Recombinant or Peptide Antigens in the Serology of Lyme Arthritis in Children

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Lyme arthritis (LA) is a manifestation of disseminated Lyme borreliosis (LB) [1, 2] and is caused by the spirochete Borrelia burgdorferi sensu lato. The diagnosis of disseminated LB is clinical, but laboratory confirmation is often needed. For clinical practice, both culturing B. burgdorferi from clinical samples other than erythema migrans lesions [3] and performing the polymerase chain reaction (PCR)-based detection methods are disappointingly insensitive [4, 5]. The mainstay of laboratory diagnosis for LB has been serological assays—ELISA and Western blotting—of antibodies to B. burgdorferi species, although the performance of these assays, in terms of their sensitivities and specificities, is highly variable [6–8]. ELISA with either whole cell lysates or, in Europe, flagella, as antigen, is widely used as a screening test. In the standard 2-tiered approach, Western blotting is used to confirm the specificity of the antibodies, but it is not without shortcomings. Western blotting is a labor-intensive laboratory procedure prone to subjective interpretation of band intensities. In European conditions, the 3 borrelial species—B. burgdorferi sensu stricto, B. afzelii, and B. garinii—as causative agents of LB, further complicate reliable interpretation of Western blots [9].

In recent years, several recombinant borrelial proteins have been tested as targets for serodiagnosis of LB [10–14]. Some of them have been shown to be sensitive and specific antigens, but none of the reported proteins has proved superior to those used in the current assays. Some of the new antigens have been used in commercial ELISAs or Western blots [15], but the usefulness of these assays in clinical practice has not been thoroughly evaluated. In a recent study, Liang et al. [16] showed that an ELISA based on a peptide antigen corresponding to the invariable region 6 (IR6) of the borrelial VlsE protein has high sensitivity and specificity. We have recently evaluated the borrelial recombinant proteins BBK32, decorin-binding protein A (DbpA), and DbpB, as antigens in the serology of LB [12–14]. In preliminary analyses, these antigens have shown potential as sensitive and spe-
specific serodiagnostic targets. However, sequence heterogeneity of variant proteins between B. afzelii, B. garinii, and B. burgdorferi sensu stricto implies that, in the serology of LB, >1 variant of these protein antigens may be needed to cover all relevant pathogenic borrelial species [12–14].

The purpose of the present study is to evaluate the usefulness of recombinant antigens BBK32, DbpA, and DbpB and a peptide antigen IR₆ in the serodiagnosis and follow-up of well-characterized patients with LA, and to compare the performance of these antigens in ELISAs with the performance of flagella as antigen in ELISAs.

**SUBJECTS, MATERIALS, AND METHODS**

**Serum samples.** A series of serum samples was collected from a group of German children with LA (age range, 2–17 years; median age, 12 years; 32 boys and 20 girls) in whom LA had been diagnosed in a national study [2]. The diagnosis was based on articular symptoms, elevated IgG antibodies to whole cell lysate in ELISA, and a confirmatory Western blot with whole cell lysate antigen [2]. Of the 62 patients with LA who were enrolled in the original study [2], sequential samples were available from 52 patients. For 43 patients, the first serum sample was obtained at the time of diagnosis of active LA, and 2–4 convalescent samples were obtained during follow-up. For the remaining 9 patients, the first available sample was drawn 6–12 months after the diagnosis of LA. At diagnosis, the clinical course of the 43 patients was defined as acute (n = 9) if there was a single episode of arthritis, as episodic (n = 17) if there were ≥2 episodes of self-limiting arthritis, or as chronic (n = 17) if the arthritis had persisted for ≥3 months [2]. All patients were treated with antibiotics. After the LA diagnosis was established, 47 of the 52 patients were intravenously treated with ceftriaxone for 2 weeks, and the remaining 5 patients were treated with penicillin, cefuroxime, roxithromycin and cotrimoxazole, doxycycline, or clindamycin. Twenty-six patients with episodic or chronic arthritis received 2–5 courses of antimicrobial therapy. From the same national study, serum samples from 22 children (age range, 1–15 years; median age, 9 years; 8 boys and 14 girls) with other arthritides (juvenile rheumatoid arthritis and reactive arthritis) were used as controls. We also used serum samples from 20 healthy Finnish blood donors as negative controls.

**Recombinant antigens.** DbpA, DbpB, and BBK32 each originated from 3 different pathogenic borrelial species, B. burgdorferi sensu stricto strain IA, B. afzelii strain A91, and B. garinii strain 40. The organisms were initially isolated from Finnish patients with LB [12]. The cloning of the genes and the production and purification of the respective recombinant proteins have been described elsewhere [12–14].

**ELISA.** Flagella ELISA (K0416 kit; DAKO) was performed according to the manufacturer’s instructions. DbpA, DbpB, and BBK32 ELISAs were performed as described elsewhere [12–14]. In brief, separate microtiter plates were coated with 100 μL of 2 μg/mL variant recombinant proteins. From each of these recombinant proteins, 3 variant antigens were used in parallel. The samples were diluted 1:100 in PBS. The secondary antibody was alkaline phosphatase–conjugated rabbit anti-human IgG (Jackson Immuno Research Laboratories) at a 1:5000 dilution. As substrate, 1 mg/mL 4-nitrophenylphosphate (Boehringer Mannheim) at pH 10.0 was used. Optical density (OD) values were obtained at 405 nm by use of a Multiscan photometer (Thermo Labsystems).

The IR₆ peptide of the VlsE protein, corresponding to the IR₆ sequence of B. garinii IP90 strain [16], was produced, as amino terminally biotinylated synthetic peptide, at the Core Facility of the Haartman Institute, University of Helsinki. For IR₆ ELISA, the microtiter plates were first coated with 100 μL of 1 μg/mL recombinant streptavidin (Roche) overnight. The plates were postcoated with 5 mg/mL human serum albumin in PBS. Biotinylated IR₆ at 20 ng/well, in PBS with 0.04% Tween 20 buffer was added to the wells. Background wells with streptavidin, but without IR₆, were included. The samples were diluted 1:100 in 0.5% human serum albumin with 0.04% Tween 20 buffer. The subsequent steps were performed as described above. The cutoff levels for all ELISAs were calculated as means + 3SD of the serum values of the blood donors. The data were expressed as ratios of sample ODs, divided by the cutoff ODs (OD/cutoff). OD/cutoff ratios >1 were considered positive.

**Sensitivity and specificity of ELISAs.** The specificities of DbpA, DbpB, and BBK32 ELISAs have been analyzed elsewhere with serum samples from patients with syphilis (n = 10), systemic lupus erythematosus (n = 8), rheumatoid factor positivity (n = 8), anti-streptolysin O positivity (n = 8), or Epstein-Barr virus infection (n = 10), and from healthy blood donors (n = 20) [12–14]. Total specificities with these samples were as follows: 98% with DbpA, 93% with DbpB, and 93% with BBK32. In the present study, the sensitivities and specificities of ELISAs with various recombinant antigens were calculated for each variant antigen. The figures were also combined for total sensitivity and specificity results. In these calculations, each serum sample was defined as positive if it recognized any of the 3 variant recombinant antigens.

**Statistical methods.** The antibody levels at diagnosis and during the follow-up were analyzed by analysis of variance. A graphic presentation of the antibodies during follow-up was created by a locally weighted scatterplot smoother method [17].

**RESULTS**

**IgG antibodies to different antigens.** The first serum samples obtained from each patient were analyzed in IgG ELISAs with 11 different antigens (flagella, IR₆, and 3 variant recombinant...
proteins from each of BBK32, DbpA, and DbpB). Use of variants of the BBK32, DbpA, and DbpB proteins increased the number of positive samples (table 1). The differences in immunoreactivities were probably due to the sequence heterogeneity of the variant proteins [12–14]. The positivity for a given recombinant antigen in subsequent analyses was based on the highest OD/cutoff value. Of the 52 patients, 50 (96%) were positive when BBK32 was used as an antigen; 51 (98%), 50 (98%), and 52 (100%) were positive when DbpA, DbpB, IR6, and flagella, respectively, were used (figure 1). Thirty-nine (75%) of the 52 patients with LA had antibodies to all tested antigens—DbpA, DbpB, BBK32, and IR6. In 10 additional cases, serum samples were positive for 3 of the 4 antigens. Of these 10 patients, 9 were negative for DbpB. The highest OD/cutoff ratios were observed for IR6 (figure 1). Of the 42 control subjects, 4 (10%) were positive for DbpB, 4 (10%) for BBK32, 3 (7%) for DbpA, 1 (2%) for flagella, and 0 for IR6. All positivities in control subjects were of low degree (figure 1). The overall specificities of the ELISAs were 90%, 93%, 90%, 100%, and 98%, for BBK32, DbpA, DbpB, IR6, and flagella, respectively.

**IgG antibodies during follow-up.** Of the 43 patients from whom serum samples at diagnosis were available, antibody levels during the posttreatment follow-up were analyzed against DbpA, DbpB, BBK32, IR6, and flagella. For the antibodies to DbpA, DbpB, and BBK32, results obtained with the same variant antigen were used throughout the follow-up. The antibody levels in DbpA and BBK32 ELISAs were higher at diagnosis in patients with acute LA than in those with episodic or chronic LA ($P = .001$ and $P = .030$, respectively). In ELISAs with DbpB, IR6, and flagella, no significant differences were observed between the patient groups ($P = .566, P = .531$, and $P = .736$, respectively). During follow-up, all antibody levels waned slowly in patients with acute, episodic, and chronic LA (figure 2). For any of the antibodies analyzed, the rate of decline did not differ significantly between the patient groups.

Of the 9 patients with acute LA, at the convalescent phase, antibody levels to IR6, DbpA, and flagella that were either higher than or at the same level as those at diagnosis were observed in 1 (11%), 2 (22%), and 0 patients, respectively; of the 17 patients with episodic LA, in the first sample after treatment, antibody levels to IR6, DbpA, and flagella that were higher than or at the same level as those at diagnosis were observed in 3 (18%), 6 (35%), and 5 (29%) patients, respectively; and, of the 17 patients with chronic LA, in the first sample after treatment, antibody levels to IR6, DbpA, and flagella that were higher than or at the same level as those at diagnosis were observed in 4 (24%), 6 (35%), and 4 (24%) patients, respectively. During further follow-up of the 9 patients with acute LA, the antibody levels to IR6 remained positive in 6 (67%) patients, both at 2 years and after 4 years. In flagella ELISA, the antibodies remained positive in 8 (89%) patients at 2 years and in 4 (44%) patients after 4 years. For DbpA, the antibodies remained positive in all 9 patients at 2 years and in 7 (78%) patients after 4 years.

The mean OD/cutoff values at diagnosis were 1.75 for DbpB and 1.67 for BBK32. The rate of decline of anti-DbpB and anti-BBK32 antibodies during follow-up was slow, with no differences between patient groups (data not shown).

**Species-specific antibodies.** Antibodies to the 3 variant DbpAs showed a distinct species-specific pattern. Of the 51 anti-DbpA–positive samples, 25 (49%) reacted with 1 antigen only; 10 samples reacted with DbpA from *B. afzelii,* 14 samples reacted with DbpA from *B. garinii,* and 1 sample reacted with DbpA from *B. burgdorferi* sensu stricto. In the remaining 26 DbpA–positive serum samples, antibodies were observed against 2 or 3 DbpA variants. In 13 of these 26 serum samples, the OD/cutoff ratio was twice as high against 1 antigen as it was against the other 2 antigens; the origin of the antigen with the highest OD/cutoff was *B. afzelii* in 7 patients, *B. garinii* in 5 patients, and *B. burgdorferi* sensu stricto in 1 patient. In the remaining 13 patients, the antibody levels for all 3 variant DbpA antigens were indistinguishable. In total, in 38 (73%) of 52 serum samples, the antibody levels against the DbpA variant antigens suggested that the infective borrelian species was *B. afzelii* in 17, *B. garinii* in 19, and *B. burgdorferi* sensu stricto in 2 patients. In 14 (61%) of 23 patients with chronic LA, the infective species appeared to be *B. garinii,* whereas, in patients with acute and with episodic LA, the proportions of *B. garinii* as the putative causative agent

| Table 1. Sensitivity and specificity of IgG ELISA with recombinant decorin-binding protein A (DbpA), DbpB, and BBK32 variants, as antigens. |
|-------------------------|-------------------------------|------------------------------|---------------------|------------------------------|-------------------|------------------------------|
| Origin of antigen      | DbpA                          |                              | DbpB                |                              | BBK32             |                              |
|                        | Sensitivity | Specificity | Sensitivity | Specificity | Sensitivity | Specificity |
| *Borrelia* species     |                          |                              |                      |                          |                  |
| *afzelii*              | 36 (69)   | 40 (95)    | 20 (38)    | 42 (100)   | 50 (96)   | 38 (90)      |
| *garinii*              | 38 (73)   | 39 (93)    | 23 (44)    | 41 (98)    | 43 (83)   | 41 (98)      |
| *burgdorferi*          | 19 (37)   | 40 (95)    | 30 (58)    | 38 (90)    | 11 (21)   | 42 (100)     |
| Any species            | 51 (98)   | 39 (93)    | 40 (77)    | 38 (90)    | 50 (96)   | 38 (90)      |

**NOTE.** Data are no. (%). Sensitivity was analyzed in serum samples from 52 patients with Lyme arthritis. Specificity was analyzed in serum samples from 42 control subjects, including 22 children with other arthritides and 20 healthy blood donors.
Figure 1. IgG antibody levels determined by ELISA (optical density [OD]/cutoff), by use of recombinant BBK32, decorin-binding protein A (DbpA), DbpB, peptide IR6, and purified flagella (Fla), as antigens. Serum samples were obtained from 52 children with Lyme arthritis (LA) and from 42 control subjects (C) (22 children with other arthritides and 20 healthy blood donors). The horizontal line indicates OD/cutoff value 1 (cutoff = mean + 3 SDs of samples from healthy blood donors).

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were 1 (10%) of 10 and 4 (21%) of 19, respectively. Antibody levels to variant BBK32 and DbpB antigens were mostly equal, a finding providing no further clues to the infecting species (data not shown).

DISCUSSION

In the present study of serologically preselected patients with LA, both the recombinantly produced borrelial protein antigens and a synthetic IR6 peptide antigen seemed to perform well. The results from this study, which are consistent with recent studies from our group and from others [12, 13, 16], show that especially IR6 and also DbpA and BBK32 have potential as serodiagnostic targets for LB. In the present study, IR6 had the highest discriminatory power between samples from patients with LA and from control subjects. Sensitivity with DbpA and BBK32 as antigens seems to be equal to that of the commercial reference ELISA with the flagella antigen, provided that variants from B. afzelii, B. garinii, and B. burgdorferi sensu stricto are included in the antigen set.

IR6 has recently been shown to be highly sensitive and specific in the serology of LB [16]. This was also observed in our series. Philipp et al. [18] have suggested that antibodies to IR6 could also be used as markers for the treatment response in LB. In our study, antibody levels to IR6 did not seem to predict the clinical course of LA. The rate at which the anti-IR6 antibodies declined failed to differentiate patients with acute LA from those with an episodic or chronic course of LA. Moreover, the majority of patients were still positive for IR6 even 4 years after diagnosis. This indicates that individual elevated anti-IR6 antibodies cannot be used as markers of disease activity. The reason for the discrepancy between the present study’s results and those reported by Philipp et al. [18] is not known. Mechanisms for a chronic or treatment-resistant course of LA may, however, differ in the United States and Europe. It is not known at present which borrelial species in Europe are responsible for the chronic course of LB, but the only pathogenic species prevalent in the United States is B. burgdorferi sensu stricto. It is also possible that the course of treatment-resistant LA may differ in children and adults. Nevertheless, our results imply that, in children with LA, antibodies to IR6, or to DbpA, DbpB, or BBK32 do not appear to be useful as markers for either disease activity or response to therapy.

The obvious explanation for the need for variant recombinant antigens is the sequence heterogeneity of borrelial proteins in isolates of B. burgdorferi sensu lato [12–14]. In keeping with our previous results [12], antibody responses to DbpA variant antigens might be used for species-specific serodiagnosis. In Europe, this information may be useful, at least for epidemiological purposes. Because some borrelial strains or species are associated with more invasive or severe clinical manifestations [19], knowledge of the infective species might also have therapeutic implications. If vaccines against Borrelia species are to be designed, it is crucial to know the prevalent infecting species. Thus, from the standpoint of serology and vaccine development, the interspecies variation of DbpA implies that a polyvalent antigen is needed to
cover all of the relevant pathogenic borrelial species. The prevalence of species-specific immune reactions in this series seems to reflect the European epidemiological situation in general [20]. In other studies, conflicting results on the causative borrelial genospecies of LA in Europe have been published [21–24]. PCR studies of synovial fluid or synovial tissue have demonstrated either that the causative species is almost exclusively *B. burgdorferi* sensu stricto [21, 22] or that there is no predominance of certain genospecies causing LA [23, 24]. It can be speculated that the variable results of these rather small studies may reflect epidemiological situations in different regions or methodological differences. The present study provides indirect evidence that LA can be caused by all 3 pathogenic borrelial species. Of interest, species-specific immune reactions suggest that *B. garinii* may be associated with the chronic course of LA more frequently than are other species. Although serological evidence is only indirect, further studies on the interaction of *B. garinii* and immunologic mechanisms in humans with chronic Lyme disease are warranted.

Akin et al. [25] have studied BBK32 antibodies in disseminated LB and have suggested that high anti-BBK32 levels in IgG ELISA might correlate with mild and brief arthritis. Our results seem to concur with those of Akin et al., because patients with high levels of anti-BBK32 antibodies at diagnosis had a single episode of arthritis (acute LA). A similar association was observed with anti-DbpA antibodies. However, in the present study, the possibility that, in patients with episodic and chronic
who had longer duration of symptoms before diagnosis and antibiotic treatment, the antibodies measured at diagnosis may have decreased from higher initial levels cannot be overlooked. Alternate to that, as suggested by Akin et al. [25], a marked antibody response to a given protein early in the course of illness may reduce the number of spirochetes in the joints, resulting in milder arthritis and shorter duration of symptoms.

The persistence of borrelial antibodies for years after active LA, shown here, emphasizes the difficulty in distinguishing active infection from previous immunity, a problem that has recently been discussed by Kalish et al. [26]. They reported that up to 62% of patients who had had LA 10–20 years earlier still had IgG immune reactivity without active infection [26]. Our results were consistent with their finding. Even in the majority of apparently cured patients who had had a single episode of arthritis, antibodies were still detectable for >4 years. Moreover, irrespective of the clinical course, the rates of decline in antibody levels in patients with acute, episodic, or chronic LA did not differ. Thus, these results might support the hypothesis that, in children with treatment-resistant LA, the chronic course may be due to immunologic factors contributing to the prolongation of LA [27]. Gross et al. [28] have suggested that autoimmune mechanisms may explain why some patients with LA do not show a response to therapy. Similar to that, a recent study showed that patients with persistent symptoms of Lyme disease do not benefit from prolonged antibiotic treatment [29], a finding supporting the theory that immunologic mechanisms are operative during the chronic course of LA.

In conclusion, especially IR, but also DbpA and BBK32 seem to have diagnostic potential as antigens in the serology of LB. In the future, it is essential to test these antigens in clinical practice in an unselected population among whom, in healthy persons, cross-reactive antibodies and elevated levels of anti-borrellial antibodies, especially in regions of endemicity, complicate the interpretation of the currently used serological assays [7, 26]. The present results suggest that, of the tested antigens, IR, may have the greatest potential to be used universally in the serology of LB. Another option would be the use of several specific borrellial antigens, in parallel, to improve the accuracy of serology of LB.

Acknowledgments

We thank Hannu Kautianen for statistical assistance and Jean Margaret Perttunen for reviewing the English.

References

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