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A Decline in C_6 Antibody Titer Occurs in Successfully Treated Patients with Culture-Confirmed Early Localized or Early Disseminated Lyme Borreliosis

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C_6, a *Borrelia burgdorferi*-derived peptide, is used as the antigen in the C_6-Lyme disease diagnostic test. We assessed retrospectively whether a fourfold decrease or a decrease to a negative value in anti-C_6 antibody titer is positively correlated with a positive response to treatment in a sample of culture-confirmed patients with either early localized (single erythema migrans [EM]; $n = 93$) or early disseminated (multiple EM; $n = 27$) disease. All of these patients had been treated with antibiotics and were free of disease within 6 to 12 months of follow-up. Results show that a serum specimen taken at this time was either C_6 negative or had a ≥4-fold decrease in C_6 antibody titer with respect to a specimen taken at baseline (or during the early convalescent period if the baseline specimen was C_6 negative) for all of the multiple-EM patients ($P < 0.0001$) and in 89% of the single-EM patients ($P < 0.0001$). These results indicate that a decline in anti-C_6 antibody titer coincides with effective antimicrobial therapy in patients with early localized or early disseminated Lyme borreliosis.

Lyme borreliosis, a tick-borne disease that is caused by the spirochete *Borrelia burgdorferi*, is endemic in North America, Europe, and Asia. It is the most common vector-borne illness in the United States (3). Clinically, the progression of Lyme borreliosis is divided into early localized, early disseminated, and late stages. During the early localized phase, the disease usually manifests itself by a characteristic skin lesion, erythema migrans (EM). After several days or weeks, the spirochete may spread, likely hematogenously, and patients may develop early disseminated disease with dermatologic, cardiac, neurologic, or rheumatologic involvement. Dermatologic signs during the early disseminated phase appear as multiple EM. Late disease presents chiefly as arthritis or neurologic symptoms (18).

Lyme disease is treated successfully with antibiotics in the majority of cases, and patients with objective evidence of treatment failure are very rare (20). Most of the patients with EM who receive antibiotic therapy have excellent outcomes (14). The response to treatment of patients with late manifestations is typically slower (19); it may take weeks or months and sometimes remains incomplete.

About 10% of the patients treated for EM have persistent or intermittently subjective musculoskeletal, cognitive, or fatigue complaints of mild to moderate intensity at 12 months of follow-up; the appearance of these symptoms correlates retrospectively with disseminated disease and a greater severity of illness at presentation (14). The underlying mechanism for these subjective symptoms is currently unknown. Arguing against the hypothesis of residual *B. burgdorferi* infection are the facts that these patients do not develop objective manifestations of late Lyme disease (e.g., Lyme arthritis), lack evidence of persistence of infection by several different microbiological testing methods, and do not objectively benefit from further antibiotic treatment (9).

Despite the absence of evidence of persistent infection, it would be desirable to have an objective test to assess therapy outcome in individual patients who complain of nonspecific symptoms after antibiotic treatment. No such test is currently available.

The detection of antibodies to C_6, a peptide that reproduces the sequence of the sixth invariable region within the central domain of the VlsE protein of *B. burgdorferi*, is used currently for the serologic diagnosis of Lyme disease in humans (1, 8b, 8c, 9a, 11, 13, 13b, 13c, 14b, 15a) and in canines (6a, 8a, 10a, 10b, 12, 13a, 14a, 19a). We have reported that levels of antibody to C_6 decline after successful antibiotic treatment of either Lyme disease patients or animals experimentally infected with *B. burgdorferi* (16). Furthermore, in a recent study, we quantified retrospectively the change in the anti-C_6 antibody reciprocal geometric mean titer (C_6-rGMT) in a group of 45 patients with Lyme disease. Eleven of these patients had EM, and 34 had disseminated disease (arthritis or neurologic manifestations). Overall, 80% of these patients experienced at least a fourfold decrease in C_6-rGMT. Patients with EM were more likely to experience a fourfold C_6-rGMT decrease (100%) than patients with manifestations of disseminated disease (73.5%). While the difference did not reach statistical significance ($P = 0.0867$, two-tailed Fisher's exact test), it seemed to indicate that antibiotic treatment was less likely to produce a decline in C_6 titers in patients that have been in-

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TABLE 1. Sensitivity of the C_6 test and the standard Lyme ELISA (historically tested) in culture-confirmed single- and multiple-EM patients

Time point	No. (%) of patients diagnosed positive by indicated test					
	Patients with single EM (n = 93)		Patients with multiple EM (n = 27)		Total patients (n = 120)	
	C_6 test (n = 93)	Standard ELISA[a] (n = 91)	C_6 test (n = 27)	Standard ELISA (n = 26)	C_6 test (n = 120)	Standard ELISA (n = 117)
Baseline	38 (41)	24 (26)	23 (85)	21 (81)	61 (51)	45 (38)
Convalescence	41 (44)	25 (27)	3 (11)	3 (11)	44 (37)	28 (24)
Total	79 (85)	49 (54)	26 (96)	24 (92)	105 (88)	73 (62)

[a] The standard ELISA is the commonly used diagnostic ELISA for Lyme disease that makes use of whole-cell extract as antigen.

fected for longer periods prior to treatment (17). This contention was supported by another study of posttreatment decline in the anti-C_6 antibody response in Lyme disease patients with both early and late disease (15). In the patients with late disease, 18 of a total of 21 (86%) had a less-than-fourfold decrease in anti-C_6 antibody titers at 4 to 6 months posttreatment.

To shore up the notion that a fall in C_6-rGMT correlates with a positive response to treatment in patients with early localized or early disseminated disease, we retrospectively assessed a cohort of patients whose infection status, disease phase at presentation, serum collection regimen, and clinical response to treatment were all rigorously defined. Patients in this study presented either with a single EM (early localized) or with multiple EM (early disseminated), were all *B. burgdorferi* culture positive, and were considered cured of the disease at 6-month follow-up or later. Our hypothesis was that for those patients with early disease who responded to therapy, the C_6-rGMT either becomes negative or decreases fourfold after at least 6 months of follow-up. Here we describe the results of this assessment.

MATERIALS AND METHODS

Patient population. The study population consisted of 120 patients who presented to the Lyme Disease Practice of the Westchester Medical Center between June 1991 and July 2000 with either a single EM (early localized disease; n = 93) or multiple EM (early disseminated disease; n = 27). A previous study of ours (17) had indicated that these sample sizes would yield 80% power, with an alpha value of 0.05, if the success rate was 75% and 90% power if the success rate was 80%.

The median age was 45 years (range, 16 to 75 years). There were 45 female and 75 male patients. Skin biopsy or blood specimens from all patients were shown to contain cultivable *B. burgdorferi* spirochetes, and each patient fulfilled the case definition of Lyme disease according to the Centers for Disease Control and Prevention clinical definition (4). Serum specimens obtained at the time of presentation and at 6 or 12 months thereafter ("posttreatment specimens"), depending on availability, were analyzed for the presence of anti-C_6 antibody. Two multiple-EM patients had follow-up specimens collected at about 15 and 21 months postpresentation. For patients in whom C_6 antibody was not detectable in the baseline serum specimen, an additional serum specimen that was collected during the early convalescent period was analyzed. Samples were obtained in accordance with protocols approved by the Institutional Review Board of the New York Medical College. All patients received antibiotic therapy for Lyme disease and were free of the signs and symptoms shown at presentation by the time the posttreatment serum specimen was obtained. All serum specimens were coded such that C_6 antibody titers were determined in a blinded fashion with respect to serum collection time or patient information.

Determination of anti-C_6 antibody index and titer. The anti-C_6 antibody index was determined using the C_6 enzyme-linked immunosorbent assay (ELISA) from Immunetics, Inc. (Cambridge, MA), as per the manufacturer's instructions. The test simultaneously detects both immunoglobulin M (IgM) and IgG antibodies. The Food and Drug Administration approved this test for human use. C_6 ELISAs were performed in duplicate for all of the specimens. The result of the C_6 ELISA is expressed as an index, which is calculated by dividing the optical density value of a given sample (diluted 1:20) by that of a positive control included in the plate. The sample is considered positive for C_6 antibody if the index value is $\geq$1.10, negative if it is $\leq$0.90, and equivocal if it is between 0.91 and 1.09. All of the serum specimens derived from any given patient were always assessed simultaneously on the same ELISA plate. If the result for any patient's specimen was positive or equivocal, all of this patient's specimens were titrated. If all of the specimens of a given patient were C_6 index negative, they were not titrated, but an early convalescent phase-specimen was sought, and its index was determined. If the convalescent phase-specimen was C_6 positive, it was titrated. All of the titrations were performed in duplicate, and results are reported as C_6-rGMT.

As mentioned above, anti-C_6 antibody indices are determined, as per the manufacturer's instructions, at a serum dilution of 1:20. Therefore, in order to use the kit to determine antibody titers, the initial serum specimen dilution of 1:20 was subsequently serially diluted twofold with the buffer provided but supplemented with normal human serum at a dilution of 1:20. Thus, the total human serum concentration was maintained at 1:20 at all dilutions of the patient specimen. Normal human serum (Sigma Chemical Co., St. Louis, MO) was tested with the C_6 kit to ensure that it did not contain anti-C_6 antibody. Apart from this modification, the manufacturer's instructions to obtain anti-C_6 antibody indices at each serum dilution were followed verbatim. The serum titer was defined as the last serum dilution at which the C_6 test yielded a positive index. The lowest titer that could be determined was 1:20.

Standard Lyme disease ELISA. Serum specimens also were tested by use of a whole-cell lysate polyvalent (IgM/IgG) ELISA (Wampole Laboratories, Princeton, NJ), or a similar assay, performed in accordance with the manufacturer's instructions.

Data analysis. To assess whether a fourfold decrease and/or a decrease to a negative value in anti-C_6 antibody titer is positively correlated with a positive response to treatment, we used a two-tailed test and standard binomial theory to compare the observed proportion of patients (p_1) who had a fourfold decrease or a decrease to a negative titer to the expected proportion under the null hypothesis of no association (p_0 = 0.5). Individual rates of decline were calculated by simple algebra for those patients with only two observations, or by simple linear regression otherwise, and group comparisons were made by Student's t test. Statistical significance was defined as a P value of less than 0.05.

RESULTS

Sensitivity of the C_6 test versus standard Lyme disease ELISA. Of the 93 patients with early localized infections, 38 (41%) were C_6 positive at baseline (presentation). Of the 55 patients that were C_6 negative at baseline, 41 were positive during early convalescence (for 1 of these 55 patients there was no convalescent-phase specimen available). Hence, the overall sensitivity of the test was 85% (38/93 patients [41%]) (Table 1). All but one of the serum specimens from the convalescent period were collected between weeks 1 and 8 postpresentation, with 94% of these specimens collected between weeks 1 and 4.

The sensitivity was higher for the multiple-EM patient population. A total of 23 in this group of 27 patients (85%) had positive C_6 test results at baseline, with an additional 3 converting to positive test results between weeks 2 and 6 of the

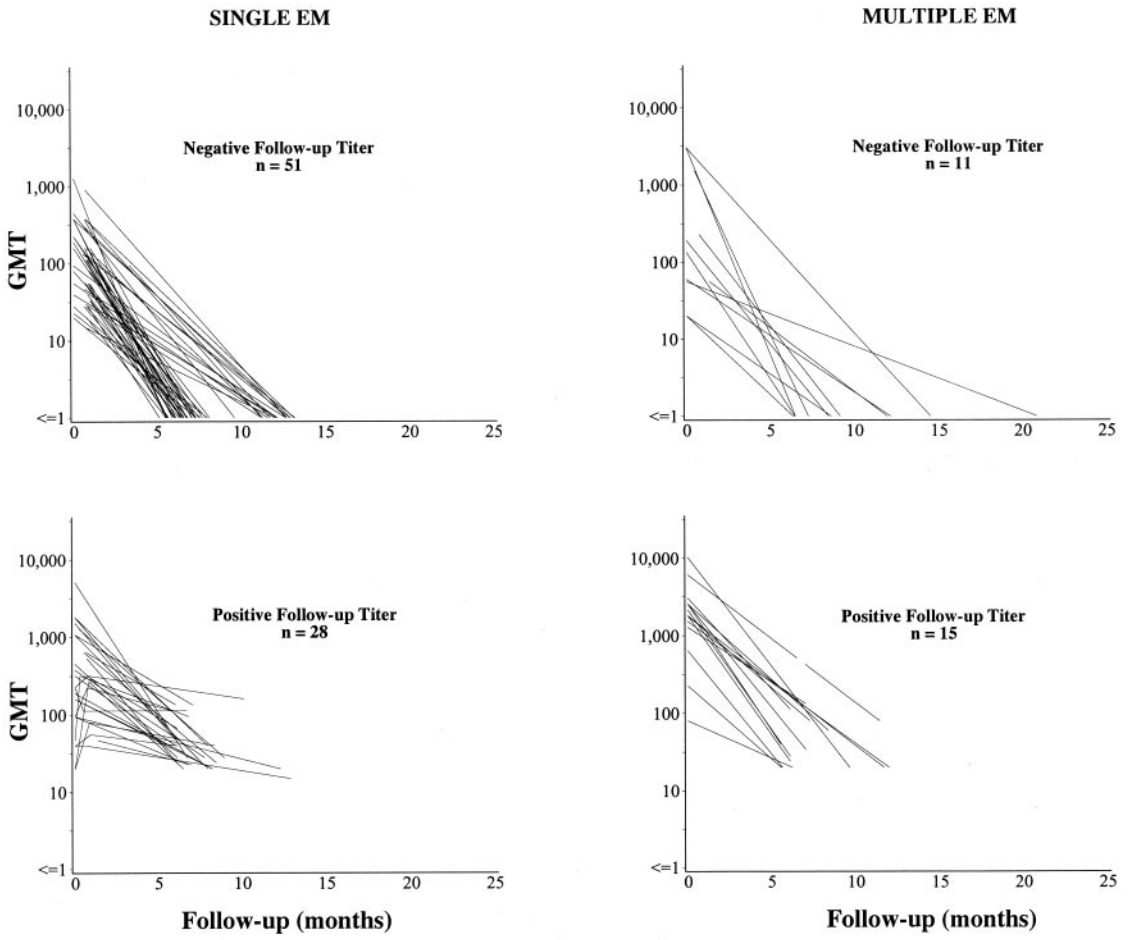

FIG. 1. C_6 antibody titer as a function of time postpresentation. For each of the two study populations (single EM and multiple EM), titers are shown separately for patients whose follow-up titers were negative (i.e., <20; top panels) and positive (≥20; bottom panels). Titers are depicted at baseline, early convalescence (for some patients), and follow-up. The follow-up time point was at approximately 6 to 12 months, except for two of the multiple-EM patients, whose follow-up serum specimens were obtained at a later date. Patients for whom all titers were negative were not included in the figure. Negative baseline titers that were followed by positive early-convalescent-phase titers were also excluded, for clarity.

convalescent period. Thus, a total of 26 patients (96%) in this population were C_6 positive. For the single- and multiple-EM groups together, the diagnostic sensitivity of the C_6 test was 88% (Table 1).

Archival results of a standard Lyme disease ELISA performed on the study samples were reviewed. The sensitivity of this test was lower than that of the C_6 test. Two of the 93 single-EM patients did not have the standard ELISA performed. Of the remaining 91, 24 patients had a positive standard ELISA at baseline (26%), and an additional 25 converted to seropositivity based on this test during convalescence. The overall sensitivity of the standard ELISA was 54% (Table 1).

One of the 27 patients with multiple EM did not have a standard ELISA performed. At baseline, 21 of the 26 patients had positive standard ELISA results (81%). Three additional patients became positive by this test during convalescence, bringing the total number of ELISA-positive multiple-EM patients to 24 (92%). These sensitivity values are slightly lower than those observed with the C_6 test in this population. For the single- and multiple-EM groups together, the diagnostic sensitivity of the standard ELISA was 62%, lower than the 88% observed with the C_6 test (Table 1).

Response to treatment as assessed with the C_6 test. As indicated in the previous section, 14 of the 93 single-EM patients did not have detectable anti-C_6 antibody at either the baseline or early convalescence time points. Seventy-nine patients of this study population (93 − 14 = 79) were therefore available for assessment of their C_6 antibody responses to treatment. Only 1 of the 27 multiple-EM patients had undetectable C_6 antibodies at the baseline and early convalescence time points. Therefore, the sample for the assessment of the C_6 antibody-versus-treatment outcome correlation comprised 26 patients.

In all of these 105 patients, the C_6 antibody titers declined posttreatment with respect to the titers at baseline or in the convalescent period (Fig. 1). The C_6 antibody titers of 51 (64.5%) single-EM patients declined to undetectable ("negative") values in the 6- to 12-month follow-up period, whereas values for 28 patients in this group declined to positive second titers (Fig. 1). Of these, 19 declined in titer by a factor of ≥4

TABLE 2. C_6 antibody titer response to treatment

Outcome	Single-EM patients (n = 79)			Multiple-EM patients (n = 26)			Single- and multiple-EM patients (n = 105)		
	Frequency[a]	%	P	Frequency	%	P	Frequency	%	P
Negative follow-up titer	51	64.5		11	42.3		62	59.0	
Declining follow-up titer[b]	19	24.1		15	57.7		34	32.4	
Total	70	88.6	<.0001	26	100	<.0001	96	91.4	<.0001

[a] Frequency is given in number of patients.
[b] Patients whose follow-up titer declined fourfold or more with respect to the baseline (or early-convalescent-phase titer when the baseline titer was negative).

(Table 2). Thus, 70 (51 plus 19) of 79 single-EM patients (89%) experienced a decline in C_6 antibody titer that, as per our hypothesis, was considered commensurate ($P < 0.0001$) with the cured clinical status exhibited by this study population in the follow-up period (Table 2). In the multiple-EM group, 11 patients (42.3%) experienced a decline in titer to negative values in the follow-up period (Fig. 1; Table 2). The titers of the remainder (15 patients) declined by a factor of four or more. Thus, the C_6 antibody titers of all of the patients in this group declined as predicted by our hypothesis ($P < 0.0001$) (Table 2). Of the 105 patients in the single- and multiple-EM groups combined, 96 (91.4%) patients experienced either a decline of more than fourfold or a decrease to negative values in their C_6 antibody titers ($P < 0.0001$) (Table 2).

A greater proportion of single-EM patients (64.5%) experienced a decline to negative titers than did the multiple-EM group (42.3%) ($P = 0.065$). This could have been due to a greater rate of titer decline and/or to a lower peak titer in the single-EM group. A comparison of the mean slopes of the plots of log titer versus time in Fig. 1 yielded a value of -0.238 for the single-EM group ($n = 79$) and -0.226 for the multiple-EM group ($n = 26$). This difference, however, is not statistically significant ($P = 0.676$). On the other hand, the mean logarithmic baseline titer of the single-EM group was 2.15 (a titer of approximately 1/140), whereas that of the multiple-EM group was 2.8 (approximately 1/640). This difference was statistically significant ($P < 0.001$). Therefore, the rates of C_6 antibody titer decline in the two groups were comparable, and patients in the single-EM group reached negative titers more frequently than those of the multiple-EM group because their baseline titers were lower.

DISCUSSION

The C_6 test is currently used for the serologic diagnosis of Lyme disease both in humans and in dogs (for examples, see the canine SNAP 3Dx and the Lyme quantitative C_6 antibody tests [IDEXX Laboratories, Inc.] and the C6 Lyme disease ELISA [Immunetics, Inc.]) (1, 6a, 8a, 8b, 8c, 9a, 10a, 11–13, 13a, 13b, 13c, 14a, 14b, 15a, 19a). For human serology, it is employed as part of a two-tier algorithm. This diagnostic algorithm was introduced in 1995 at the recommendation of the Centers for Disease Control and Prevention (5). Customarily, the first tier is an ELISA based on *B. burgdorferi* whole-cell antigen extract. When results are positive or equivocal, it is followed by Western blot analysis, which is evaluated by using the banding criteria developed by Dressler et al. (6) and Engstrom et al. (7). Presently, the C_6 test has been approved by the Food and Drug Administration to be used like the whole-cell ELISA, as the first tier. In our study, with a population composed of individuals undoubtedly infected with *B. burgdorferi*—insofar as their infections were culture confirmed—the sensitivity of the C_6 test in patients with early localized infections was over one and a half times higher than that of a whole-cell standard ELISA. This difference in sensitivity held true for the patients whose results were positive at baseline as well as for those whose results became positive during early convalescence. The C_6 test and the standard ELISA were similarly sensitive for antibody detection in patients with early disseminated Lyme borreliosis. The C_6 test thus emerges as a notably sensitive test for early localized *B. burgdorferi* infection.

Our study demonstrates that in a clinically cured patient population, which had presented with an early (localized or disseminated) *B. burgdorferi* infection, the C_6 antibody titer decreases either fourfold or to undetectable levels in a highly significant proportion of this population and within 6 to 12 months posttreatment. This decline was observed in 89% of the patients with single EM and in 100% of the multiple-EM patients. It should be pointed out, in addition, that even though the 15 patients (14 with single EM and 1 with multiple EM) whose baseline and early convalescent-phase C_6 titers were negative were formally excluded from the study, their 6- to 12-month follow-up specimens also were C_6 negative. Thus, if a follow-up C_6 titer that is below a certain value, as yet to be determined, were the sole serologic evidence sought for successful treatment, these patients would have satisfied it.

The results indicate that in patients treated shortly after infection, the C_6 antibody titers are more likely to decline to an undetectable level than in patients who received treatment after the infection has had time to disseminate. Thus, the C_6 antibody titer declined to undetectable levels in 51 of the 91 single-EM patients (64.5%) but in only 42.3% (11/26) of the multiple-EM patients. This difference was not due to a faster decline in titers of patients from the single-EM group, as the mean values of the titer decline rate were statistically comparable for the two groups; it was due, rather, to a significantly lower mean C_6 antibody titer at baseline for the single-EM patients. As mentioned in the introduction, antibodies to C_6 may be more likely to persist posttreatment, albeit at lower-than-baseline titers, in patients who received treatment later in the infection process.

We had hypothesized that the decline in anti-C_6 antibody titers following antibiotic treatment might be due to properties

we attributed to VlsE and its stimulation of immunological memory (16). We argued that this antigenic variation molecule would elicit B-lymphocyte memory at a lower rate than nonvariant antigens due to the high level of turnover we putatively ascribed to it. Inevitably, the longer an infection remained untreated, the more this trend would be reversed (16). This hypothesis thus predicts that patients with late disease should be more likely to show posttreatment persistence of C_6 antibody titers than patients with early Lyme borreliosis and perhaps also a decreased rate of C_6 antibody titer decline (15, 20).

As mentioned in the previous section, a titer decline of less than fourfold was observed in eight of the single-EM patients. It may be of interest that five of these eight patients had had prior episodes of EM. Previous exposure to the pathogen may have increased the C_6 immunological memory in a manner that affected the C_6 antibody titer decline. In contrast, of the 112 other subjects in the study, only 12 had had prior episodes of EM. This is a significant difference ($P = 0.001$).

The serologic correlates of a positive response to treatment that we chose for this study, namely, C_6 seronegativity or a fourfold decline in C_6 antibody titer, with either of these end points occurring at 6 to 12 months posttreatment, are not without precedent. Quantitative nontreponemal antibody tests are used in a very similar way for the evaluation of syphilis treatment with antibiotics (10). Adequate therapy for primary and secondary syphilis correlates with a fourfold decline in antibody titer by the third or fourth month and an eightfold decline at 6 to 8 months posttreatment (2). For most patients treated in early stages of syphilis, the titers decline and reach seronegativity after the first year (8). In contrast, as with Lyme disease patients with late-stage disease and C_6 antibody titers, treatment in the latent or late stages of syphilis results in a less frequent decline in titers to negative values, with low titers persisting in about 50% of this patient population after 2 years. Moreover, these patients are likely to remain unchanged serologically (serofast) even when subjected to additional antibiotic therapy (10). Duration of infection, or length of exposure to antigen prior to treatment, seems thus to be directly related to the persistence of the antibody response and inversely related to the rate of its decline after treatment with both the cardiolipin antigen used in the quantitative nontreponemal antibody test and C_6.

As with VlsE, it is possible that the cardiolipin antigen form that elicits the nontreponemal antibody response is in short supply and is thus a slow inducer of B-cell memory. The fact that both antigens, although chemically of very different natures, are relatively small in size and may comprise perhaps just a single B-cell epitope (cardiolipin probably aggregates or forms lamellar structures) also may be important, albeit in a manner we do not as yet understand.

We were unable to study Lyme disease patients in whom antibiotic therapy had been objectively demonstrated to be unsuccessful, as such patients are extremely rare. Their scarcity makes it difficult to prove that C_6 antibody titers do not decrease in patients who fail therapy. Therefore, absence of posttreatment decline in C_6 antibody titer may not be equated to treatment failure.

As infection progresses, a quantitative C_6 test used as a correlate to assess response to treatment may decline in sensitivity. However, for patients with early localized or disseminated Lyme disease involving the skin, our data convincingly show that a decline (of fourfold or to zero values) in C_6 antibody titer significantly correlates with a successful treatment outcome.

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