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Immunoblot Analysis of the Seroreactivity to Recombinant *Borrelia burgdorferi* sensu lato Antigens, Including VlsE, in the Long-Term Course of Treated Patients with Erythema Migrans

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Key Words
Lyme borreliosis · *Borrelia burgdorferi* · Immunoblot · Erythema migrans · Variable major protein-like sequence expressed antigen

Abstract
Objective: We evaluated whether immunoblotting is capable of substantiating the posttreatment clinical assessment of patients with erythema migrans (EM), the hallmark of early Lyme borreliosis. ***Methods:*** In 50 patients, seroreactivity to different antigens of *Borrelia burgdorferi* sensu lato was analyzed by a recombinant immunoblot test (IB) in consecutive serum samples from a minimum follow-up period of 1 year. Antigens in the IgG test were decorin-binding protein A, internal fragment of p41 (p41i), outer surface protein C (OspC), p39, variable major protein-like sequence expressed (VlsE), p58 and p100; those in the IgM test were p41i, OspC and p39. Immune responses were correlated with clinical and treatment-related parameters. ***Results:*** Positive IB results were found in 50% before, in 57% directly after therapy and in 44% by the end of the follow-up for the IgG class, and in 36, 43 and 12% for the IgM class. In acute and convalescence phase sera, VlsE was most immunogenic on IgG testing (60 and 70%), and p41i (46 and 57%) and OspC (40 and 57%) for the IgM class. By the end of the follow-up, only the anti-p41i IgM response was significantly decreased to 24%. ***Conclusions:*** No correlation was found between IB results and treatment-related parameters. Thus, immunoblotting does not add to the clinical assessment of EM patients after treatment.

Introduction

Lyme borreliosis (LB) is a multisystem infectious disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato (*Bb*sl) complex [1]. Erythema migrans (EM) is defined as the hallmark of early LB that is associated with *B. afzelii* infection in the majority of cases [2]. It is associated with unspecific extracutaneous signs and symptoms in 30–40% of European patients and up to 70% in North American patients [2]. In untreated cases, spirochetal dissemination may lead to other organ manifestations, including neurological, musculoskeletal or cardiac disorders [2]. Adequate antibiotic treatment usually resolves EM as well as extracutaneous symptoms within days to

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weeks, although (intermittent) complaints may sometimes clear slowly [2, 3]. Subsequent objective neurological, cardiac or joint manifestations of LB develop in a minority of patients [2, 4]. Patients with true treatment failure (persistence or recurrence of EM, major sequelae and/or survival of *Bb*sl) have to be retreated [5], whereas post-LB syndrome (i.e. long-lasting subjective symptoms, such as fatigue, musculoskeletal pain and cognitive dysfunction), which develops in about 10–50% of primarily American patients, does not respond to (repeated) antimicrobial retreatment [6].

So far, no reliable laboratory test is available to substantiate the clinical assessment of EM patients after antibiotic treatment. Analyses of biopsy samples for the persistence of *Bb*sl or *Bb*sl-specific DNA in lesional skin by cultivation or polymerase chain reaction are very specific but rather insensitive [7]. Cultivation is a laborious and tedious method, and both procedures are invasive and would not detect possible residual borrelial infection of other tissues. Culture or polymerase chain reaction analyses of blood or urine are less valuable and not established for routine diagnosis [8]. In clinical practice, serum anti-*Bb*sl antibody analyses would be most convenient, but for enzyme-linked immunosorbent assay (ELISA) testing, it has been shown recently that repeated examinations of anti-*Bb*sl antibodies after antibiotic therapy of patients with EM do not provide additional information about the efficacy of treatment to the clinician [9].

The immunochemical detection of antibodies against a variety of individual proteins of *Bb*sl by immunoblot (IB) tests provides a superior specificity in the serological testing for LB [10, 11]. Advanced IB tests with additional excellent sensitivity rely on recombinant antigens instead of whole-cell lysates and the inclusion of antigenic proteins that are expressed primarily in vivo, such as variable major protein-like sequence expressed (VlsE) [12, 13]. In Europe, LB is caused by at least 3 different species of *Bb*sl, *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto. Thus, recombinant IB tests should contain combinations of antigenic proteins from different borrelial species and strains, especially if the respective proteins are immunologically heterogenous. The aim of the present study was to evaluate by immunoblotting whether changes of antibody responses to different borrelial antigens over time allow for the assessment of the efficacy of antibiotic therapy, which has not been examined specifically so far. In this study a recombinant IB which allows for the combination of immunodominant proteins from the 3 major European *Borrelia* species was used [12].

Patients and Methods

Patients

We carried out a retrospective study of serum anti-*Bb*sl IgG and IgM antibody responses before and after antibiotic therapy in 50 patients with EM by IB testing. These 50 patients were selected from a series of 554 consecutive EM cases, seen between 1998 and 2001 at the Department of Dermatology in Graz, Austria, based on the following inclusion criteria: (i) EM was diagnosed by a dermatologist according to the clinical definition of an expanding, sharply demarcated, red to bluish-red erythema of at least 5 cm in diameter with or without central clearing [2, 14, 15]. (ii) The patient had been treated with standard oral antibiotic therapy [2, 16] following the clinical diagnosis and had been followed for at least 1 year after therapy. (iii) At least 3 serum samples from the patient were available for the analysis of anti-*Bb*sl antibodies, including a sample from directly before therapy and at least 2 posttreatment samples. The last sample had to be obtained at least 1 year after therapy. (iv) Information on the following clinical parameters was available: age, sex, duration of EM before therapy, type of EM (solitary vs. multiple), presence of associated extracutaneous signs and symptoms, type and duration of antibiotic therapy, duration of EM and associated extracutaneous signs and symptoms after therapy. Exclusion criteria for our study were: (i) reinfection, i.e. history of a previous EM episode or development of a second independent EM during the follow-up period; (ii) antibiotic treatment before first serum sampling or during the follow-up period; (iii) concurrent conditions or therapies with potential influence on the development of anti-*Bb*sl antibodies (e.g. autoimmune disease, malignancy, corticosteroid medication).

Recombinant Proteins

To analyze anti-*Bb*sl serum IgG and IgM antibodies, an IB test was applied that contained a set of recombinant immunodominant *Bb*sl antigens, which were produced by genetic engineering as described previously [12, 17–21]. For most antigens, variants from 2–4 *Bb*sl strains representing the 3 main human pathogenic species of *Bb*sl were used (table 1). The antigens were: (1) decorin-binding protein A (DbpA), a lipoprotein that acts as adhesin to decorin, a collagen-associated proteoglycan [22]; (2) p41i, a 14-kDa internal fragment of the 41-kDa subunit (p41) of flagellin, a polypeptide that makes up the flagella of *Bb*sl [23] – the use of p41i instead of whole p41 provides a higher specificity, because it is not cross-reactive; (3) outer surface protein C (OspC), the major outer surface lipoprotein expressed by *Bb*sl in early infection [24, 25]; (4) p39, an immunodominant membrane-associated protein [19, 26]; (5) VlsE, a 35-kDa surface lipoprotein that frequently undergoes antigenic variation, an important immune evasion mechanism of *Bb*sl [27, 28]; (6) p58, oligopeptide permease, an immunodominant membrane-associated protein not to be confused with the heat shock protein hsp60 [21, 29]; (7) p83/100, a prominent antigen localized to the periplasmic space of *Bb*sl with yet undefined function [30].

Immunoblot Testing

Of the 50 included patients, a median number of 5 (range 3–8) serum samples, which had been obtained directly before and during a median follow-up period of 535 days (range 368–1,156 days) after therapy and stored at –70°C, were available for IB analyses of IgG and IgM antibodies to *Bb*sl. All samples from each patient

Table 1. Recombinant antigens from different strains of *Bb*sl used in IB testing of patients with EM

	IgG							IgM		
	DbpA	p41i	OspC	p39	VlsE	p58	p100	p41i	OspC	p39
*Bb*sl strain	PKo PBr	PKo	PKo	PKo			PKo	PKo	PKo	PKo
		PBi	PBi 20047	PBi		PBi		PBi	PBi 20047	PBi
			PKa2	PKa2	PKa2				PKa2	PKa2

DbpA = Decorin-binding protein A; p41i = internal fragment of p41; OspC = outer surface protein C; VlsE = variable major protein-like sequence expressed. Strain PKo corresponds to the species *B. afzelii*, strains PBr, PBi and 20047 correspond to *B. garinii*, and strain PKa2 corresponds to *B. burgdorferi* sensu stricto.

were tested together at the same time on serial IB strips from the same electrophoresis gel. IB analyses were performed as described previously [12, 19, 21] with different combinations of test antigens for the IgG and the IgM class, respectively (table 1). Briefly, IB strips were placed into incubation trays with 10 wells each. Strips were first incubated with patients' sera on an orbital shaker overnight at room temperature in 1:200 and 1:100 dilutions for IgG and IgM, respectively (dilution buffer: 0.9% sodium chloride, 10 mM Tris-OH/HCl, pH 7.4, 0.2% Tween 20, 0.4% thimerosal, 1% nonfat dried milk). Afterwards, strips were washed for 4 × 5 min (wash buffer: 0.9% sodium chloride, 10 mM Tris-OH/HCl, pH 7.4, 0.2% Tween 20). Bound antibodies were detected with conjugate consisting of horseradish-peroxidase-labeled anti-human immunoglobulin (Dako, Glostrup, Denmark) at a dilution of 1:1,000 for IgG and 1:500 for IgM detection. After 2 h of conjugate incubation, strips were washed for 4 × 5 min with wash buffer. Bound conjugate was visualized by addition of a chromogenic substrate (diaminobenzidine, 10 mM Tris-OH/HCl, pH 7.4, 30% H_2O_2). The staining reaction was stopped after 5 min with 1 N H_2SO_4. For IgM testing, sera were preincubated with rheumatoid factor absorbent (Behring, Marburg, Germany) for 30 min at room temperature to eliminate rheumatoid factor activity as well as possible competitive inhibition by specific IgG antibodies. For validation, negative and positive control sera were included in each test run. IB strips were read independently by one of us (M.G.) and a research associate of our laboratory. A band was reported as positive in case its intensity was at least as strong as the weakly positive bands of the positive control. For band location, assay strips were compared with control strips. Our criteria for a positive IgG IB result of a patient were the recognition of at least 2 of the investigated antigens, and for a positive IgM IB result recognition of at least 2 investigated antigens or presence of a single strongly positive OspC band [31, 32].

ELISA Testing

Sera of all patients were also analyzed for anti-*Bb*sl IgG and IgM antibodies by an ELISA test, using purified, native flagella of *B. afzelii*, strain DK-1, as test antigen (Dako Lyme Borreliosis ELISA Kit; Dako) [33]. This is a highly immunogenic, phenotypically stable flagellum antigen that elicits an early and strong immune response [34] and shows no variation between strains of different geographic origins [33]. All samples of each patient were tested together at the same time and on the same well microtitration plate in order to avoid intertest variability.

Statistics

The χ^2 test was used to assess the following categorical comparisons of serological results: number of patients with a positive IgG or IgM total IB versus ELISA result before therapy, by the end of therapy and by the end of follow-up; and the number of patients with a positive IgG or IgM total IB or specific antigen result before therapy versus the end of therapy and versus the end of follow-up, and between the end of therapy or 1 month after therapy and the end of follow-up. Furthermore, a set of clinical parameters (age, sex, type, size and duration of EM before therapy, presence of extracutaneous signs and symptoms, type and duration of therapy, duration of EM and extracutaneous symptoms after therapy) were compared with pretreatment total or antigen-specific IB results as well as with the serological courses (decline vs. persistence of anti-IgG or -IgM *Bb*sl antibodies from before or directly after therapy to the end of follow-up) by the appropriate test (χ^2 test or Mann-Whitney rank sum test); p values less than 0.05 were considered statistically significant, and all tests were two tailed. Statistical analyses were performed on a personal computer with the statistical package Primer of Biostatistics for Windows, version 4.0 (McGraw-Hill, New York, N.Y., USA).

Results

Clinical Characteristics

Fifty patients with EM with a median age of 52.5 years (range 12–78 years) were investigated in this study. Thirty patients (60%) were females and 20 (40%) were males. The clinical diagnosis was substantiated by either a typical histopathological picture plus a positive polymerase chain reaction for *Bb*sl-specific DNA in 48% of the patients or by histopathology plus cultivation of *Bb*sl in 29% of the patients. Thus, the clinical and histopathological

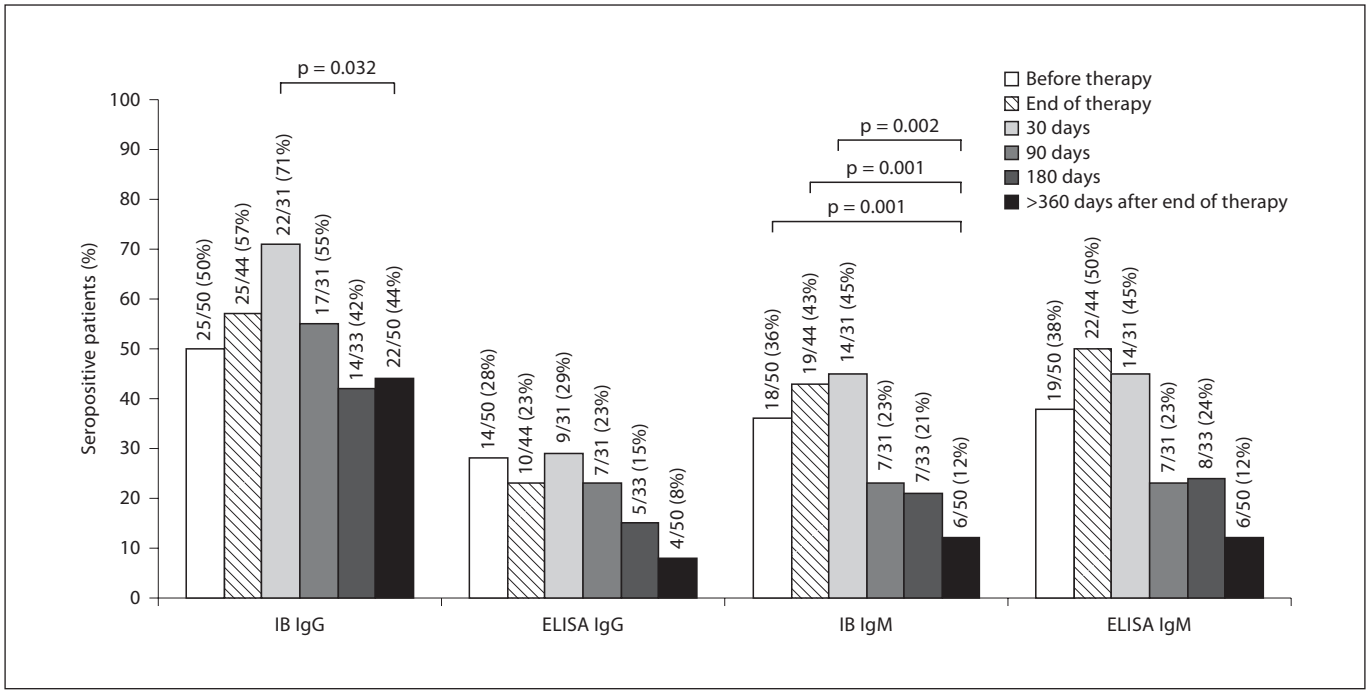

Fig. 1. ELISA and IB IgG and IgM seroreactivity of patients with EM.

diagnosis was supported by direct detection of *Bb*sl (-specific DNA) in 77% of all included patients.

The median duration of EM before therapy was 9.5 days (range 1–112 days). The median size of the EM lesions (largest diameter) was 12 cm (range 5–40 cm). Forty-four patients (88%) had a solitary EM lesion, 6 patients (12%) had multiple EM lesions. Nineteen patients (38%) reported 1 or more extracutaneous signs and symptoms of the following distribution: headache (n = 10), elevated temperature (n = 7), arthralgias (n = 12), myalgias (n = 6), malaise (n = 9) and fatigue (n = 10), whereas 31 patients (62%) had no other signs and symptoms. Thirty-six patients (72%) were treated with tetracyclines (33/36 patients, 92%, with peroral minocycline and 3/36 patients, 8%, with doxycycline), 14 patients (28%) received betalactam antibiotics (10/14 patients, 72%, received peroral phenoxymethyl penicillin, 3/14 patients, 21%, peroral amoxicillin and 1/14 patients, 7%, intravenous ceftriaxone). Duration of therapy was 2 weeks in 25 patients (50%) and 3 weeks in the other half of patients. Overall, EM cleared within a median of 14 days (range 1–126 days) after initiation of antibiotic therapy. In 29/50 patients (58%), it resolved during antibiotic therapy, in 14/50 patients (28%) within 1 month and in 7/50 patients (14%) within 2–5 months after the end of therapy. Extracutaneous signs and symptoms disappeared completely within a median of 8.5 days (range 1–350 days) after initiation of antibiotic therapy in all 19 patients. Resolution was observed in 15/19 patients (79%) during antibiotic therapy, in 3/19 patients (16%) within 1 month after the end of therapy, and persisted for 12 months in 1 patient (5%). Cutaneous or extracutaneous recurrences were not seen in any of the patients within the median observation period of 535 days (range 368–1,156 days).

Immunoblot Results

The IgG IB test results showed an increase in sensitivity from before therapy, where half of the patients were seroreactive to *Bb*sl, to the end of therapy (convalescence phase) and further to 1 month after the end of therapy, where serological testing yielded positive results in more than two thirds of patients (fig. 1). Thereafter, the percentage of patients with a positive IgG IB declined to the end of the follow-up period, but still almost one half of the patients remained seropositive. The decline from the percentage of patients with a positive IgG IB between 1 month after therapy and the end of follow-up was significant, but the proportion of positively tested patients between before therapy or the convalescence phase did not differ significantly from that by the end of follow-up.

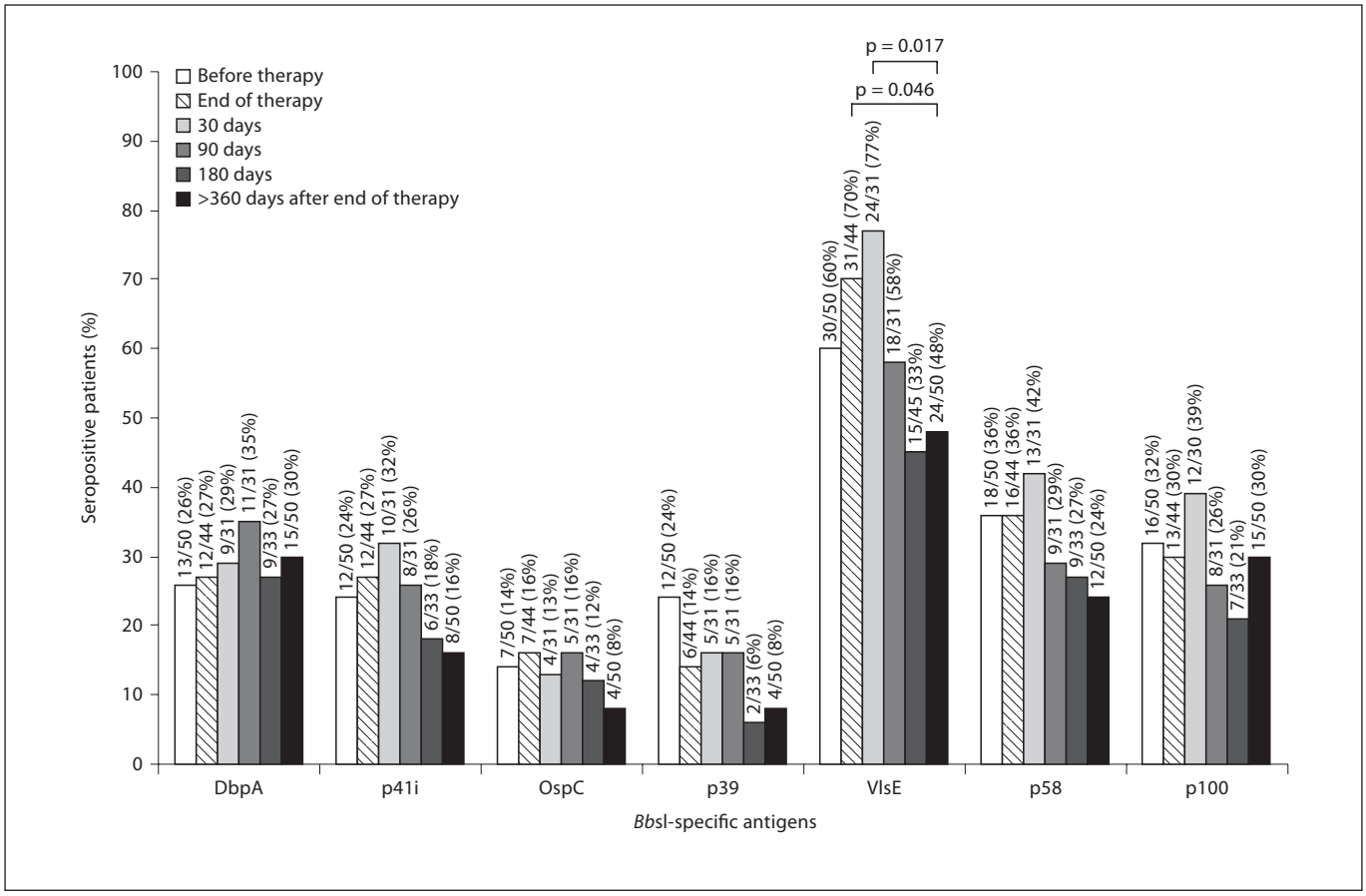

Fig. 2. IB IgG seroreactivity of patients with EM to distinct antigens of *Bb*sl.

The IgM IB showed the same development as the IgG IB until 1 month after therapy. However, significantly more patients were seroreactive before therapy and in the convalescence phase compared to the end of follow-up. At all time points, sensitivity of IgG IB testing was greater than of IgM testing, which was constantly less than 50%. A comparison between IB and ELISA testing revealed a significantly higher sensitivity for IgG IB testing over ELISA testing at all times, whereas for the IgM class, ELISA testing was comparable to the IB with a trend for higher sensitivity.

Subanalysis of IgG seroreactivity to different proteins of *Bb*sl revealed that before therapy antibodies were significantly more often produced against VlsE than against all other proteins ($p \leq 0.028$; fig. 2). The second most immunogenic antigen was p58. However, this protein elicited a significantly greater reactivity only compared to OspC ($p = 0.021$), which had the lowest reactivity with IgG antibodies. Analyses of IgG antibody kinetics revealed that for all different *Bb*sl proteins, with the exception of p39, the number of seropositive patients was greater in the convalescence phase or 1 month after treatment than before therapy. Thereafter, seroreactivity declined to the end of follow-up for most antigens, although significantly only for VlsE. The continuous decline of seroreactivity to p39 from before therapy to the end of follow-up was not significant ($p = 0.056$). Thus, a significant drop of IgG seroreactivity from before therapy to the end of follow-up could not be observed for a single *Bb*sl protein. In IgM analyses, significantly more patients showed antibody production to p41i ($p < 0.001$) or OspC ($p \leq 0.003$) than to p39 before therapy (fig. 3). Also, IgM reactivity to p41i ($p = 0.036$) or OspC ($p = 0.007$) was significantly greater than the respective IgG responses before therapy, whereas IgM reactivity to p39 ($p = 0.193$) was smaller than IgG reactivity. Changes of IgM antibodies to different *Bb*sl proteins over time showed an increase in the percentage of seropositive patients from before ther-

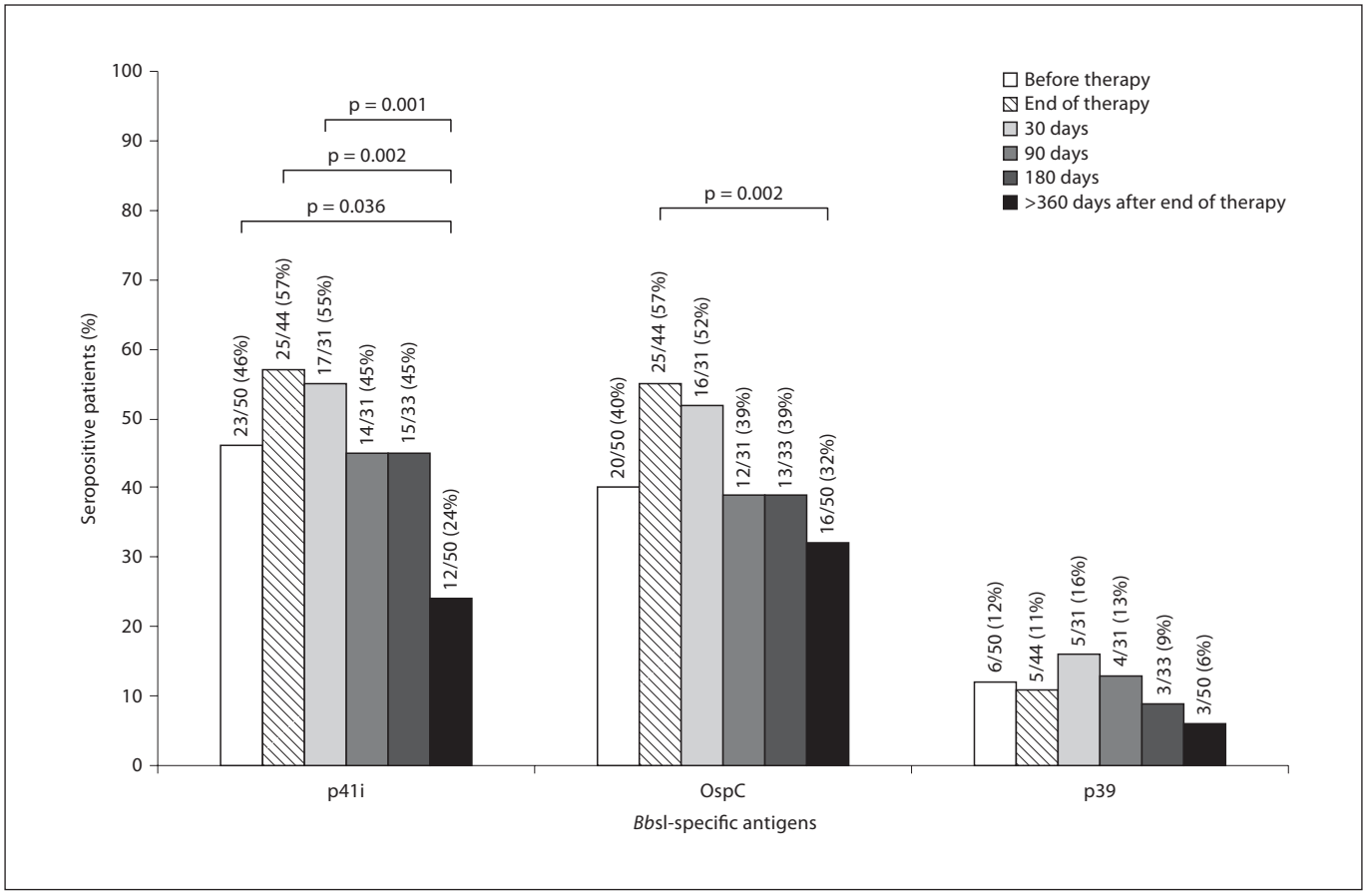

Fig. 3. IB IgM seroreactivity of patients with EM to distinct antigens of *Bbs*l.

apy to the convalescence phase or during the following month for all antigens tested. Afterwards, reactivity to p41i and OspC dropped significantly to the end of follow-up, but only slightly for p39. However, comparing the number of reactive patients before therapy and the end of follow-up, a significant decline was found only for p41i ($p = 0.036$).

Correlation of Clinical Characteristics with Immunoblot Results

Patients with a positive IgG IB result before therapy had a significantly shorter duration of extracutaneous signs and symptoms after therapy than patients with a negative pretreatment IgG IB (median, 5.5 vs. 16 days, $p = 0.040$). In patients with IgG reactivity to the VlsE or p100 antigen before therapy, duration of EM before therapy was significantly longer than in patients with no reactivity to these antigens (VlsE: median, 17.5 vs. 8 days, $p = 0.048$; p100: median, 26 vs. 8 days, $p = 0.049$). Patients with a positive IgM IB or IgM reactivity to p41i alone before therapy were significantly younger than nonreactive patients (total IgM: median, 47 vs. 59 years, $p = 0.036$; p41i: median, 47 vs. 58.5 years, $p = 0.030$). Those with a positive IgM IB also had more often multiple EM lesions (5/13 vs. 1/31 patients, $p = 0.034$). Comparison of the 6 serological constellations with a significant decline of IB results from before or directly after therapy to the end of follow-up (fig. 1–3) with the clinical variables were significant in the following instances: the age of the patients was significantly lower in patients with a declining versus a persistent IgM IB result from before therapy to the end of follow-up (median, 40 vs. 57.5 years, $p = 0.006$) or declining versus persistent anti-VlsE IgG antibodies from directly after therapy to the end of follow-up (median, 46 vs. 58 years, $p = 0.049$). Multiple EM lesions, compared to solitary lesions, were significantly more often found in patients with a significant decline of their IgM IB or of anti-p41i antibodies from before therapy to the end of fol-

low-up (total IgM: 5/9:1/35, p = 0.006; p41i: 4/8:2/36, p = 0.036). None of the treatment-related parameters (type and duration of therapy, duration of EM or extracutaneous symptoms after therapy) was significantly different among patients with declining or persistent IB results.

Discussion

We investigated the long-term IgG and IgM immune response to *Bb*sl and some different proteins in EM patients by IB testing. The immunodominant proteins throughout the 1-year follow-up period were VlsE in the IgG IB and OspC and p41i in the IgM IB. A significant decline of the antibody response over time was only found against those most antigenic proteins, but decline or persistence of serological responses was not correlated with any therapy-related parameter.

Despite the frequent use of serological control examinations of LB patients following antibiotic therapy, there is no larger controlled study that explicitly examined the development of immunoreactivity to different *Bb*sl proteins in EM patients during an extended posttreatment course. In 9 studies [15, 35–42] which have analyzed anti-*Bb*sl antibodies over time in patients with various manifestations of LB by IB tests, a total of 382 subjects with EM were included. In these investigations, the follow-up was limited to the convalescence phase or subgroups of study patients [36, 38, 40, 41], serological responses were either not discriminated by IgG or IgM antibodies [36, 41] or different specific *Bb*sl proteins [15, 37, 40, 41] or patients were not seronaïve [15]. Of importance, correlations of IB results with clinical parameters after therapy have been performed in only two American studies with a total of 61 patients [35, 42]. The present study is the first to analyze long-term IgG and IgM seroreactivity to *Bb*sl and its immunodominant proteins in EM patients before and after therapy and its correlation with a comprehensive set of clinical and therapy-related parameters. The recombinant test system employed in this study combines several advantages. As only specific antigens of the 3 major pathogenic *Bb*sl species present in Europe are included in the assay, one test indicates all possible infections and allows for exact identification of the respective antigens on the IB strips. In contrast, whole-cell lysate tests which are capable of covering infections with different *Bb*sl species as used in earlier studies [40, 41] require additional application of monoclonal antibodies to exactly identify the various antigenic proteins [43]. In addition, primarily in vivo expressed antigens, like VlsE, OspC and DbpA, are often not present in sufficient amounts in cultured borreliae [44]. Very recently described borrelial antigens, such as BB0323 and complement regulator-acquiring surface protein 3, increase the sensitivity of IB investigations in neuroborreliosis or Lyme arthritis but not in EM [45]. So far, no data exist about kinetics of serological responses to these antigens after therapy. Future studies have to show whether analyses of these antibodies over time could add information to the clinical assessment after therapy.

In general, our IB test was significantly more sensitive than the ELISA in detecting IgG *Bb*sl antibodies during the whole observation period, whereas the IgM ELISA was comparable to the IgM IB with a trend for higher sensitivity (fig. 1). Differences in sensitivities of the two methodologies have been repeatedly described [36, 38, 46, 47] and may be due to the time into infection or differences in antigen composition and/or concentration between the assays. In case of IgG, the better reactivity of the IB is apparently due to VlsE which is not a test antigen in our ELISA. ELISA tests based on C6, a peptide representing an invariable region of VlsE, reach a sensitivity of up to 60% [48] which is well comparable to the sensitivity of our recombinant IB test.

In IB investigations at baseline, IgG reactivity was observed most frequently to the VlsE antigen followed by antibody production to the p58 and the p100 antigen (fig. 2). For each of the other antigens tested (DbpA, p41i, OspC, p39), antibodies were detected in only a quarter of patients or less. The same distribution of IgG antibody positivity (VlsE > p58 > p100) was observed in the convalescence phase as well as 1 month after therapy. IgM antibodies at baseline as well as in the convalescence phase and 1 month after therapy were most frequently observed against the p41i and OspC antigens, whereas antibodies against p39 were present in a minority of patients only (fig. 3). The IgM response to the p41i protein preceded the respective IgG response, but a converse IgG/IgM reactivity was observed for the p39 protein, whereas peak seroreactivity to OspC occurred at the same time after baseline for the IgG as well as the IgM class (fig. 2, 3). In previous IB studies, the frequencies of individual reaction bands at baseline vary widely depending on the applied test system (whole-cell lysate vs. recombinant assay) and differences of clinical parameters of the investigated patients, particularly disease duration [32, 38, 39, 43, 49]. IgG (or IgM) antibodies to p41 before therapy were present in 7–94% (19–82%) of patients, to OspC in 6–77% (41–84%), p39 in 2–85% (0–84%), p58 in 2–54% (2–48%), DbpA in 2–53% (2–7%) and p100 in 0–27%. The

ranking of seroreactivity to these antigens remained the same in the convalescence phase [38, 39]. The percentages of patients who tested positive for the respective antigens at baseline and in the convalescence phase in our study were within the reported ranges. Also, the most sensitive antigens on IgM testing in our study (p41i, OspC) complied with those from the literature [24, 32, 38, 39, 43, 49]. In contrast, the most sensitive antigens on IgG testing were different between our study and former investigations, in which p41 turned out to be most sensitive. The difference may be explained by the use of the whole flagellin (p41) as test antigen in former studies [38, 39, 49], which is relatively unspecific and cross-reactive, and elicits an immune response in a large proportion of patients [17, 50]. In contrast, the *Bb*sl-specific internal fragment of this protein (p41i) was applied in our study, to which only few patients develop antibodies [32, 49]. IgG seroreactivity of EM patients to VlsE, an immunodominant surface protein of *Bb*sl, first introduced in borrelial diagnosis in 1999 [51], has been investigated by IB analysis only once [32]. As in our study (sensitivity of 60%), in this series of 15 untreated EM patients, VlsE was the most reactive protein (80%). Several IgG ELISA or chemiluminescence immunoassay-based investigations confirm the high sensitivity of antibodies to VlsE or its immunodominant conserved internal region C6 in EM patients before therapy (up to 80% of patients) [52–55]. Thus, IgG antibodies to VlsE and IgM antibodies to OspC and p41 appear to be the most sensitive markers in IB testing in early LB.

Regarding long-term development of anti-*Bb*sl antibodies, we found that IgG as well as IgM seroreactivity over the 1-year follow-up period displayed a uniform decrease following the initial rise during and shortly after therapy. This decline was significant only for IgM reactivity, whereas almost half of the patients remained seropositive on IgG testing during the whole follow-up period. The rise of IgG or IgM seroreactivity during therapy or the persistence of IgG antibodies in IB tests after 1 year of follow-up might be interpreted as a sign of failure of antibiotic therapy. However, correlations of serological courses with a comprehensive set of clinical parameters before and after therapy did not show any relationship between serological test results and clinical outcome of patients. A comparable peak of positive serological results by the end of therapy or shortly thereafter followed by a fall over the next 12 months, but still with one third to half of patients being seropositive, has been described in other studies [38, 39]. There are even descriptions of longer persistence [35, 42] and/or higher percentages [35] of IB positivity in EM patients. In accordance with our results, patients with direct postbaseline seroconversion or long-term persistence of antibody production had no distinct clinical features in the investigations by Aguero-Rosenfeld et al. [39] and Feder et al. [35]. Except for these two studies, IB follow-up results were not correlated with clinical characteristics elsewhere. Furthermore, it has been shown that antibiotic therapy of early LB does not necessarily suppress the development of antibodies to *Bb*sl [56]. It could rather be speculated that augmented presentation of *Bb*sl proteins to the host's immune system due to spirochetal disruption by an antimicrobial results in an enhanced antibody response. Thus, an increase in antibody titers during or shortly after treatment or long-term serological positivity should not be interpreted as evidence for inefficiency of the antibiotic therapy.

In line with the literature [38, 39] and consistent with the maximum total IB reactivity during or shortly after therapy and its consecutive partial decline in our study, IgG as well as IgM reactivity to almost all separately analyzed proteins of *Bb*sl exhibited analogous peaks around therapy and a drop of the numbers of reactive patients' sera between 1 and 3 months afterwards. Of importance, the long-run decline (pretreatment period to the end of the 1-year follow-up) of IgG and IgM seroreactivity to the different *Bb*sl proteins was not significant with one exception. More patients recognized the p41i protein fragment before therapy than by the end of follow-up in IgM tests. Further significant differences in antibody kinetics were only observed when convalescence phase results were compared with the end of follow-up, namely for IgM anti-OspC and for IgG anti-VlsE antibodies. Three earlier studies with a total of about 100 EM patients [35, 39, 42] have analyzed the development of immunoreactivity detailed by separate *Bb*sl proteins over 1 year or more. In general, only a slight decrease in IgM and even less of IgG seroreactivity to various distinct proteins, including DbpA, OspC, p39, p41, p58 and p100, was observed from before therapy to the end of follow-up. Use of the whole p41 antigen may be responsible for the persistent anti-flagellin seroreactivity in those studies compared to the significant decrease in antibody production to the more specific truncated p41i protein as used in our study. In accordance with these results, the few studies investigating seroreactivity by IB after treatment in other manifestations of LB, in particular Lyme arthritis, showed that reaction bands remained essentially unchanged for even many years [35, 42].

For the first time, this study shows by immunoblotting persistence of anti-VlsE antibodies for a longer period of

time, irrespective of the clinical outcome. ELISA and chemiluminescence immunoassay studies on the course of antibodies to VlsE or its C6 peptide gave contradicting results of either a fourfold or greater decline of titers or persistence of the antibody response for months or years after antibiotic treatment [55, 57, 58]. Collectively, IgG and IgM immunoreactivity to separate *Bb*sl proteins as analyzed by IB appears to show a uniform course with a transient peritherapeutic peak, followed by a mostly insignificant drop of antibody production resulting in long-term persistence of seroreactivity.

So far, only very few studies have specifically addressed correlations of clinical characteristics and IB results in patients with EM. To investigate whether IB results can facilitate clinical decisions, particularly regarding the efficiency of antibiotic therapy, we compared a comprehensive set of clinical variables with IB results in the present study. We found that patients with longer disease duration before therapy more often produced anti-VlsE and -p100 IgG antibodies. In accordance, Aguero-Rosenfeld et al. [36, 39] found a higher percentage of seroreactivity to all *Bb*sl antigens investigated, including p100, among patients with EM of longer disease duration. We and others have found the same phenomenon by ELISA testing [9, 39]. In the study by Aguero-Rosenfeld and coworkers, patients with longer disease duration also remained positive for a longer period of time in IgM IB analyses [39]. It thus seems that the probability for a positive pretreatment IB result and persistence of an anti-*Bb*sl immune response is higher in case of a longer pretreatment interaction between the host's immune system and the spirochete. We also found that younger age of patients was more frequently associated with a positive pretreatment IgM IB result and seroreactivity to the p41i antigen, and a more frequent VlsE-specific IgG IB after therapy. In ELISA investigations of patients with EM [9], elder patients had a higher probability to remain persistently seronegative. Thus, younger patients seem to develop a more vigorous immune response to *Bb*sl. Finally, multiple EM lesions were correlated with a positive total IgM IB result before therapy. Also other authors have described – based on IB and/or ELISA testing – that patients with disseminated EM are more prone to produce antibodies to *Bb*sl at baseline [39, 59]. Of importance, neither the type of therapy nor outcome-related parameters were significantly correlated with any serological constellations in IB testing before or after therapy in the present study. Consistent with these results, in another series of 32 patients with EM persistent or recurrent symptoms were not correlated with distinct IB patterns on a follow-up investigation after a median of 16 months [35]. Indirect support of these findings comes from a study by Plorer et al. [60], who did not find differences in IB patterns among adequately or inadequately treated patients with EM on a single endpoint IB test up to 8 years after the disease. Thus, as with ELISA tests [9, 35], follow-up IB analyses of anti-*Bb*sl antibodies are not helpful to control for the efficiency of antibiotic treatment.

In conclusion, IgG and IgM immune responses to different antigenic proteins of *Bb*sl in patients with EM after antibiotic therapy show a uniform temporary peak followed by long-term persistence of seroreactivity. No correlation was found between serological courses and type or duration of antibiotic therapy or clinical course after therapy. Thus, as with ELISA testing, IB analyses in the follow-up period of EM do not add to the clinical assessment of treatment response.

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