significantly improved accuracy of diagnosis of early Lyme disease by peptide enzyme-linked immunosorbent assay based on the borreliacidal antibody epitope of *Borrelia burgdorferi* OspC. Clin. Vaccine Immunol. 15:981–985.

16. Jobe, D. A., S. D. Lovrich, R. F. Schell, and S. M. Callister. 2003. C-terminal region of outer surface protein C binds borreliacidal antibodies in sera from patients with Lyme disease. Clin. Diagn. Lab. Immunol. 10:573–578.

17. Kowalski, T. J., S. Tata, W. Berth, M. A. Mathiason, and W. A. Agger. 2010. Antibiotic treatment duration and long-term outcomes of patients with early Lyme disease from a Lyme disease-hyperendemic area. Clin. Infect. Dis. 50:512–520.

18. LaFleur, R. L., et al. 2010. One-year duration of immunity induced by vaccination with a canine Lyme disease bacterin. Clin. Vaccine Immunol. 17:870–874.

19. Ledue, T. B., M. F. Collins, J. Young, and M. E. Schriefer. 2008. Evaluation of VlsE-based chemiluminescence immunoassay for detection of *Borrelia burgdorferi* and diagnosis of Lyme disease. Clin. Vaccine Immunol. 15:1796–1804.

20. Liang, R. F., et al. 1999. Specificity and sensitive serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. J. Clin. Microbiol. 37:3990–3996.

21. Lovrich, S. D., D. A. Jobe, R. F. Schell, and S. M. Callister. 2005. Borreliacidal OspC antibodies specific for a highly conserved epitope are immunodominant in human Lyme disease and do not occur in mice or hamsters. Clin. Diagn. Lab. Immunol. 12:746–751.

22. Marangoni, A., et al. 2006. A decrease in the immunoglobulin G antibody response against the VlsE protein of *Borrelia burgdorferi* sensu lato correlates with the resolution of clinical signs in antibiotic-treated patients with early Lyme disease. Clin. Vaccine Immunol. 13:525–529.

23. Mathiesen, M. J., et al. 1998. Peptide-based OspC enzyme linked immunosorbent assay for serodiagnosis of Lyme borreliosis. J. Clin. Microbiol. 36:3474–3479.

24. Mathiesen, M. J., et al. 1998. The dominant epitope of *Borrelia garinii* outer surface protein C recognized by sera from patients with neuroborreliosis has a surface-exposed conserved structural motif. Infect. Immun. 66:4073–4079.

25. Mitchell, P. D., K. D. Reed, M. F. Vandermouse, and J. W. Melski. 1993. Isolation of *Borrelia burgdorferi* from skin biopsy specimens of patients with erythema migrans. Am. J. Clin. Pathol. 99:104–107.

26. Mogiljansky, E., C. C. Loa, M. E. Adelson, E. Mordechai, and R. C. Tilton. 2004. Comparison of western immunoblotting and the C6 Lyme antibody test for laboratory detection of Lyme disease. Clin. Diagn. Lab. Immunol. 11:924–929.

27. Nowakowski, J., et al. 2003. Long-term follow-up of patients with culture-confirmed Lyme disease. Am. J. Med. 115:591–96.

28. Padilla, M. L., et al. 1996. Characterization of the protective borreliacidal antibody response in humans and hamsters after vaccination with an outer surface protein A vaccine. J. Infect. Dis. 174:739–746.

29. Pelotoma, M., M. McGilugh, and A. C. Steere. 2003. Persistence of the antibody response to the VlsE sixth invariant region (IR6) peptide of *Borrelia burgdorferi* after successful antibiotic treatment of Lyme disease. J. Infect. Dis. 187:1178–1186.

30. Philipp, M. T., et al. 2005. A decline in C6 antibody titer occurs in successfully treated patients with culture-confirmed early localized or early disseminated Lyme borreliosis. Clin. Diagn. Lab. Immunol. 12:1069–1074.

31. Preuc-Mursic, V., et al. 1989. Survival of *Borrelia burgdorferi* in antibo}

tically-treated patients with Lyme borreliosis. Infection 17:355–359.

32. Rouselle, J. C., et al. 1998. Borreliacidal antibody production against outer surface protein C of *Borrelia burgdorferi*. J. Infect. Dis. 178:733–741.

33. Steere, A. C., J. Coburn, and L. Glickstein. 2004. The emergence of Lyme disease. J. Clin. Invest. 113:1093–1101.

34. Steere, A. C., et al. 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. N. Engl. J. Med. 339:209–215.

35. Wormser, G. P., et al. 2006. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin. Infect. Dis. 43:1089–1134.