Complement Split Products C3a and C4a in Chronic Lyme Disease

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Abstract

Complement split products C3a and C4a are reportedly elevated in patients with acute Lyme disease. We have now examined these immunologic markers in patients with chronic Lyme disease compared to appropriate disease controls. The study population consisted of 29 healthy controls, 445 patients with chronic Lyme disease, 11 patients with systemic lupus erythematosus (SLE) and six patients with AIDS. The Lyme disease patients were divided according to predominant musculoskeletal symptoms (324 patients) or predominant neurologic symptoms (121 patients). C3a and C4a levels were measured by radioimmunoassay. All patients with chronic Lyme disease and AIDS had normal C3a levels compared to controls, whereas patients with SLE had significantly increased levels of this marker. Patients with predominant musculoskeletal symptoms of Lyme disease and AIDS patients had significantly increased levels of C4a compared to either controls, patients with predominant neurologic symptoms of Lyme disease or SLE patients. Response to antibiotic therapy in chronic Lyme disease was associated with a significant decrease in the C4a level, whereas lack of response was associated with a significant increase in this marker. In contrast, AIDS patients had persistently increased C4a levels despite antiretroviral therapy. Lyme patients with positive single-photon emission computed tomographic (SPECT) scans had significantly lower C4a levels compared to Lyme patients with normal SPECT scan results. Patients with predominant musculoskeletal symptoms of Lyme disease have normal C3a and increased C4a levels. This pattern differs from the increase in both markers seen in acute Lyme disease, and C4a changes correlate with the response to therapy in chronic Lyme disease. C4a appears to be a valuable immunologic marker in patients with persistent symptoms of Lyme disease.

Introduction

Lyme disease caused by the spirochete *Borrelia burgdorferi* is the most common tick-borne illness in the world today [1, 2]. Although prompt diagnosis and treatment results in a favourable outcome in most patients with *B. burgdorferi* infection, tick exposure and acute infection with the Lyme spirochete often go unrecognized, and patients with untreated infection may go on to develop a chronic debilitating multisystem illness that is difficult to diagnose and treat [1, 2].

Testing for Lyme disease remains unreliable, and recognition of this complex illness suffers from a lack of immunologic markers of disease activity [3–5]. Shoemaker *et al.* [6] recently reported increased levels of complement split products C3a and C4a in patients with acute Lyme disease. The authors suggested that C3a and C4a may be useful markers for acute infection with *B. burgdorferi* following a tickbite. Because immunologic markers would also be valuable for diagnostic and therapeutic purposes in chronic Lyme disease [4, 5], we performed a prospective study of C3a and C4a levels in patients with persistent symptoms of this tick-borne illness.

Materials and methods

A total of 491 patients were enrolled in the study (Table 1). Twenty-nine normal subjects served as controls, while 445 patients with clinical and serologic evidence of chronic Lyme disease (symptoms lasting more than 3 months and reactive Lyme Western blot) were

Table 1 Study patients NA, not applicable.

Patient group	Number	Mean age ± SD, years (range)	Sex (M/F)	Duration of disease (mean ± SD, months)
1. Controls	29	45.3 ± 17 (8–75)	13/16	NA
2. Chronic Lyme disease	445	45.1 ± 15 (7–86)	130/315	36 ± 17
A. Predominant musculoskeletal symptoms	324	45.4 ± 15 (7–86)	100/224	42 ± 21
B. Predominant neurologic symptoms	121	44.5 ± 14 (9–78)	30/91	27 ± 13
3. Systemic lupus erythematosus	11	43.3 ± 19 (18–68)	0/11	32 ± 14
4. AIDS	6	57.2 ± 8 (51–72)	6/0	72 ± 22

divided according to predominant musculoskeletal symptoms (324 patients) or predominent neurologic symptoms (121 patients), as previously described [4].

Patient ages ranged from 7 to 86 years, with a mean of 45.1 years. The distinction between the two Lyme patient groups was based on clinical symptoms, single-photon emission computed tomographic (SPECT) brain scanning and treatment protocols that differed according to symptomatology: Patients with predominant musculoskeletal symptoms were treated with oral antibiotics (doxycycline monotherapy or combination therapy with a macrolide plus amoxicillin, cefdinir or metronidazole), while patients with predominant neurologic symptoms received parenteral antibiotic therapy (intramuscular benzathine penicillin or intravenous ceftriaxone). Symptoms were graded on a 0–3 scale, as previously described [7]. Changes in symptom scores were assessed at 3–6 months after initiation of oral or parenteral antibiotic therapy. The changes were then correlated in a blinded fashion with changes in complement split product measurements, as described below.

Eleven patients with systemic lupus erythematosus (SLE) and six patients with AIDS served as disease controls. The SLE patients were receiving varying doses of steroid medication, while the AIDS patients were treated with standard doses of highly active antiretroviral therapy (HAART). Informed consent was obtained from all subjects or their legal guardians prior to testing.

All C3a and C4a testing was performed by radioimmunoassay (Institute of Isotopes, Budapest, Hungary) at the National Jewish Medical and Research Center in Denver, CO, USA, as previously described [8]. In order to avoid complement activation *in vitro*, samples were collected in tubes containing EDTA and centrifuged within half an hour after collection. The cell-free EDTA-plasma was transferred to a clean tube, labelled and frozen at −70 °C. Samples were shipped to the laboratory on dry ice by overnight delivery. All samples were received frozen and in good condition and were stored at −70 °C until they were thawed for testing. Levels of C3a and C4a were calculated according to the standard curves supplied with the assay kits. Normal ranges were calculated based on a sampling of 60 healthy individuals.

Testing was performed at baseline for all patients and 3–6 months after initiation of antibiotic therapy in the Lyme disease patients. Follow-up testing at 3–6 months was also performed in the AIDS patients who were receiving standard antiretroviral therapy.

Statistical analysis was performed using GraphPad InStat Version 3.0b software (GraphPad Software, La Jolla, CA, USA). Kruskall–Wallis analysis of variance was used for non-parametric variables in grouped sample testing, and the two-tailed Wilcoxon signed-rank test was used for matched pair testing.

Results

The characteristics of the study subjects are shown in Table 1. The mean patient age was similar in the control group, the two Lyme patient groups and the SLE group. The AIDS patient group was significantly older. The female:male ratio was 2.4:1 in the Lyme groups. In contrast, all patients in the SLE group were women, while all patients in the AIDS group were men. The estimated duration of disease was similar in the Lyme groups compared to the SLE group. However, Lyme patients with predominant musculoskeletal symptoms had significantly longer duration of disease compared to Lyme patients with predominant neurologic symptoms ($42 ± 21$ versus $27 ± 13$ months, $P = 0.01$). The duration of disease in the AIDS patient group was significantly longer than in the Lyme and SLE groups ($P = 0.0015$ and $P = 0.0036$ respectively).

The results of baseline C3a and C4a testing are shown in Fig. 1. C3a was found to be normal compared to controls in patients with predominant musculoskeletal and neurologic symptoms of chronic Lyme disease. While AIDS patients also had normal levels of C3a, patients with active SLE were found to have significantly increased levels of this complement split product compared to controls ($P < 0.001$; Fig. 1A).

C4a was found to be significantly increased in Lyme disease patients with predominant musculoskeletal symptoms ($P < 0.001$) and in AIDS patients ($P < 0.05$) compared to either controls, Lyme disease patients with

(A)

C3a in Lyme and other diseases

(B)

C4a in Lyme and other diseases
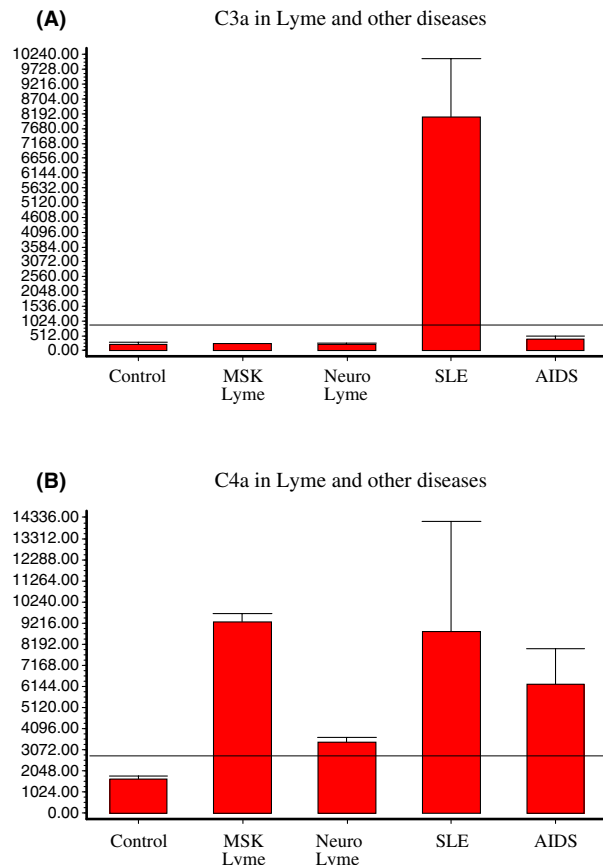
Figure 1 C3a and C4a levels in chronic Lyme disease (mean and SE). (A) Baseline levels of C3a (ng/ml) and (B) baseline levels of C4a (ng/ml). Horizontal line represents the upper limit of normal for C3a or C4a (mean + 2 SD). Lyme patients were divided according to predominant musculoskeletal symptoms (MSK) or predominant neurologic symptoms (Neuro). Only the C3a level in systemic lupus erythematosus (SLE) patients was significantly increased compared to controls ($P < 0.001$). Levels of C4a were significantly increased in patients with MSK Lyme disease ($P < 0.001$) and AIDS ($P < 0.05$) compared to controls. C4a levels were also significantly higher in MSK Lyme patients compared with Neuro Lyme patients ($P < 0.001$) and SLE patients ($P < 0.01$).

predominant neurologic symptoms or SLE patients (Fig. 1B). Although patients with predominant musculo-skeletal symptoms of Lyme disease had higher levels of this complement split product compared to AIDS patients, the difference was not statistically significant. C4a levels in SLE patients showed the widest variation (range: 1455–61,200 ng/ml) reflecting the variation in disease activity and treatment response in these patients.

At follow-up, Lyme disease patients who responded clinically to antibiotic therapy according to the symptom scale had a significant decrease in C4a level ($P < 0.0001$). In contrast, patients who failed to respond to treatment had a significant increase in this immunologic marker ($P = 0.0328$) (Fig. 2). In contrast, AIDS patients on anti-retroviral therapy had persistently elevated levels of C4a (Fig. 2). Longer follow-up in the Lyme disease patients

C4a changes in Lyme disease and AIDS
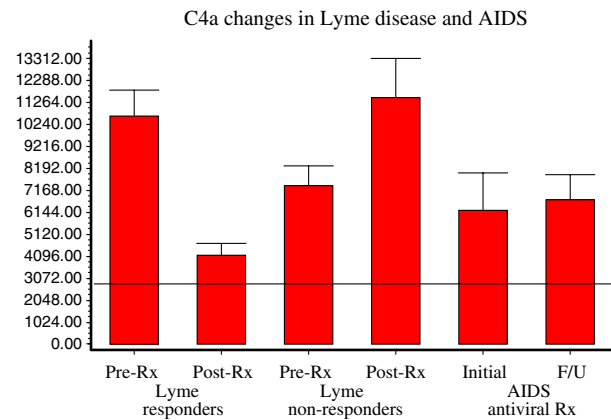
Figure 2 Change in C4a level (mean and SE) associated with treatment in chronic Lyme disease and AIDS. Lyme patients who responded to treatment had a significant decrease in C4a ($P < 0.0001$), while Lyme patients who failed treatment had a significant increase in C4a ($P = 0.0328$). In contrast, AIDS patients had no change in their C4a levels while on stable antiretroviral treatment ($P = NS$).

confirmed the decrease in C4a levels in response to anti-biotic therapy and indicated an inverse correlation with CD57 natural killer (NK) cell levels (data not shown).

Evaluation of a subset of patients with predominant neurologic Lyme symptoms and positive versus negative SPECT scans is shown in Fig. 3. Fifteen patients had positive scans, while six patients had normal scans despite significant neurocognitive deficits. All patients also complained of musculoskeletal symptoms. Patients with negative SPECT scans had significantly higher C4a levels than controls ($P < 0.001$) or patients with positive scans ($P < 0.05$). From a clinical standpoint using the symptom scale, patients with negative scans showed a better response to antibiotic therapy compared to patients with positive scans (data not shown).

C4a levels in neurologic Lyme disease
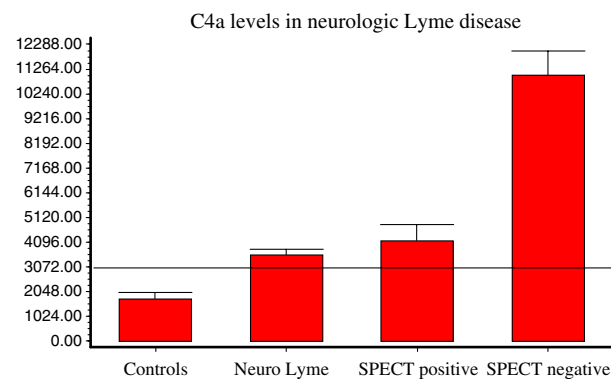
Figure 3 C4a levels in neurologic Lyme disease (mean and SE). Patients were divided according to positive or negative single-photon emission computed tomographic (SPECT) scans. Patients with negative SPECT scans had significantly higher C4a levels compared with controls ($P < 0.001$) or patients with positive SPECT scans ($P < 0.05$).

Discussion

C3a, C4a and C5a have been designated as anaphylatoxins, and phylogenetic studies suggest that these immune activation products have been conserved in the animal kingdom for over 300 million years [8–10]. Recent studies, however, have cast doubt on the role of C4a as an anaphylatoxin, and the function of this molecule is presently unclear [11]. Whereas C4a is generated by the classical or the lectin complement activation pathway, C3a is generated by the classical, alternative and lectin pathways [8, 10]. C5a, which is a product of the terminal pathway of complement activation, has a very short half-life that makes it difficult to measure in routine blood samples. In contrast, C4a levels are selectively increased in adult insulin-dependent diabetes mellitus [12], while C3a and C4a are reportedly increased in normal pregnancy, active SLE requiring immunosuppressive therapy and other forms of vasculitis [13–16]. C4a was also found to be increased in a model of chronic fatigue syndrome [17]. Of note, C3a appears to play a significant role in central nervous system inflammation associated with ischaemic stroke and subarachnoid haemorrhage [18, 19], while C4a appears to have only a minor role in brain inflammation. The reason for this discrepancy is unclear, but it may reflect decreased constitutive production of the parent C4 compound or a diminished response to cytokines such as interferon-gamma in brain tissue [20–22].

In a recent study using an enzyme-linked immunosorbent assay technique, Shoemaker *et al.* [6] demonstrated that C3a and C4a levels were increased in 31 patients with acute Lyme disease who developed musculoskeletal and/or neurologic symptoms within 96 h of a tickbite. These patients were diagnosed and treated promptly following the onset of symptoms, and complement activation was not evaluated beyond the acute phase of the disease. In contrast to these fortunate patients, many individuals who contract Lyme disease do not know that they are infected because they are unaware of a tickbite and/or they do not have the characteristic erythema migrans Lyme rash. Subsequently these individuals develop a multisystem illness that can be difficult to diagnose and treat. Commercial laboratory testing at this stage of Lyme disease has a sensitivity of only 56% or less [3, 23, 24], so that approximately half the patients with chronic Lyme disease will not be diagnosed by standard testing. In this setting, other laboratory markers would be extremely useful to support the diagnosis of chronic Lyme disease.

To date, the only clinically useful immunologic marker of chronic Lyme disease is the CD57 NK cell level [4, 5]. Although this test has been used extensively by clinicians who treat chronic Lyme patients, the test has not been extensively validated. Therefore additional immunologic testing would be useful to validate the CD57 NK assay and to monitor the treatment of patients with persistent symptoms of Lyme disease. As C3a and C4a were shown to be increased in acute Lyme disease, these immunologic markers were logical candidates for evaluation in the chronic phase of the illness.

Using a radioimmunoassay technique, we found that C3a levels were normal in patients with chronic Lyme disease. In contrast, C4a levels were significantly increased in patients with predominant musculoskeletal symptoms but normal in patients with predominant neurologic symptoms of the disease. This pattern differs from the increase in both C3a and C4a levels that was observed in acute Lyme disease by Shoemaker *et al.* [6]. Although the different outcomes in the two studies might be due to the different techniques used to measure C3a and C4a, our results were validated by the levels of complement activation in our SLE patients that were similar to levels reported previously in this autoimmune disease [14, 15]. In chronic Lyme disease, C4a levels decrease in patients who respond to antibiotic therapy but increase in patients who fail to respond to treatment. Thus C4a appears to be a useful marker for both clinical diagnosis and response to treatment in patients with chronic Lyme disease.

In contrast to C4a, an increase in C3a was only seen in patients with active SLE. As stated previously, increased C3a correlates with active autoimmunity [14, 16], and this immunologic marker may help to distinguish chronic autoimmune pathology from persistent tick-borne infection [25]. As increased C4a was also found in patients with AIDS and to a variable degree in patients with SLE, this marker by itself would not be sufficient to diagnose chronic Lyme disease. In the absence of a positive AIDS test or autoimmune serology and the presence of significant musculoskeletal symptoms, however, the pattern of normal C3a and increased C4a appears to correlate with the presence of chronic tick-borne disease.

Sorensen *et al.* [17] demonstrated that C4a is increased in patients with chronic fatigue syndrome following exercise challenge. Chronic fatigue and fibromyalgia are prominent symptoms of chronic Lyme disease [1, 2], and there appears to be significant overlap in these clinical syndromes. It is noteworthy that patients with predominant neurologic symptoms of Lyme disease had normal levels of C4a despite the presence of chronic fatigue in most of these patients. A comparison between chronic fatigue patients who are seronegative for Lyme disease and seropositive Lyme patients would be of interest to help distinguish these disease entities.

It is unclear why C4a but not C3a is increased in patients with predominant musculoskeletal symptoms of chronic Lyme disease. The pattern suggests that chronic infection with *B. burgdorferi* is associated with activation of the classical complement pathway rather than the alternative and lectin pathways. Support for this hypothe-

sis comes from *in vitro* studies showing that the Lyme spirochete activates complement via both the classical and alternative pathways, but the spirochetes are capable of inactivating the alternative pathway, thereby allowing the infection to persist [26, 27]. Alternatively, increased C4a with normal C3a may reflect the inhibition of immune precipitation rather than solubilization of immune complexes in chronic Lyme disease [28]. Thus elevated C4a may be a marker of a failed immune response against the Lyme spirochete. Conversely return of this complement component to normal suggests clearance of the organism by antibiotic therapy.

To our knowledge, our study represents the first examination of circulating C3a and C4a levels in AIDS patients. Mondino *et al.* [29] demonstrated increased C3a and C4a in vitreous humour from AIDS patients with retinitis in the pre-HAART era. The persistent elevation in C4a despite the use of HAART in our patients appears to reflect the ongoing inflammation in this condition, as measured by genetic markers of complement activation [30]. Our study only included a small sample of long-term AIDS patients taking antiretroviral therapy, and additional studies at various stages of HIV infection may show differences in complement activation levels. Further evaluation of complement split products in AIDS patients may be clinically enlightening.

As stated previously, C4a does not appear to play a significant role in inflammation of the central nervous system [18, 19]. This may explain why patients with predominant neurologic symptoms of chronic Lyme disease have relatively normal levels of this complement split product. The difference in C4a levels associated with positive or negative SPECT scan results is intriguing. One explanation is that predominant neurologic symptoms may reflect generalized inflammation rather than direct brain involvement in some patients with concomitant musculoskeletal symptoms. Alternatively, increased C4a may be associated with inflammation in peripheral nerves rather than the central nervous system in chronic Lyme disease. We have observed elevated levels of C4a in patients with Lyme-associated motor neuron disease that resembles amyotrophic lateral sclerosis (data not shown). In this condition, however, the increase in C4a may reflect the involvement of both upper and lower motor neurons. The interaction between nervous system inflammation and complement activation requires further study.

In summary, chronic Lyme disease with predominant musculoskeletal symptoms is associated with increased levels of C4a but not C3a, and elevated C4a levels decrease with successful treatment. In contrast, increased levels of C3a are associated with active autoimmunity. Larger clinical trials are needed to confirm these observations in chronic Lyme disease and other inflammatory conditions.

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