Enzyme-Linked Immunosorbent Assay Using Recombinant OspC and the Internal 14-kDa Flagellin Fragment for Serodiagnosis of Early Lyme Disease

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The outer surface protein C (OspC) and the internal 14-kDa flagellin fragment of strain GeHo of Borrelia burgdorferi sensu stricto were expressed as recombinant proteins in Escherichia coli and were purified for use in an immunoglobulin M (IgM) enzyme-linked immunosorbent assay (OspC–14-kDa antigen ELISA). No hint at disturbing protein-protein interferences, which might influence the availability of immunoreactive epitopes, was found when the recombinant antigens were combined in the ELISA. The recombinant OspC–14-kDa antigen ELISA was compared to a commercial IgM ELISA that used a detergent cell extract from Borrelia afzelii PKo as the antigen. According to the manufacturer’s information, the cell extract contains, in addition to other antigens, the following diagnostically relevant antigens: the 100-kDa (synonyms, 93- and 83-kDa antigens), 41-kDa, OspA, OspC, and 17-kDa antigens. The specificity was adjusted to 95% on the basis of data for 154 healthy controls. On testing of 104 serum samples from patients with erythema migrans (EM), the sensitivity of the recombinant ELISA (46%) for IgM antibodies was similar to that of the commercial ELISA (45%). However, when 42 serum samples from patients with polyclonal B-cell stimulation due to an Epstein-Barr virus infection were tested, false-positive reactions were significantly less frequent in the recombinant ELISA (10%) than in the whole-cell-extract ELISA (23%). OspC displays sequence heterogeneity of up to 40% according to the genomospecies. However, when the reactions of serum specimens from controls and EM patients with OspC from representative strains of B. burgdorferi sensu stricto (strain GeHo) and B. afzelii (strain PKo) were compared in an ELISA, almost no differences in specificity and sensitivity were seen. This demonstrates that the sera predominantly recognize the common epitopes of OspC tested in this study. In conclusion, we suggest that the OspC–14-kDa antigens ELISA is a suitable test for the detection of an IgM response in early Lyme disease.

Serological testing is the most common way of confirming a clinical diagnosis of Lyme disease (12, 29). Indirect fluorescent-antibody staining or enzyme-linked immunosorbent assays (ELISAs) with whole cells or extracts thereof as antigens have adequate sensitivity but are affected by a lack of specificity (3, 7, 21). This is due mainly to the reactivity of conserved antigens like heat shock proteins in the range of 60 to 70 kDa and parts of the 41-kDa flagellin, which cross-react with those of other bacterial species (4, 19). Western blotting (WB) performed with whole-cell lysates or recombinant antigens of Borrelia burgdorferi offered the advantage of being able to differentiate between specific and nonspecific bands. Some investigators claim that WB with either native (5, 18, 31, 36, 37) or recombinant (33, 34) antigens is sufficiently sensitive and specific. WB on the other hand, is a nonquantitative method and difficult to standardize, resulting in some controversy on interpretation criteria (5, 6, 14, 18, 38). Results are subjective, particularly in cases of faint bands. Comigration of different antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as the potential loss of conformational epitopes are limitations of immunoblotting. ELISAs performed with isolated borrelial antigens showed increased specificity. Hansen et al. (13) established an ELISA with flagellins isolated from B. burgdorferi. That ELISA appears to be, in terms of diagnostic sensitivity and specificity, superior to WB (13, 16, 22). We have reported on a highly specific borrelia immunoglobulin G (IgG) ELISA performed with recombinant p83; this assay has value for the serodiagnosis of advanced-stage Lyme disease (26). Such assays are appropriate alternatives to WB because they are quantitative and easy to standardize. In addition, they can be performed more conveniently and less expensively than WB.

In the early stages of Lyme disease, IgM antibodies are predominantly reactive with the outer surface protein C (OspC) or the flagellin of B. burgdorferi, or both (2, 5, 6, 9, 36). In some of these patients the antibody response is also directed against the 39-kDa protein (6, 14). Epitope mapping of the B. burgdorferi flagellin indicated that the internal region of the molecule comprises the variable, genus-specific immunodominant domains (10, 17). Rasiah et al. (25) purified and characterized from this central region a tryptic 14-kDa peptide which reduces cross-reactivity in immunoblots and ELISA.

There is broad consensus in the literature that OspC is a specific and an IgM-sensitive antigen that can be used for testing (2, 6, 20, 23, 34). It displays sequence identity ranging from 60.5 to 77% among the three delineated genomospecies (15, 30, 35). The objective of this study was to establish a combined IgM ELISA with recombinant OspC and the internal 14-kDa flagellin fragment. Its suitability for routine diagnostic use was evaluated, and it was compared to a proven commercial IgM ELISA that uses a detergent cell extraction.
from *Borrelia afzelii* as the antigen. In addition, we tested whether recombinant OspCs from two different borrelial genomicspecies produced in the IgM ELISA disrepe nt results due to sequence heterogeneity.

**MATERIALS AND METHODS**

**Bacterial strains.** *B. burgdorferi* sensu stricto (strain GeHo; passage number, 28; provided by K. Pelz, Freiburg, Germany) was grown in modified BSK medium at 33°C for 4 days. Spirochetes were centrifuged at 5,000 × g for 15 min at 20°C, and solution of DNA was done as described previously (1). *Escherichia coli* HB101 was used for transformation and expression of the recombinant OspC. *E. coli* HB101 was used for expression of recombinant flagellin.

**Construction of recombinant OspC.** Recombinant OspC was constructed as a nonfusion protein by methods previously described for other borrelial antigens (24). The oligonucleotides used for the amplification of the entire open reading frame of OspC by PCR were derived from published sequences (8, 30) (sense primer, 5′-AGGCCAAATCCATGGAAAAGAATACA-3′; the NcoI site is underlined); antisense primer, 5′-CTTATAATATGATCTTAAATAGATT-3′ [the BamHI site is underlined]). PCR products were ligated into the expression vector pJLa602 (deilmac) by standard methods.

**Expression and purification of recombinant OspC from *B. burgdorferi* GeHo.** E. coli transformed with recombinant *B. burgdorferi* Luria broth medium (GIBCO) containing ampicillin at 100 μg/ml. The culture was then diluted 1:50 in the same medium and was grown for an additional 3 h at 37°C. The expression of OspC was induced by raising the temperature to 42°C for 8 h. The cells were then harvested at 5,000 × g for 15 min and washed twice in phosphate-buffered saline. After the final wash, the cells were resuspended in buffer containing 8 M urea and was enriched by anion-exchange chromatography (SP-Sephadex C50; Pharmacia). Trypsin (sequencing grade; Boehringer Mannheim) digestion of the 41-kDa flagellin yielded the 14-kDa flagellin fragment by gel filtration on Superdex 75 (Pharmacia). Fractions containing OspC were dialyzed against buffer A, which contained 20 mM NaCl, 20 mM Tris-HCl (pH 8.0), and protease inhibitors (see above). Final purification was achieved by anion-exchange chromatography on Resource Q (Pharmacia). Elution buffer B was the same as start buffer A except that buffer B contained 1 M NaCl. OspC eluted at 10% buffer B. The protein content for the OspC antigen produced by this method was about 100 μg/ml. It was determined with the Coomassie Plus Protein Assay Reagent (Pierce).

**Purification of recombinant tryptoph 14-kDa fragment.** Purification of the recombinant 14-kDa fragment from *B. burgdorferi* was done as described previously (25). Briefly, the 41-kDa flagellin expressed by *E. coli* HB101 was extracted with 8 M urea and was enriched by anion-exchange chromatography (DEAE-Cellulose; Serva). Enriched flagellin was precipitated by dialysis against distilled water. Pure 41-kDa flagellin was obtained following cation-exchange chromatography (SP-Sephadex C50; Pharmacia). Trypsin (sequencing grade; Boehringer Mannheim) digestion of the 41-kDa flagellin yielded the 14-kDa flagellin peptide, which was finally isolated by gel filtration on Superdex 75 (Pharmacia).

**ELISA.** ELISA was done by standard methods. Each well of 96-well flat-bottom plates (Greiner) was coated at 4°C overnight with 100 μl of recombinant OspC (1.0 μg/ml) or recombinant 14-kDa fragment (2.0 μg/ml) diluted in coating buffer (15 mM Na2CO3, 35 mM NaHCO3 [pH 9.5]). After washing with PBS (0.15% [vol/vol] Tween 20) (3 times), coating buffer (0.15% [vol/vol] Tween 20, 1 M NaCl, 3 mM KCl, 10 mM Na2HPO4, 1 mM KH2PO4), the plates were incubated with patient sera diluted 1:200 in dilution buffer (washing buffer containing 1% [wt/vol] Tween [Bayer], 2% [vol/vol] Tween 20, and 0.5% [vol/vol] Trispropan phageoides sonicate [BAG]) for 1 h at 37°C. The plates were washed again. Bound antibodies were detected with peroxidase-conjugated goat-anti-human IgG (Dako), diluted 1:10,000 in the same buffer used for the sera, and incubated for 30 min at 37°C. After a second washing step, substrate solution (0.4 mg of ortho-phenylenediamine/ml; tablets; DAKO) in substrate buffer (20 mM Na2HPO4, 100 mM K2HPO4) was added, and the mixture was incubated for 15 min in a dark chamber at 4°C. The reaction was stopped with 2 M H2SO4, and the optical density (OD) was read at 492 nm with an SLT reader (type 340 ATC). The cutoff for optical density readings at OD492 was set 2 standard deviation above the mean for 154 samples from healthy controls. For comparison, sera were tested in a commercial IgM ELISA with a detection limit of 0.25 OD/ml for OspC and 2.0 μg/ml for the 14-kDa peptide. On testing of sera from local students with no current clinical symptoms and with no prior history of Lyme disease, cutoff values were 0.42 and 0.26, respectively. Sensitivities were 38% (IgM ELISA with OspC of strain GeHo) and 36% (IgM ELISA with OspC of strain PKo) for the group of 104 patients with EM. Indices (ratio of the OD to the cutoff value) for samples with clearly discrepant results by both ELISAs are given in Table 1. One index of >1 indicates a positive result.

**RESULTS**

The cutoff values for positive results were adjusted to a diagnostic specificity of 95%, estimated by examination of 154 serum samples from local students with no current clinical symptoms and with no prior history of Lyme disease. In the IgM ELISA with *B. burgdorferi* GeHo and OspC of strain PKo, cutoff values were 0.42 and 0.26, respectively. Sensitivities were 38% (IgM ELISA with OspC of GeHo) and 36% (IgM ELISA with OspC of PKo) for the group of 104 patients with EM. Indices (ratio of the OD to the cutoff value) for samples with clearly discrepant results by both ELISAs are given in Table 1. One index of >1 indicates a positive result.

**TABLE 1. Indices for sera with clearly discrepant results by the ELISA with recombinant OspC from *B. burgdorferi* GeHo and recombinant OspC from *B. afzelii* PKo**

<table>
<thead>
<tr>
<th>Serum sample no.</th>
<th>Index by ELISA with OspC from GeHo</th>
<th>Index by ELISA with OspC from PKo</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.7</td>
<td>3.4</td>
</tr>
<tr>
<td>14</td>
<td>2.8</td>
<td>0.6</td>
</tr>
<tr>
<td>19</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>61</td>
<td>1.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* The sera were from patients with EM.
six times. The reproducibilities of the ODs from run to run were ± 10%. The study was performed without involving the definition of a borderline range. However, in routine diagnostic use, when clinical decisions depend on the results of the ELISA, the use of a borderline range is essential.

In order to investigate possible protein-protein interference between the proteins used in combination in the recombinant ELISA, we examined 40 preselected serum samples from patients with various manifestations of Lyme disease (n = 25 patients with EM; n = 12 patients with neuroborreliosis; n = 3 patients with acrodermatitis chronica atrophicans). They revealed a positive result in OspC IgM ELISA (30 of 40 serum samples) and/or in the 14-kDa antigen IgM ELISA (27 of 40 serum samples). A total of 39 of 40 (98%) of the serum samples were positive and 1 (2%) serum sample was borderline in the combined OspC–14-kDa antigen ELISA. In addition, we compared the indices estimated with the antigens used singly in the ELISA with that estimated with the antigens used in combination ELISA. Results for 10 representative serum samples are given in Fig. 2. There is a clear additive effect regarding the indices obtained by the OspC–14-kDa antigen ELISA compared to those obtained by ELISAs performed with each antigen alone.

Sensitivity (defined as the number of correct positive results divided by the total number of serum samples from Lyme disease patients) was estimated by testing 104 serum samples from patients with a clinical diagnosis of EM. By the recombinant OspC–14-kDa antigen IgM ELISA, 48 (46%) of 104 serum samples were positive, whereas by the commercial IgM ELISA, 47 (45%) of the serum samples were positive (Fig. 3). The mean of the differences between the ODs obtained by both ELISAs indicated significantly higher ODs by the recombinant ELISA (mean OD, 0.98) than by the whole-cell-extract ELISA (mean OD, 0.64) (P < 0.0001; paired Wilcoxon rank test).

To investigate false-positive reactions in both ELISAs with cross-reactive antibodies due to an EBV infection, we tested 42 serum samples from patients with acute EBV infection. Four (10%) revealed a positive result by the recombinant ELISA and 10 (24%) were positive by the commercial ELISA (P < 0.002; chi-square test). Furthermore, we screened a larger panel of EBV-positive sera by the recombinant ELISA. A total of 11 positive serum samples were also tested by immunoblotting with recombinant OspC and the 14-kDa antigen (Fig. 4). Three serum samples revealed antibodies to both recombinant antigens. Six serum samples showed a positive result exclusively with the 14-kDa band, and two serum samples reacted only with the OspC band.

On testing of 44 serum samples from patients with a history of syphilis, 5 serum samples (11%) were positive by each ELISA. Of these, four serum samples were positive by both ELISAs. In addition, 12 serum samples from healthy controls and from patients with syphilis which were reactive in the OspC–14-kDa antigen ELISA were also tested by immuno-
infection. Lanes 3, 4, 5, and 6 display both the OspC and the 14-kDa antigen from patients with a history of syphilis; 4, 5, and 6, sera from patients with EBV gens are presented. Lanes: 1, positive control; 2, negative control; 3, 7, and 8, sera B. burgdorferi biffinant OspC and the 14-kDa flagellin fragment of

detection of IgM antibodies in patients with early Lyme disease

As antigens. In addition, we investi-

ment from GeHo as anti-

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smear does not eliminate all cross-reactive antibodies. On comparing the immunoreactivities of recombinant OspC from two different borrelial genomospecies in an ELISA would increase the sensitivity of IgM antibody assessment in Lyme serology.

On comparing the immunoreactivities of recombinant OspC from two different genomospecies in an IgM ELISA (B. burgdorferi sensu stricto and B. afzelii), very few discrepancies were noted. The low level of serological heterogeneity of both OspCs by ELISA is a surprising finding, particularly if one considers the low degree of amino acid identity between the two genomospecies of 61.5 to 74.0% (15, 30). On the other hand, our results are in agreement with those of a study by Wilske et al. (34). Those investigators observed that IgM antibodies from patients with neuroborreliosis mainly recognized epitopes of the recombinant OspC, which were conserved among the three genomospecies. According to our data, sensitivity would not increase significantly by combining OspC from strain PKo and GeHo in ELISA. Thus, we used only OspC from strain GeHo in the combined IgM ELISA.

To determine the possible protein-protein interference between recombinant antigens in ELISA, we tested 40 prese-

lected serum samples positive either in the ELISA with OspC from GeHo or in the 14-kDa antigen IgM ELISA. With the exception of one serum sample (which showed a borderline OD), all sera revealed higher ODs by the ELISA with both antigens, indicating a higher sensitivity of the ELISA with both antigens compared to those of ELISAs with the single antigens. This can be further confirmed when the indices obtained by the three ELISAs are compared (Fig. 2). Most sera showed an increased index by the combined OspC–14-kDa antigen ELISA. Only for serum sample 9 did the index of the combined ELISA seem to be slightly lower compared to the index of the 14-kDa ELISA. Both indices, however, represent extremely high ODs. It is possible that slight differences in the indices for highly positive sera (serum samples 2, 9, and 10) are not significant because of the nonlinear dependence of high ODs (>2.0) on the serum dilution. Nevertheless, from these data it can be concluded that there is an additive effect regarding the ODs of the single-antigen ELISAs when both antigens are combined in an ELISA. The additive effect regarding the ODs in the combination ELISA might be explained by a broader spectrum of relevant epitopes per microtiter well compared to those for the ELISAs performed with single antigens. Furthermore, we infer that there are no disturbing interferences between the recombinant antigens used in the ELISA; such interference would influence the availability of immunoreactive sites.

The incidence of false-positive reactions by cross-reactive antibodies due to EBV infection was markedly high in the commercial ELISA, with 23% positive results. The recombinant ELISA revealed only 10% positive results for this group of serum specimens. Increased cross-reactivity of EBV-positive sera in the commercial ELISA is not surprising because B. burgdorferi sensu lato contains many cross-reactive antigens like conserved parts of the 41-kDa flagellin, which share epitopes with other bacterial species (4, 19).

The 46% sensitivity of the recombinant OspC–14-kDa antigen ELISA at the level of 95% specificity was similar to the specificity of the commercial ELISA. Considering the results presented in Fig. 3, the high concordance of the OD values for single serum samples by both ELISAs can be observed. There are no discrepancies regarding positive sera with ODs of more than 1.0 and 0.7 by the recombinant and the whole-cell-extract ELISAs, respectively. This indicates that the sensitivity of an ELISA with only two recombinant antigens is at least as good as the sensitivity of a whole-cell-extract ELISA, while the specificity of the recombinant ELISA is improved. Gerber et al. (11) observed a clearly improved sensitivity of a recombinant OspC-IgM ELISA compared to that of a whole-cell-extract IgM ELISA. OspC is known to be expressed in various amounts by different borrelial strains in culture (30). We suggest that the discrepancies in the sensitivities of IgM ELISAs performed with whole-cell extracts mainly depend on the various levels of expression of OspC.

The high concordance of ODs by both ELISAs with sera from patients with syphilis was a surprising result. We expected a clearly improved specificity of the recombinant ELISA because of suspected cross-reactive epitopes in the whole-cell-extract ELISA. This observation might be explained by the quite effective adsorption of cross-reactive antibodies with the T. phagedenis sonicate in the commercial ELISA. When the recombinant ELISA was performed without preadsorption of the sera with T. phagedenis sonicate, no significant effect on the specificity and the sensitivity was observed (data not shown). This suggests that high concentrations of cross-reactive epitopes do not exist between T. phagedenis and either of the recombinant antigens tested in this study. On the other hand, these data show that preadsorption of sera with T. phagedenis sonicate does not eliminate all cross-reactive antibodies.

By additionally testing false-positive sera (sera from controls and patients with EBV infection and syphilis) by immunoblotting and by ELISAs performed with single antigens, these sera were reactive with the 14-kDa antigen and with OspC. Cross-reactive antibodies had a tendency to be slightly more frequently directed against the 14-kDa antigen than against OspC. These data are in line with those presented in previous reports (20, 34).

As can be seen in Fig. 3 the mean of the positive ODs was significantly higher by the recombinant ELISA than by the whole-cell-extract ELISA. From these data, one may conclude...
that the ability to discriminate between real-positive and real-negative sera is superior for the recombinant ELISA due to a presumed higher concentration of relevant epitopes per microtiter well.

There are several reports on the use of recombinant OspC for the serological diagnosis of Lyme disease (11, 23, 33, 34). This, however, is the first study in which the whole sequence of recombinant OspC is used as a nonfusion protein in combination with the internal flagellin fragment in an ELISA. Padula et al. (23) reported on the results of studies with recombinant OspC expressed as a fusion protein. Tests with sera from 74 individuals with culture-confirmed EM revealed a sensitivity of approximately 65% (23), which was clearly higher than that in our study. This discrepancy could be explained by differences in evaluation of the cutoff OD value and by variations in the criteria used for case definition.

In conclusion, the recombinant OspC–14-kDa antigen IgM ELISA is a suitable test for the sera from patients with EBV infection. We suggest that the specificity to the whole-cell-extract ELISA on the testing of ability to discriminate between true-positive and true-negative sera is superior for the recombinant ELISA due to a high immunoblot compared to purified flagellum-ELISA in early Lyme disease. J. Clin. Microbiol. 31:2133–2135.


