Complement Split Products C3a and C4a Are Early Markers of Acute Lyme Disease in Tick Bite Patients in the United States

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Abstract

Background: Current laboratory markers do not readily detect acute Lyme disease. We assessed the utility of complement and its split products as markers of Lyme disease in patients shortly after a tick bite. *Methods:* Thirty-one consecutive acute Lyme disease patients, 14 with and 17 without erythema migrans (EM) skin rash, seen by a physician within 96 h of a tick bite were matched with 24 consecutive tick bite patients without Lyme disease symptoms and 46 healthy control subjects. Complement and split products measured included factor B, Bb, C4, C3c, C3a$_{des Arg}$, C4a$_{des Arg}$, C1q- and C3d-containing immune complexes, and C2. *Results:* C2, C4, C3 and factor B levels were within normal ranges in all groups. C3a and C4a levels were significantly higher in acute Lyme disease patients than in tick bite and healthy control groups (both p < 0.001). All acute Lyme disease patients, regardless of EM, had elevated levels of C3a or C4a. Few tick bite controls had elevated levels of C3a (2/20) or C4a (5/24) and only 1 of the healthy control subjects had elevated C3a (0/46) or C4a (1/32). *Conclusions:* These findings suggest that C3a and C4a may be useful markers of Lyme disease in patients seen shortly after tick bite, even in those without EM.

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Introduction

Lyme disease, the most prevalent arthropod-borne illness in the United States, is caused by *Borrelia burgdorferi* and spread by the bite of ticks of the *Ixodes ricinus* complex [1]. In North America, *B. burgdorferi* sensu stricto is the sole etiologic agent of Lyme disease, whereas *Borrelia garinii* and *Borrelia afzelii* are more commonly associated with disease in Europe [2]. Since its identification over 30 years ago, Lyme disease has continued to spread, with more than 23,000 cases reported in the United States in the year 2002 [3]. The rising number of reported cases of Lyme disease in the United States and Europe [4], along

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with evidence of rapid dissemination of Lyme disease in some patients, demands that protocols often used for diagnosis and treatment of acute tick bites be revisited.

Diagnosis without laboratory testing is possible if a typical skin lesion, erythema migrans (EM), appears within several days to weeks after the infected tick bite, accompanied by flu-like symptoms of fever, malaise, headache, joint pain, frank arthritis and muscle aches. The typical EM rash appears as a target lesion, though the rash can be pleomorphic. However, EM is seen in fewer than 70% of acute cases of Lyme disease. Diagnosis of acute Lyme disease without EM on clinical grounds alone is potentially fraught with error in patients without classic manifestations of illness temporally associated with a tick bite [5].

Recommendations for laboratory testing from the Centers for Disease Control and Prevention (www.CDC.gov) include an enzyme-linked immunosorbent assay (ELISA) with confirmation of positive results by Western blot [6, 7]. However, this approach is inadequate for diagnosis in patients with a bite occurring within several days of presentation for care. An ELISA will not be positive for at least 2 weeks, resulting in underestimation of the true number of cases by testing too early. ELISA results will also be negative in patients who do not make antibody but who do have Lyme disease (so-called seronegative Lyme disease) [8]. Other approaches to diagnostic testing, such as measurement of immune complexes [9–12], will not be positive within a few days of a tick bite, as they rely on detection of antibody complexed with antigen and complement.

Current approaches to acute care of patients with tick bites suffer drawbacks. Observation risks potential complications, from underdiagnosis, due to lack of follow-up. One-time dosing with doxycycline may provide less-than-adequate treatment of true cases [13, 14], and treatment of all patients with 3 weeks of antibiotics, including doxycycline, results in overtreatment of patients without Lyme disease.

Studies of the innate immune response to Lyme disease in patients with EM have shown activation of neutrophils and macrophages, and dendritic cells also showed activation markers [15]. Serum levels of IFN-γ, TNF-α and IL-2 were higher in patients with multiple EM lesions than in those with a single EM lesion. Monocytes displayed increased Toll-like receptors TLR1 and TLR2, while some dendritic cells showed increased expressions of TLR2 and TLR4 [15].

Borrelia activates innate immune responses [15–18], particularly complement, with circulating C1q-binding complexes found in nearly all patients with EM [12]. Complement responses are not always adequate to clear the organism, making the ability of *Borrelia* to evade complement-mediated killing a subject of intense interest [12, 19–23]. Activation of complement by any pathway produces the anaphylatoxin C3a, whereas C4a is a marker of activation of the classical and lectin complement pathways.

To understand innate immune responses to early infection in Lyme disease, we explored the complement system in patients with clinical symptoms of Lyme disease, with or without EM, seen within 4 days of a tick bite. We further investigated the acute complement responses in vitro by adding pure cultures of *B. burgdorferi* or *Borrelia hermsii* to human serum. *B. hermsii* was studied for complement activation because it is a causative agent of relapsing fever and has 30–44% structural homology with *B. burgdorferi* [2].

Our results show that C4a and C3a are markedly elevated in nearly all patients with acute Lyme disease, but rarely in control subjects with or without tick bite. Further, the elevation of complement split products in patients with Lyme disease was reproduced in vitro when *B. burgdorferi* or *B. hermsii* was added to cell-free samples of normal human serum (NHS).

Methods

Patients

Thirty-one consecutive patients with acute Lyme disease (14 EM positive and 17 EM negative) were included in this study. Patients ranged in age from 18 to 72 years. Sixteen (52%) were male and 15 (48%) were female. All patients exhibited at least 3 symptoms, including fatigue, muscle aches, joint pain, headaches or new-onset impairment of cognitive function occurring within 96 h of a tick bite. The patients were examined by an experienced physician and compared to 24 consecutive patients with a tick bite but without EM or Lyme disease (tick bite controls) and 46 healthy control subjects. Tick bite controls ranged in age from 27 to 71 years; 13 (54%) were male and 11 (46%) were female. No patient had a prior history of Lyme disease or other acute health problems identified at the visit. Because all patients with acute Lyme disease were treated soon after diagnosis, laboratory tests for antibody responses would likely have been negative and were not performed. Use of data from these patients was approved by the Copernicus Group Institutional Review Board, Cary, N.C., USA. All subjects provided written informed consent.

Complement Determinations

Plasma samples for C3a and C4a were prepared from Vacutainer™ EDTA-anticoagulated whole blood. The tubes were centrifuged within 10 min of collection and frozen at –70°C for analysis by the Complement Laboratory at the National Jewish Medical and Research Center, Denver, Colo., USA. C3a was measured

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using kits purchased from Quidel (San Diego, Calif., USA) and C4a with kits from Pharmingen BD (San Jose, Calif., USA). Reference ranges of C3a and C4a by these methods were <368 ng/ml and 101–745 ng/ml, respectively (means ± 2 standard deviations, n = 60). Serum C2, C3, C4, C1q- and C3d-containing immune complexes, and factor B were measured by nephelometry or immunoassays at Quest Diagnostics Nichols Institute, San Juan Capistrano, Calif., USA. All complement determinations were performed without the laboratory's knowledge of the subject's clinical status.

Borrelia Cultures

B. burgdorferi and *B. hermsii* were cultivated in BSKII culture medium containing CMRL, peptone, yeast extract, bovine serum albumin and 12% rabbit serum [24]. The rabbit serum was incubated for 1 h at 60°C to heat inactivate complement before adding to the final medium. Bacteria were grown at 37°C in airtight 6-ml culture tubes to a density of approximately 10^7 cells/ml and then passaged into a new 6-ml culture by inoculating 50 μl of cell culture into fresh media. Spirochetes were counted in culture using a Petroff-Hausser counting chamber (Hausser Scientific Partnership, Horsham, Pa., USA). High-density cultures were diluted 1:10 with phosphate-buffered saline containing 5 mmol/l MgCl before counting. To obtain an accurate measure, each culture was counted a minimum of 4 times and the average was used. Experimental samples of the different strains were always equal densities.

In vitro Complement Testing

For studies of in vitro activation by *Borrelia* cultures, serum (NHS) was obtained from a healthy individual who had not been exposed to *Borrelia*. C3a and Bb were measured using ELISA kits from Quidel and C4a and C5a were measured with ELISA kits from Pharmingen BD. Dilutions of pure cultures of *B. burgdorferi* and *B. hermsii* starting at 7×10^7 organisms/ml were added to NHS in duplicate, keeping the proportions of the culture volume and NHS volume constant. The negative control for the assay was culture medium plus NHS and the positive controls were boiled yeast cell walls (zymosan) and heat-aggregated γ-globulin. After 60-min incubation at 37°C, the incubation mixtures were centrifuged to pellet the bacteria and zymosan particles, and C3a, C4a, C5a and Bb were measured in the supernatants. Concentrations of C3a, Bb and C5a produced were calculated as the relative percent of NHS activated.

Statistics

t test determinations of significance were performed using differences in proportion analyses between the Lyme disease and control groups. Wilcoxon signed rank tests were used for analysis of nonparametric paired data. All reported p values are 2-sided.

Results

Complement Determination

The presence or absence of EM was documented in all 31 cases of acute Lyme disease. To study complement activation, we measured major initiating components of the classical (C2 and C4) and alternative (factor B and C3) pathways, as well as their respective split products, C4a, Bb and C3a. Circulating immune complexes (CIC) binding C1q or containing bound C3d were also tested. We did not measure C5a levels because (1) it is cleared very rapidly from the circulation and (2) our preliminary studies indicated C5a was rarely increased in our study group (data not shown).

Levels of C2, C4, C3 and factor B, as well as CIC, did not differ substantially between Lyme disease patients and control groups (data not shown). C4d levels were not increased in any group. In the acute Lyme disease patients and the 2 control groups, levels of C2, C4, C3, factor B and C4d were within the normal range in all samples tested.

C3a and C4a Levels

C4a and C3a levels were significantly higher in Lyme disease patients than in the tick bite and normal control subjects (both p < 0.001). All tested patients with acute Lyme disease, regardless of EM status, had elevated levels of either C4a or C3a. All tested patients with EM-positive acute Lyme disease had elevated levels of C3a (12 of 12) and C4a (10 of 10; fig. 1, 2). Most patients with EM-negative acute Lyme disease also had increased levels of C3a (10 of 17) and C4a (15 of 17). In contrast, C3a was elevated in only 2 of 20 tick bite and none of 46 healthy control subjects, and C4a was elevated in 5 of 24 tick bite control subjects and 1 of 32 healthy control subjects (fig. 1, 2).

To assess the effect of antibiotic treatment on C3a and C4a elevations, we compared measurements before and after 3 weeks of antibiotic treatment in a group of 31 patients with suspected acute Lyme disease. Because pre- or posttreatment values of either C3a or C4a were not available for several patients, the analyses included 24 patients (12 with and 12 without an EM rash) for C3a and 24 patients (7 with and 17 without an EM) for C4a (fig. 3). Of 20 patients with elevated pretreatment C3a levels, 11 (55%) showed normalization after treatment. Similarly, of 22 patients with elevated C4a levels before treatment, 17 (73%) exhibited normalized levels after treatment. Overall, levels of both markers decreased significantly after treatment; median values fell from 887 ng/ml before treatment to 290 ng/ml after treatment for C3a, and from 2,520 to 495 ng/ml for C4a (both p < 0.001, Wilcoxon signed rank test).

In vitro Testing

Incubation of *B. burgdorferi* and *B. hermsii* (7×10^7/ ml) with NHS resulted in activation of complement, as
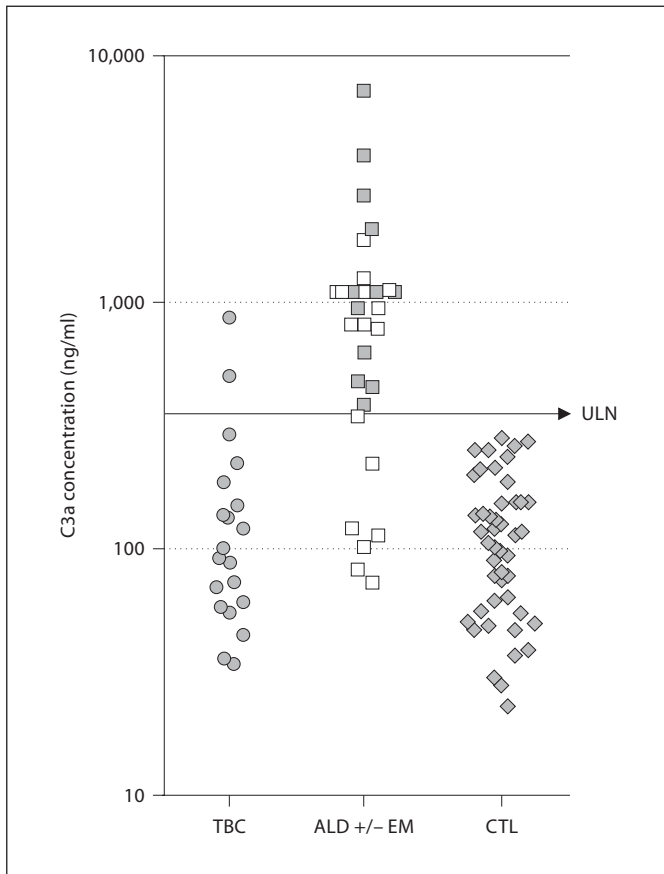
Fig. 1. C3a levels in EDTA plasma of patients with acute Lyme disease (ALD) and control subjects. Lyme disease patients with (n = 12) or without (n = 17) EM had significantly higher levels of C3a than tick bite and healthy control subjects (p < 0.001). All EM-positive (12/12) and most EM-negative (10/17) patients had elevated C3a. None of the 46 healthy controls and 2 of 20 tick bite controls had elevated levels of C3a. Data for 46 healthy controls are included. Upper limit of normal (ULN; mean + 2 SD): 368 ng/ml. Shaded squares indicate EM-positive, open squares indicate EM-negative patients. TBC = Tick bite controls; CTL = healthy controls.
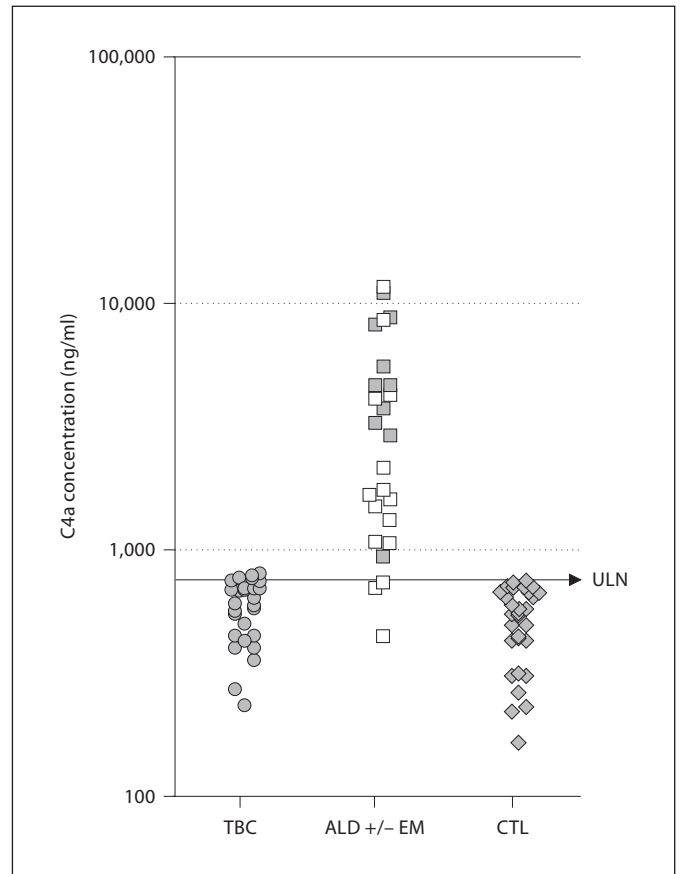

Fig. 2. C4a levels in EDTA plasma of patients with acute Lyme disease (ALD) and control subjects. Lyme disease patients with (n = 10) or without (n = 17) EM had significantly higher levels of C4a than tick bite and control subjects (p < 0.001). C4a was elevated in all 10 EM-positive and 15 of 17 EM-negative patients with acute Lyme disease. Five of the 24 tick bite controls and 1 of the 32 healthy controls had elevated C4a levels. Upper limit of normal (ULN; mean + 2 SD): 745 ng/ml. Shaded squares indicate EM-positive, open squares indicate EM-negative patients. TBC = Tick bite controls; CTL = healthy controls.

demonstrated by the appearance of C4a, C3a, Bb and C5a (fig. 4). Dilution of the spirochetes produced a dose-related reduction in the amount of complement split products. Activation of the alternative pathway of complement predominated in this in vitro experiment, as evidenced by the greater concentrations of C3a, Bb and C5a produced. The classical and/or lectin pathways were also triggered, as documented by C4a generated in the same experiment. Figure 4 is representative of 1 of 4 similar experiments in which the spirochetes were incubated with NHS.

Discussion

The results of this study demonstrated activation of complement in Lyme disease, as evidenced by elevations of C3a and C4a in plasma of patients with acute Lyme disease, but rarely in tick bite and healthy control subjects. All patients with acute Lyme disease had elevated levels of either C3a or C4a, whereas only 2 of 20 tick bite controls had elevated C3a and 5 of 24 had elevated C4a. Taken with the results of the in vitro experiments, these data indicate that *B. burgdorferi* and *B. hermsii* spiro-
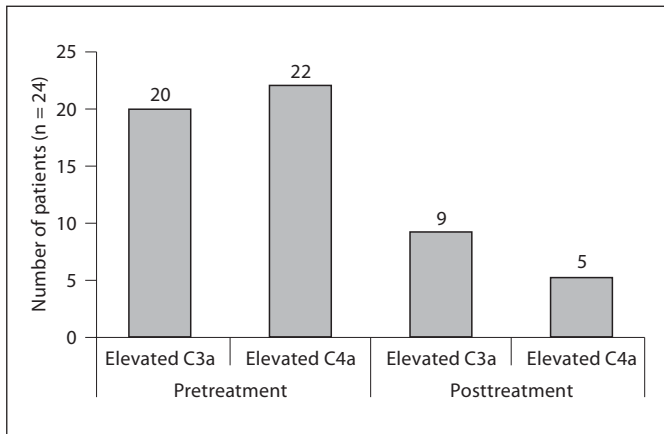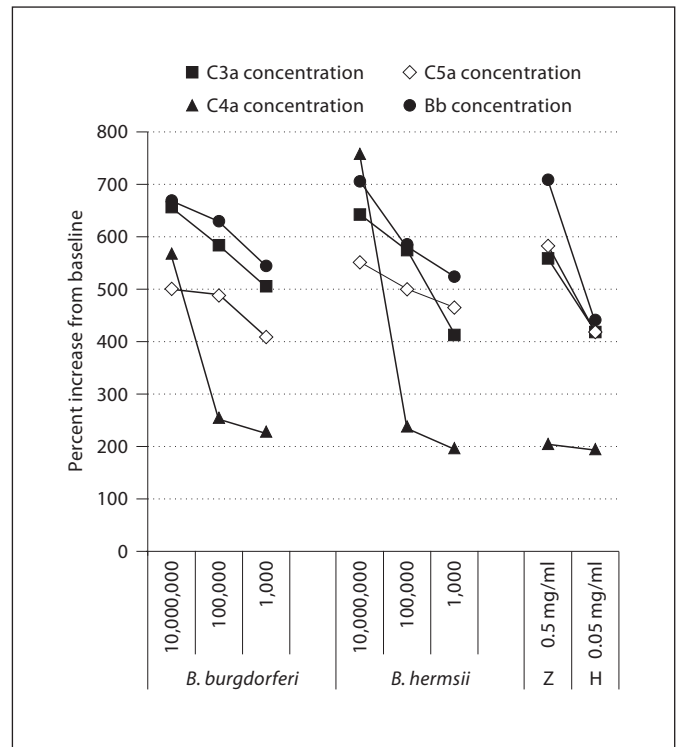
Fig. 3. Decrease in C3a and C4a levels after antibiotic treatment in patients with acute Lyme disease. Levels normalized after treatment in 11 of 20 (55%) patients with initially elevated C3a and 17 of 22 (77%) patients with initially elevated C4a.

Fig. 4. Activation of the classical or lectin (C4a), alternative (Bb) and terminal (C3a and C5a) complement pathways by *B. burgdorferi* and *B. hermsii* in NHS. Replicate samples varied by less than 5%. Z = Zymosan, a control activator of the alternative complement pathway; H = heat-aggregated γ-globulin, an activator primarily of the classical complement pathway.

chetes activate complement to the point that measurement of such activation might become a useful adjunct in the early diagnosis of Lyme disease in tick bite patients.

In vivo activation of the classical or lectin pathway, as occurs in systemic lupus erythematosus, results in decreased levels of C1, C4, C2 and sometimes C3. These decreases were not observed in our patients with acute Lyme disease. When activation proceeds through the alternative pathway, decreases in C3 and the later components generally predominate. In immune complex diseases, elevations of circulating C1q-binding and C3d-containing complexes often correlate with low complement levels. Quantitative measurements of the complement proteins (C2, C3, C4, C4d and factor B) and the CIC (CIC-C1q, CIC-C3d) were similar in Lyme disease patients and control subjects, suggesting that measurement of these proteins alone is not useful. C3 and C4 levels by themselves can be misleading. Because the ranges of C3 and C4 concentrations are relatively broad, cleavage of 10–20% would still leave enough C3 to be well within the normal range. Thus, only by looking at the cleavage products of these proteins can one estimate the extent of an ongoing activation process in its early stages.

As expected, both species of *Borrelia* activated complement in vitro when added to NHS, with the alternative pathway predominating. Since cell wall proteins and lipoproteins may differ between the cultured and wild-type strains of *Borrelia*, the extent of activation may differ from the infectious *B. burgdorferi*. In addition, the presence of rabbit serum factor H in the culture medium may have affected the binding of human factor H to the spirochete surface, thus altering the modulation of control of the alternative pathway activation. It is risky to assume that activation of complement by an organism or substance will be the same in vivo as it is in vitro. Additional factors present in vivo may be absent in serum or even whole blood used for in vitro testing. Such factors include cell-associated complement receptors and control proteins as well as additional complement-cleaving enzymes released from activated neutrophils, monocytes and the bacteria themselves.

Laboratory testing for acute Lyme disease has been hampered by difficulty in culturing *B. burgdorferi* organisms from the blood. *B. burgdorferi* has been cultured from skin biopsies of about 50% of the EM lesions of acute Lyme disease patients, but this procedure is time-consuming, subject to sampling error and expensive. IgM and IgG antibodies are not measurable until 2–4 weeks

after infection and are present in at most 80% of infected patients, a rate that can be further reduced by early antibiotic therapy [24, 25]. In the present study, early treatment of identified Lyme disease cases precluded antibody testing.

In this study, C4a and/or C3a levels were elevated in patients with acute Lyme disease tested within 4 days of tick bite. Both C3a and C4a were elevated in all patients with EM-positive acute Lyme disease. In the absence of an EM rash, definitive diagnosis of acute Lyme disease is difficult. However, the characteristic clinical findings of myalgia, headache, arthralgia and malaise, associated with a recent tick bite, make acute Lyme disease a likely diagnosis in our EM-negative patients; other tick-borne illnesses found in this geographic region (mid-Atlantic) are relatively low in prevalence. Moreover, C3a or C4a levels were rarely elevated in our tick bite control subjects lacking these clinical symptoms. Since the clinical symptoms of acute Lyme disease are similar to those of other infectious diseases, it is possible that acute upper respiratory infections or other tick-borne organisms could activate complement. Thus, although C3a and C4a elevations are found in Lyme disease, they may not be specific and results should be correlated with clinical findings, including history of tick bite.

Not surprisingly, levels of C3a and C4a appeared to normalize after antibiotic treatment in our preliminary study. Although not all patients exhibited normalized levels after treatment, median values of both markers decreased significantly. Again, although similar decreases would be expected with other infectious etiologies, the clinical history of the patients was most consistent with acute Lyme disease.

The use of complement split product measurements in the management of potentially infected tick bite patients must also take into account elevation of C4a and C3a caused by other clinically recognized illnesses, such as systemic lupus erythematosus, rheumatoid arthritis, pancreatitis and other immune complex or inflammatory diseases [26, 27]. Acute Lyme disease usually has a temporal association with a tick bite and distinct clinical findings, including skin rash and flu-like symptoms, that differ from those of immune complex diseases.

Other spirochetes such as *Treponema pallidum* activate the complement system. Fitzgerald [28] has shown that incubation of *Treponema* organisms with NHS results in generation of C3a and C4a. The relative concentration of *T. pallidum* added to NHS (10^8/ml) was similar to that of *B. burgdorferi* tested in our experiments. Whether acute syphilis with chancres could be associated with elevated complement split products has yet to be investigated and is worthy of future study. However, the clinical symptoms are sufficiently different from Lyme disease that the diagnoses should not be confused.

The results of this study are striking enough to suggest that C3a and C4a could provide useful laboratory markers for determining which patients with recent tick bites require antibiotic treatment. Since the number of acute Lyme disease patients in this study was small, these results should be confirmed in a larger study with clinical follow-up. The duration of elevation of C3a and C4a in the course of untreated Lyme disease is not known. We are currently investigating the effect of 3 weeks of oral antibiotic treatment on C3a and C4a in Lyme disease patients with persistent symptoms following antibiotic therapy compared with those who are rendered asymptomatic. Although our findings reflect the effects of *B. burgdorferi* infection on the complement system in patients from the eastern United States, additional studies are needed to assess the effects of *B. garinii* and *B. afzelii*, which are more common in Europe.

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