Acta Derm Venereol (Stockh) 1985; 65: 509–514

Serologic Studies of Erythema chronicum migrans Afzelius and Acrodermatitis chronica atrophicans with Indirect Immunofluorescence and Enzyme-linked Immunosorbent Assays

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Åsbrink E, Hovmark A, Hederstedt B. Serologic studies of erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans with indirect immunofluorescence and enzyme-linked immunosorbent assays. Acta Derm Venereol (Stockh) 1985; 65: 509–514.

To determine whether antibodies to Borrelia spirochetes were present, sera from 88 patients with uncomplicated erythema chronicum migrans Afzelius (ECMA), from 9 patients with ECMA-related extracutaneous complications and from 26 patients with acrodermatitis chronica atrophicans (ACA) were submitted to an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescence (IF) assay. The assays were calculated to be 95% specific. There was good correlation between the IF test with a polyvalent conjugate and IgG ELISA. Of patients with uncomplicated ECMA, 18% were seropositive by IgG ELISA and 11% by IgM ELISA, and 15% showed elevated IF titers. Elevated serum antibody levels of IgG as measured by ELISA and elevated IF titers were found in all patients with extracutaneous complications and in the patients with ACA. Declining IgG titers were observed at follow-up 6–12 months after therapy, but the majority of the patients with ACA were still seropositive. (Received April 30, 1985.)

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Erythema chronicum migrans Afzelius (ECMA) and acrodermatitis chronica atrophicans (ACA) have recently been recognized as different manifestations of infection by Borrelia spirochetes, transmitted by the tick *Ixodes ricinus* (1, 2, 3). ECMA is an early spirochetal manifestation which, if untreated, may be followed by extracutaneous complications (4). ACA is probably a late manifestation of infection by the same spirochete that causes ECMA (2).

A spirochete, now named *Borrelia burgdorferi*, and transmitted by the tick *Ixodes dammini*, is the etiologic agent of Lyme disease in the United States (5). Both an indirect immunofluroescence (IF) test and an enzyme-linked immunosorbent assay (ELISA) have been used in the serologic diagnosis of Lyme disease (5, 6, 7, 8). We have previously used the indirect IF test with a polyvalent conjugate and found that determinations of antibody titers are useful as a laboratory method in the diagnosis of ACA (2), but are of little help for the majority of patients with uncomplicated ECMA (1).

The purpose of the present investigation was to compare an indirect IF test with an ELISA in the detection of antibodies to Borrelia spirochetes and to determine whether ELISA, including both IgM and IgG serology, can be of diagnostic help for patients with ECMA. A further aim was to carry out a serologic follow-up of ACA patients after treatment.

MATERIAL AND METHODS

Patients

The patients were investigated at the Department of Dermatology, Södersjukhuset, Stockholm, Sweden. Analyses were made of sera from 88 patients with typical, clinically uncomplicated ECMA

(duration 5 days–11 months) and from 26 patients in whom the clinical and histopathologic findings were compatible with a diagnosis of ACA. The median duration of ACA was 2.5 years (range 6 months–>10 years).

Sera from nine patients with ECMA-related extracutaneous manifestations were also studied. Four of these patients developed facial palsy after tick bites and showed increasing, high IF titers against Borrelia spirochetes in serologic tests. These patients have been described previously (9). One other patient developed meningoradiculitis and another patient arthritis after a spontaneous healing of ECMA and these patients have also been described earlier (4). Three patients with ECMA (aged 28, 43 and 53 years) showed electrocardiographic disturbances with T-wave inversions. Two of them also suffered from malaise and profound fatigue. The T-wave inversions were restored to normal after antibiotic therapy and these patients were included in the present group because of suspected cardiac involvement.

Controls

Humans aged 17–80 years served as controls. They visited our department and blood samples were drawn for routine analyses. Persons with recognized syphilis were excluded, as cross-reactivity between treponemal and borrelial antigens is known to occur. The patients and controls lived in the same area.

Therapy

The standard treatment for adult patients with ECMA was phenoxymethyl penicillin 2 g a day for 10 days, and patients with ACA received the same antibiotic in a dose of 2–3 g a day for 3 weeks. The patients with facial palsy and meningitis were treated with penicillin G intravenously 3 g every 6 h for 2 weeks.

Serologic test

In all patients serum samples were obtained before treatment. From the patients with ACA and from five of the patients with ECMA-related extracutaneous manifestations, sera were also obtained 6–12 months after therapy. The serum samples were stored at −70°C and different sera from the same patient were analysed simultaneously.

Enzyme-linked immunosorbent assay. Spirochetes isolated from the skin of patient with ACA (strain ACA I) were cultivated in modified Kelly's medium as described previously (5) and used as antigen. For preparation of ELISA antigen, two batches of 320 ml each of Kelly's medium containing about 10^7 spirochetes/ml were centrifuged at 10000 × g for 30 min. The pellets were washed four times in PBS with 5 mM $MgCl_2$ before being resuspended in PBS. The suspensions were sonicated on ice by seven 30 s blasts. After centrifugation at 10000 × g for 30 min at 4°C, the partially clarified supernatants were drawn and stored at −70°C. The protein content was determined with a micro biuret method (10). One antigen batch was used for IgM ELISA and one for IgG ELISA. With the use of titration curves the optimal coating concentration was determined. The ELISA method as described earlier (11) was used. Tests were performed in duplicates and the same positive and negative control sera were invariably used on each plate. The plates were read at 405 nM in an ELISA reader (Flow laboratories). The time for substrate incubation was adjusted to the positive control serum. The ELISA titer was expressed as the A_{405} value multiplied by the reciprocals of the serum dilution. If the sera duplicates differed by >10% from the mean, or if the background in the uncoated control wells was >25% of the value for the coated wells, the results were not accepted and the sera were retested.

Immunofluorescence test. The method used, with a polyvalent conjugate has been described previously (1). The results are presented as reciprocal titers.

A ≥four-fold change in IF titer and a ≥two-fold change in ELISA titer were considered significant.

RESULTS

A positive ELISA or IF result was defined as a titer above the 95th percentile for controls. The results of the determinations of serum antibodies against Borrelia spirochetes with the use of the ELISA technique are given in Table I. An ELISA titer level of >410 for IgG and of >800 for IgM was considered positive. With the IF method serum titers of ≥160 were found in 13 out of 252 (5%) control tests. Titers below these values are designated negative.

The Wassermann reaction was negative in all patients.

Table I. *Serum antibody levels measured by enzyme-linked immunosorbent assay in patients with uncomplicated erythema chronicum migrans Afzelius (ECMA), in patients with ECMA-related complications, in patients with acrodermatitis chronica atrophicans, and in controls*

		Erythema chronicum migrans Afzelius				
		Uncomplicated	Related complications		Acrodermatitis chronica atrophicans	
	Controls	Before treatment	Before treatment	6 months after treatment	Before treatment	6–13 months after treatment
IgG						
No	185	88	9	5	26	26
>410	9 (4.9%)	16 (18%)	9 (100%)	3	26 (100%)	24 (92%)
Median	150	210	640	530	3 600	970
Range	<100–750	<100–1 500	540–1 260	250–550	420–32 500	200–9 300
IgM						
No	185	88	9	5	26	26
>800	9 (4.9%)	10 (11%)	5 (56%)	0	7 (27%)	4 (15%)
Median	370	460	890	490	550	430
Range	<100–1 090	210–1 500	430–1 400	390–590	210–3 400	180–1 100

Erythema chronicum migrans Afzelius

In the sera from the 88 patients with uncomplicated ECMA, 16 (18%) had positive IgG ELISA titers and 10 (11%) had positive IgM ELISA titers (Table I). A positive IgG and/or IgM titer was found in sera from 25/88 (28%) ECMA patients and in 18/185 (10%) controls. Sera from 13 patients (15%) were positive in the indirect IF test. Seven of these 13 sera were also positive by IgG ELISA, but none by IgM ELISA. The median duration of ECMA in patients with elevated IgM titers was 4 weeks (range 1 week–9 months) and the median duration in patients with elevated IgG titers was 2 months (range 5 days–11 months).

ECMA-related extracutaneous manifestations

All serum samples from the group of nine patients with suspected complications showed elevated IgG ELISA titers and five also showed elevated IgM ELISA titers (Table I). Elevated indirect IF titers (160–1 280) were found in sera from all nine patients. At the time of follow-up (6 months after treatment) the five patients examined had negative serum IgM titers (Table I). All patients also displayed a tendency toward decreasing IgG ELISA titers, but this decrease was significant in only one patient. At follow-up only two of these patients had negative IgG ELISA titers.

Acrodermatitis chronica atrophicans

Before treatment, all sera from the 26 patients with ACA showed positive titers both by IgG ELISA (Table I) and in the indirect IF assay (Fig. 1). At the time of follow-up after treatment there was a tendency toward decreasing IgG ELISA titers in all cases (Fig. 1), and a significant decrease in titer as measured by IgG ELISA was found in 15 patients (58%) and as measured by indirect IF assay in 13 patients (50%). Only two of the 18 patients who underwent a follow-up examination 9–12 months after therapy had negative IgG ELISA titers at the time of follow-up, and none of the remaining eight patients in whom sera had been obtained 6–8 months after therapy had negative serum IgG titers.

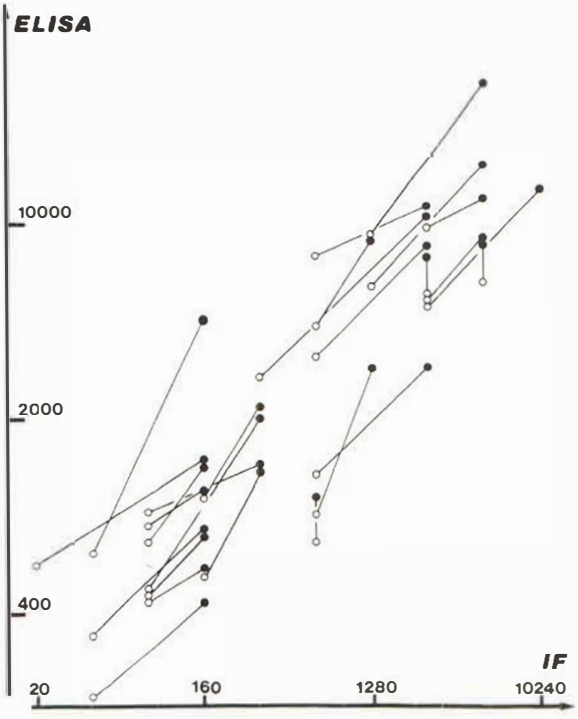

Fig. 1. Relationship between serum titers measured with an indirect immunofluorescence test with a polyvalent conjugate and an IgG enzyme-linked immunosorbent assay in 26 patients with acrodermatitis chronica atrophicans, before (●) and after (○) treatment.

There was a good correlation (correlation coefficient = 0.88) between the indirect IF titers and IgG ELISA (Fig. 1). Seven patients (27%) had positive IgM ELISA titers before treatment and four at the time of follow-up (Table I).

DISCUSSION

In previous studies we have reported on isolation of spirochetes from *Ixodes ricinus* ticks (1), from skin lesions of patients with ECMA (1, 3) and ACA (2, 3). Examinations (performed by Dr A. Barbour, Rocky Mountain Laboratories, Montana, USA) of our human isolates from patients with ECMA (strains ECMA I–VIII) and ACA (strains ACA I–II) and of tick isolates (FI–II) with four monoclonal antibodies against *Borrelia burgdorferi* (H5332, H6831, H5TS, H9724) revealed differences between our isolates and North American spirochete isolates (unpublished data). No differences were found between the spirochete isolates from ECMA and ACA lesions nor between these and one (strain FII) of the two isolates from *Ixodes ricinus* ticks. These results further support the hypothesis that ECMA and ACA are caused by the same *Ixodes ricinus*-borne spirochete. Thus, in the present study the same spirochete isolate (strain ACA I) was used as antigen in the serologic tests of patients with ECMA and with ACA.

In a previous investigation, in which serum antibodies against spirochetes isolated from Ixodes ticks were detected with the use of the indirect IF test, we found a significantly elevated median titer among 58 patients with uncomplicated ECMA (1). In the majority of these patients, however, the test was not diagnostic because of overlapping between patients and controls. These results are similar to those found in the present study, in which serologic evidence of spirochetal infection could only be proven in a minority of the 88 patients with uncomplicated ECMA with the use of the ELISA or the indirect IF test.

Similar results were obtained by Stiernstedt et al. in a study of 25 patients with ECMA (12).

In the present study it was considered that the use of ELISA for detecting IgM antibodies would improve the serologic diagnosis of uncomplicated ECMA. Positive IgM titers were demonstrated, however, in only 11% of the patients, as compared with 4.9% of the controls. There may be several reasons for this serologic failure. One may be that the number of spirochetes involved in uncomplicated ECMA is often too small to give rise to detectable amounts of antibodies. The serologic results indicate that small amounts of antibodies reacting with the Borrelia spirochete antigens may also have been present in many of the control sera. We have previously found that preabsorption of sera with Reiter spirochetes may increase the sensitivity of the indirect IF test, but we found the preabsorption procedures difficult to standardize (1). The preparation of a more specific antigen may be one way of improving the serologic diagnosis of uncomplicated ECMA.

In about half of the patients with ECMA-related extracutaneous manifestations and in about one-fourth of those with ACA, positive IgM titers were found. It may be difficult, however, to evaluate the findings of specific IgM antibodies in sera. It has been found that the presence of high titers of specific IgG may give falsely low results for IgM in ELISA systems (13). On the other hand, according to Wilske et al., false positive IgM reactions can occur in sera from ACA patients as a result of the presence of rheumatoid factor activity, despite the fact that the patients are negative in conventional rheumatoid factor tests (14). Neither fractionation of sera nor preabsorption of IgG antibodies was performed in the present study, but such procedures might answer the question concerning the frequency of specific IgM antibodies in sera from patients with ACA and high titers of specific IgG.

Sera from all the patients with ECMA-related extracutaneous manifestations displayed positive indirect IF and IgG ELISA titers. Thus both ELISA and the indirect IF assay may be of diagnostic help in patients with extracutaneous manifestations. In patients with a short duration of disease the antibody titers may be negative, however, and convalescent-phase sera ought to be investigated. Stiernstedt et al. found elevated IgG and/or IgM antibodies against *I. ricinus*-borne spirochetes in sera from 30 of 45 patients with chronic meningitis and achieved the highest sensitivity by measuring a spirochetal cerebrospinal fluid titer index (11).

Positive IgG ELISA titers and positive indirect IF titers were found in sera from all untreated patients with ACA, indicating that these serologic tests are of great diagnostic help in patients with ACA. In most cases of ACA elevated IgG ELISA titers (>410) were still found 6–12 months after treatment. Similar results have been obtained in patients with late manifestations of Lyme disease (6). Parallels may be drawn between ACA and late syphilis, in which treponemal tests may remain elevated despite antibiotic therapy. It is possible that the time required to attain seronegativity in patients with ACA depends upon the duration of the untreated infection.

Although the IgG ELISA titers remained positive in sera of the majority of the patients at the time of follow-up, all patients studied showed a tendency to decreasing serum titers. This serologic response to therapy supports the clinical finding that the antibiotic treatment given to patients with ECMA-related complications and ACA is effective in most cases. However, included among the patients with extracutaneous manifestations there was one who after untreated ECMA developed arthritis of an ankle, and who was then treated with our standard penicillin regimen for uncomplicated ECMA (4). Two weeks later the joint swelling had disappeared and the patient was still free from symptoms at reexamination 6 months later. Twelve months after therapy, the patient sought advice for recurrence of the ECMA on the same leg in which the previous ECMA and the arthritis

had occurred. There were no major differences between the serum IgG ELISA titers found at the time of the arthritis (620), 6 months after treatment (510) and at the time of the recurrence of the ECMA (560). The clinical findings in this patient with involvement of the joint indicate that the penicillin dose used in this case may have been inadequate and that spirochetes may have invaded tissues not penetrated by the drug in therapeutic concentrations. Thus further studies and a longer follow-up time are needed for definitive recommendations on the dosage, route of administration and duration of therapy for all different manifestations of infections by *Ixodes ricinus*-borne spirochetes.

ACKNOWLEDGEMENTS

We thank Elisabeth Ishizaki and Eva Jansson for technical assistance. This work was supported by grants from the Finsen and the Welander Foundations.

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