Cross-Reactive Epitopes in *Borrelia burgdorferi* p66

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Epitope mapping of the p66 outer membrane protein of *Borrelia burgdorferi* revealed that the protein contains numerous cross-reactive linear epitopes recognized by serum antibody in the majority of individuals tested, regardless of Lyme disease history, limiting the usefulness of this antigen in Lyme disease serodiagnostic assays.

The protein p66 is an outer membrane protein of *Borrelia burgdorferi* that functions as a porin and a β3-integrin binding protein (1–3). It is prominently displayed on the surface of the bacteria and is expressed in the feeding tick prior to transmission (4). p66 is also a prominent component antigen of many Lyme disease serodiagnostic assays. In the present study, we performed epitope mapping of a full-length p66 protein to define linear B cell epitopes present in this antigen, with the goal of identifying epitopes unique to *B. burgdorferi* for use as specific and sensitive antigen targets in a peptide-based serodiagnostic assay. However, we found that all patient sera, regardless of origin or disease state, contained circulating antibodies that recognized multiple linear epitopes of p66, confounding their use as specific serodiagnostics. The presence of cross-reactive epitopes in the p66 protein highlights a primary pitfall associated with the use of many, though not all, whole bacterial proteins in diagnostic assays and underscores why many *B. burgdorferi* serodiagnostics have significant issues with specificity.

Epitope mapping was performed by ProImmune, Inc. (Oxford, United Kingdom), using their proprietary ProArray Ultra peptide microarray technology as previously described (5). B cell epitopes were identified by incubating an overlapping peptide library of a full-length p66 protein (GenBank accession number AAC66949.1), consisting of 15-amino-acid (15-aa) peptides offset by 5 aa (10 aa overlap), with 8 serum samples collected from patients with physician-diagnosed Lyme disease presenting with erythema migrans (EM) at the time of initial diagnosis (n = 4) or at a follow-up visit 1 to 2 months after initial diagnosis and treatment (n = 4). Thirty-three peptides in the overlapping peptide library had detectable antibody binding in at least one of the eight patient sera. These epitopes were distributed throughout the protein and were not clustered in any particular region. All of the epitopes were smaller than 20 aa, as serum antibody binding for any one patient sample did not exceed >2 consecutive overlapping peptides. Peptides (LifeTein, South Plainfield, NJ) were synthesized for five sequences that were detected by patient antibodies in all eight serum samples, p66(56-75), p66(121-135), p66(211-230), p66(516-530), and p66(576-590), as well as a sixth sequence, p66(261-275), that was detected in 5 of 8 serum samples (3 collected during the first visit and 2 collected during the second visit). The remaining presumptive epitopes were excluded from further analysis because they were detected in <50% of serum samples, were detected predominantly (>75%) in second-visit serum samples, or were only detected at a single dilution of antibody (low affinity).

Specificity and sensitivity for each peptide were evaluated by enzyme-linked immunosorbent assay (ELISA) using serum panels collected from patients with early Lyme disease (EM patient sera), rheumatoid arthritis (RA), or syphilis, as well as healthy individuals living in regions where Lyme disease is endemic or nonendemic. Early Lyme disease serum was collected, under informed consent and approval from the relevant institutional review boards, from physician-diagnosed patients at the time of initial presentation with EM at New York Medical College in Westchester, NY (n = 73), Gunderson-Lutheran Medical Center in LaCrosse, WI (n = 37), or Stonybrook University in Long Island, NY (n = 20). All three regions are areas where Lyme disease is endemic. Healthy control sera from a region of Lyme disease endemicity (endemic healthy control sera) were collected under informed consent from field workers on the south shore of Long Island (n = 35). Healthy control sera from regions where Lyme disease is nonendemic (nonendemic healthy control sera) were obtained from New Mexico (n = 64) (Creative Testing Solutions, Tempe, AZ) and southern California (n = 40) (Bioreclamation LLC, Westbury, NY). Sera from syphilis patients (rapid plasma reagin [RPR] and antitreponemal antibody positive) (n = 23) (Bioreclamation LLC) were used as negative controls for potential cross-reactive antibodies raised against a related spirochete, *Treponema pallidium*. Sera from RA patients (n = 40) (Bioreclamation LLC) were used as negative controls for chronic inflammation marked by high antibody levels and joint damage, which can occur in Lyme disease. To assess the contribution of nonspecific interactions of serum antibodies with peptide-coated plates, sera were incubated in plates that were blocked but not coated with peptide (no-peptide), as well as plates that were coated with an unrelated nonbacterial peptide derived from the ovalbumin protein, OVA(323-339). ELISA was carried out using previously described methods (5) with the following parameters: peptide concentration, 10 µg/ml; blocking buffer, 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS); detecting antibody, 1:15,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG and IgM (Jackson ImmunoResearch).
developing solution, tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD); and stop solution, 2 N H₂SO₄. Statistical comparisons of antibody binding absorbance were carried out using a Kruskal-Wallis test followed by a Dunn’s multiple-comparison test and GraphPad Prism software (San Diego, CA). P values of <0.05 were considered significant.

Levels of nonspecific antibody binding to no-peptide or OVA(323-339)-coated plates were virtually identical. In contrast, in all serum samples, regardless of Lyme disease status, antibody binding to all six p66 peptides was significantly elevated over that for the no-peptide and OVA(323-339)-coated plates [P < 0.0001, except for P < 0.01 for p66(261-275) and p66(516-530) syphilis patient sera versus no-peptide and OVA(323-339), and p66(516-530) endemic healthy control sera versus no-peptide and OVA(323-339)]. Considerable overlap was observed in the absorbance values for antibody binding to p66 peptides when comparing early Lyme disease serum to healthy and disease control sera, though some statistically significant differences were observed (Fig. 1) [P < 0.0001 for p66(211-230) EM patient sera versus nonendemic healthy control sera and p66(576-590) EM patient sera versus endemic healthy control sera; P < 0.005 for p66(211-230) EM patient sera versus endemic healthy control sera and p66(121-135) EM patient sera versus nonendemic healthy control sera P < 0.01 for p66(121-135) EM patient sera versus syphilis patient sera; and P < 0.05 for p66(121-135) EM patient sera versus endemic healthy control sera]. A generally accepted cutoff for determining positivity of antibody binding in Lyme disease seroassays is 3 standard deviations (SD) from the mean of absorbance of healthy control serum (Fig. 1, dashed line). Due to the increased level of antibody binding in nonendemic healthy control sera, these cutoff values were very high for each of the p66 peptides, reducing the sensitivity of the assay. As a result, positive antibody binding for each peptide was very poor. p66(211-230) had the highest level of positive antibody binding at 28.2%, followed by p66(56-75) at 16.0% (Table 1). If positive and equivocal binding to p66(211-230) is considered, then sensitivity is increased to...
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IgG antibodies to p66 (9). We have made similar observations in that 20% of healthy individuals with no history of Lyme disease had significant levels of antibody binding to other Borrelia protein p66.

Lack of specificity is often discussed as a negative factor associated with many current Lyme disease serodiagnostic assays; however, actual data demonstrating nonspecificity are rarely presented in the literature. In the present study, we offer clear empirical evidence for the presence of multiple cross-reactive epitopes in p66, p66 is a component of many serodiagnostic assays for Lyme disease; it is present in whole-cell sonicate assays and is a band included in the CDC IgG Western blot diagnostic criteria (6). The data presented here demonstrate that many individuals have preexisting levels of circulating antibody that cross-react with linear epitopes in the Borrelia protein p66.

TABLE 1 Binding of Lyme disease antibody to p66 peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>No. positive/total no. (%)</th>
<th>No. equivocal/total no. (%)</th>
<th>No. negative/total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p66(36-75)</td>
<td>21/131 (16.0)</td>
<td>13/131 (9.9)</td>
<td>97/131 (74.1)</td>
</tr>
<tr>
<td>p66(121-135)</td>
<td>13/129 (10.1)</td>
<td>12/129 (9.3)</td>
<td>104/129 (80.6)</td>
</tr>
<tr>
<td>p66(211-230)</td>
<td>37/131 (28.2)</td>
<td>19/131 (14.5)</td>
<td>75/131 (57.3)</td>
</tr>
<tr>
<td>p66(261-275)</td>
<td>7/131 (5.3)</td>
<td>7/131 (5.3)</td>
<td>117/131 (89.4)</td>
</tr>
<tr>
<td>p66(516-530)</td>
<td>9/131 (6.9)</td>
<td>12/131 (9.2)</td>
<td>110/131 (83.9)</td>
</tr>
<tr>
<td>p66(576-590)</td>
<td>2/131 (1.5)</td>
<td>9/131 (6.9)</td>
<td>120/131 (91.6)</td>
</tr>
</tbody>
</table>

42.7%. While not effective in a stand-alone assay, it is conceivable that p66(211-230) may be an effective serodiagnostic target when used as a component of a multiantigen assay containing other specific protein and/or peptide antigens; however, this was not assessed in this study. Positive binding for the four remaining peptides was 10% or lower (Table 1). The similar antibody binding profiles in serum from healthy individuals and Lyme disease patients suggest that many individuals have levels of circulating antibody that cross-react with linear epitopes in the Borrelia protein p66.

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REFERENCES

