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Epitope Length, Genospecies Dependency, and Serum Panel Effect in the IR6 Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to *Borrelia burgdorferi*▽

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In the absence of erythema migrans, the basis for diagnosis of Lyme disease is the demonstration of an antibody response against *Borrelia burgdorferi* in an appropriate clinical setting. The C6 enzyme-linked immunosorbent assay, based on the IR6 region of VlsE, has become widely used in both the United States and Europe. We mapped the antigenic epitopes of IR6 to a shorter sequence that is equivalent in sensitivity and specificity to the full-length IR6 25-residue peptide. In addition, we observed significant differences in sensitivity between serum panels (60 to 100%), indicating that the selection of the serum panels can shape the apparent overall sensitivity of the assay. Contrary to prior reports, the assay sensitivity is greater when the IR6 peptide is derived from the sequence of the same infecting *Borrelia* genospecies. Using our North American panels and the two panels obtained from European Lyme disease patients, we determined that the IR6 assay that is based on a single genospecies of *Borrelia* spp. is not optimal for use as a universal diagnostic assay for Lyme disease.

Lyme disease is the most frequently reported vector-borne disease in the United States and Europe. The only clinical manifestation that is adequate for the diagnosis of Lyme disease is erythema migrans (EM) (18). When present, EM is seen for a limited period of time in early disease. Although in areas of endemicity the presence of bilateral Bell's palsy suggests Lyme disease (2), neither this nor the other clinical manifestations are specific enough, singly or in combination, to determine clinical diagnosis.

In the absence of EM, the basis for diagnosis is the demonstration of an antibody response against *Borrelia burgdorferi* in an appropriate clinical setting. In North America, a two-tier approach is recommended for serodiagnosis: a sensitive first tier assay followed by a Western blot if the first tier assay is positive or equivocal. Most of the current first tier assays are based on whole *B. burgdorferi* or recombinant proteins. The sole exception is the C6 peptide assay. This assay, which is based on the IR6 region of the variable surface antigen (VlsE) of *B. burgdorferi* (C6), is becoming more widely used in both the United States and Europe (3, 6, 8, 14, 17). It is recognized as the most specific of the first tier assays (1), and it has a high degree of sensitivity for disseminated or late Lyme disease (3). Despite its greater performance and previous suggestions that could be used as a single-tier assay (1, 4, 11), lately it has become apparent that the C6 assay is not sufficiently sensitive or specific to develop a single-tier Lyme disease assay (13, 16).

The linear B-cell epitopes within the VlsE IR6 peptide were previously mapped using sera from experimentally infected monkeys, from mice, and from humans clinically diagnosed with Lyme disease using an overlapping peptide technique. That study concluded that the full 25-residue IR6 peptide (IR6-25) was required to maintain antigenicity (5, 7). We observed that the sequence used to design the IR6 peptide was from IP90, a *Borrelia garinii* strain that has not been found to cause Lyme disease in the United States. In addition, we noted that this relatively conserved region was somewhat long for a single antigenic epitope. Analysis of the chemical properties of this peptide predicted an antigenic region within a much shorter sequence, in the N terminus of this peptide. To test this hypothesis, we remapped the C6 peptide by employing a finely detailed mapping strategy. Considering the chemical properties of this peptide and working from the natural sequence matrix of IR6 from *B. burgdorferi* sensu stricto, we designed a series of peptides and were able to define the shortest effective IR6 peptide for diagnosis of Lyme disease in the United States. This short version of the IR6 peptide could be the core of a multiantigenic peptide assay that may lead to the development of a single-tier assay for Lyme disease.

MATERIALS AND METHODS

Peptide synthesis. The synthetic peptides were custom synthesized by the Keck Biopolymer Resource at Yale University. The peptides were made by an automated solid-phase methodology using 9-fluorenylmethoxy carbonyl (FMOC) N protection protocols.

ELISA procedure. Immobilization of peptides onto enzyme-linked immunosorbent assay (ELISA) plates was performed as follows. Solutions of crude peptides in 100 mM *N,N*-methylenebisacrylamide (Bis)-Tris propane buffer (pH 9.7) were used to coat commercial microwell plates (MaxiSorp; Nunc) at 5 μg/ml as follows. One hundred microliters of the solution described above was dispensed in each well. After 1 h of incubation at room temperature, 300 μl of blocking solution (100 mM Bis-Tris propane buffer [pH 9.7], 0.10% Tween 20, 3% skim milk) was added to each well and the mixture was incubated overnight at 4°C. The blocking solution successfully saturates the high antigen binding capacity, leaving low background readings in the control channels. The contents

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TABLE 1. Clinical characterization of the serum panels used in this study

Panel	No. of serum samples	Clinical characterization
U.S. Lyme disease		
Acute	55	Early, EM, first month of infection
Acute, single EM	42	Early, acute, single EM at presentation, 33 culture positive
Acute disseminated, multiple EM	5	Early, acute disseminated, multiple EM at presentation
Acute, neurologic	6	Early, acute neurologic, meningitis, facial nerve palsy
Late Lyme disease	15	Compatible with late Lyme disease based on arthritis and/or neurologic symptoms at presentation
Lyme disease reinfected	5	Previously successfully treated for EM, continued exposure to tick bite who again presented to the Lyme disease clinic
European Lyme disease		
Austrian	30	Erythema migrans, cutaneous Lyme disease
Portuguese	100	Panel comprised of sera from patients with signs and symptoms suggestive of Lyme disease and patients for differential diagnosis with Lyme disease
Controls		
Rheumatoid arthritis	34	Rheumatoid arthritis
Syphilis	27	Syphilis, *Treponema pallidum* infection
Systemic lupus erythematosus	20	Systemic lupus erythematosus
Healthy	50	Healthy individuals from area of endemicity

were discarded, and the plate was washed three times using Tris-buffered saline-Tween (TBST) buffer.

A standard procedure for the ELISAs was followed. Human serum was diluted at 1:50 in 100 μl of TBST buffer with 1% skim milk. The samples were added to each well, and the plate was incubated for 1 h at room temperature. Plates were washed three times with TBST buffer. The alkaline phosphatase-conjugated anti-human immunoglobulin G (H+L; Jackson ImmunoResearch, West Grove, PA) antibody was diluted at 1:1,600 in TBST buffer with 1% skim milk; 100 μl of this solution was dispensed onto the plate and incubated for 30 min at room temperature. Plates were washed three times with TBST buffer, and 100 μl of substrate was added (pNPP microwell substrate system; KPL, Gaithersburg, MD) and incubated for 1 h at room temperature. Plates were read at 405 nm on a microplate reader (Spectramax 320; Molecular Devices). A sample was considered positive if it produced an average absorbance superior to the mean of five negative controls plus 3 standard deviations.

Test panels. Our screening panels consist of sera from patients with early Lyme disease and clinically defined as late Lyme disease, patients with syphilis, and patients with rheumatologic disorders as well as from normal healthy donors from an area of endemicity. The clinical characterization of the serum panels is described in Table 1. The Lyme disease serum panels are representative of the population of suburban New York and include samples from children, adults, males, females, whites, and minorities. The panels were randomly selected from our serum bank (from SUNY Stony Brook Lyme Disease Center). They were not preselected in any way.

Statistical analysis. The odds ratio (OR) method was used to assess statistical significance. The OR is a measure of effect size particularly important in logistic regression that is a statistical regression model for Bernoulli-distributed dependent variables. The OR is defined as the ratio of the odds of an event occurring in one group to the odds of it occurring in another group.

RESULTS

Epitope mapping. The sequence used to design the C6 peptide was from a *B. garinii* strain not found in North America. Because there are amino acid differences between the IR6 sequences from *B. garinii* (PT7, strain IP90) and *B. burgdorferi* sensu stricto (strain B31), a prototypic North American strain, we decided to compare the two. We synthesized the 25-mer IR6 segment from both strains with N-terminal cysteinyl residues appended for attachment of biotinyl ligands as described by Liang et al. (5, 8) and compared them against a panel of Lyme disease sera: 55 acute-phase sera characterized by EM, 33 of which were culture confirmed. The results are shown in Table 2. We observed that the peptide that was based on the *B. garinii* sequence (IP90-25) was less sensitive (41.8%) than the peptide based on the *B. burgdorferi* B31 sequence (B31-25; 56.4%). An OR of 1.787 indicates the *B. burgdorferi* sequence is ~1.8 times as likely to be more sensitive than the *B. garinii* sequence.

Analysis of the chemical properties of the IR6 peptide by the Kyte-Doolittle hydrophilicity method predicted a large antigenic region within a much shorter sequence in the N terminus of this peptide and a minimal epitope in the C terminus. In order to determine the minimum human active epitope(s) in this region for diagnostic purposes, we employed a finely detailed mapping strategy working from the natural sequence matrix of *B. burgdorferi* B31 genospecies and used 33 Lyme disease sera defined as EM and culture positive. Liang et al. speculated that the key portion of the human epitope of IR6 was located in the central area of the peptide (7), but they were unable to define it. To test this, we shortened the 25-residue sequence of B31-IR6 from the C terminus until its efficiency at detecting early Lyme disease antibodies dropped. We then returned to the shortened sequence of IR6 and began deleting residues from the N terminus. The results are shown below in Table 3. We found that shortening the C-terminal portion of

TABLE 2. Comparison between *B. garinii* and *B. burgdorferi* IR6 test sensitivities[a]

Peptide	Sequence	No. positive/ total (%)[b]
IP90-25	(C)MKKDDQIAAAMVLRGMAKDGQFALK	23/55 (41.8)
B31-25	(C)MKKDDQIAAA**IA**LRGMAKDG**K**FA**V**K	31/55 (56.4)

[a] The 25-residue segments correspond to the IR6 immunodominant regions of VlsE in the respective *B. burgdorferi* sensu lato strains: IP90-25 is *B. garinii* and B31-25 is *B. burgdorferi* sensu stricto. (C) is the cysteine appended for purposes of biothinylation. Bold residues in the *B. burgdorferi* B31 sequence differ from the *B. garinii* IP90 sequence. The Lyme disease panel used contained 55 acute-phase sera characterized by EM in the first month of infection.

[b] The OR for IP90-25 versus B31-25 was 1.787.

TABLE 3. *B. burgdorferi* B31 VlsE-IR6 shortest immunodominant region

Peptide[a]	Sequence[b]	No. positive/ total (%)[c]
B31-25	(C)MKKDDQIAAAIALRGMAKDGKFAVK	11/33 (33)*
B31-18	MKKDDQIAAAIALRGMAK	12/33 (36)
B31-17	(C)MKKDDQIAAAIALRGMA	13/33 (39)*†
B31-16a	MKKDDQIAAAIALRGM	8/33 (24)†
B31-16b	KKDDQIAAAIALRGMA	11/33 (33)

[a] The peptides correspond to the IR6 immunodominant regions of VlsE in *B. burgdorferi* sensu stricto.
[b] (C) is the cysteine appended for purposes of biotinylation. The Lyme disease panel used contained 33 acute-phase sera characterized by EM and culture positive.
[c] *, OR = 1.552; †, OR = 2.009.

TABLE 5. Comparison between the four peptides for specificity

Panel	No. of serum samples	No. of positive samples (%)[a]			
		B31-25	IP90-25	B31-17	IP90-17
Rheumatoid arthritis	34	0 (0)	2 (5.8)	1 (2.9)	3 (8.8)
Syphilis	27	2 (7.4)	1 (3.7)	1 (3.7)	3 (11.1)
Systemic lupus erythematosus	20	1 (5)	0 (0)	1 (5)	3 (15)
Healthy	50	2 (4)	3 (6)	3 (6)	4 (8)
Total	131	5 (3.8)*	6 (4.6)	6 (4.6)†	13 (9.9)*†

[a] *, OR = 2.766; †, OR = 2.288.

the peptide by 7 and 8 amino acids showed a trend toward increased sensitivity (B31-17 versus B31-25; OR = 1.552) which indicates that B31-17 is 1.5 times as likely to be more sensitive than B31-25 against the panel tested. In contrast, deletion of the 9th C-terminal residue (B31-16a) and deletion of the first N-terminal residue (B31-16b) caused a decline in B31-17 peptide antibody detection. The OR between IR6-17 and IR6-16a is 2.009, which indicates that IR6-17 is twice as likely to be more sensitive than IR6-16a. Thus, we concluded that the critical immunodiagnostic epitope of the 25-residue IR6 sequence from *B. burgdorferi* B31 is confined within a 17-residue segment of the IR6 sequence and that the N-terminal sequence is essential for maintenance of antigenicity.

Sensitivity and specificity. With these new findings, we wanted to see how this narrowly defined peptide from *B. burgdorferi* (B31-17) compared to the homologous sequence from *B. garinii* (IP90-17) as well as to the respective 25-residue peptides, B31-25 and IP90-25. The sensitivities and specificities (Tables 4, 5, and 6) of the four peptides were determined using several panels of clinically defined Lyme disease sera.

Differences in sensitivities between the four peptides were significant in detecting *B. burgdorferi* antibodies in the panel defined as acute, single EM, and early Lyme disease (Table 4 and Table 6). The peptide B31-25 is ~1.5 times as likely to be more sensitive than IP90-25 and IP90-17 and ~1.3 times as likely to be more sensitive than B31-17 against this panel. In the panel defined as late Lyme, we observed that B31-25 is 1.6 times as likely to be more sensitive than IP90-25 and no differences were observed between B31-25 and the two 17-residue peptides. Overall, we conclude that the peptide based on the *B. burgdorferi* sensu stricto sequence (B31) is more sensitive than *B. garinii* IP90, the sequence used to develop the C6 assay. There was no difference in sensitivity between the four peptides in detecting antibodies in sera from patients with specific symptoms of Lyme disease, such as the sera defined in three other panels, acute disseminated (60%), acute neurologic (83.3%), and Lyme disease reinfected (100%).

We tested the specificity of the assay against sera from patients with conditions whose clinical presentation could place Lyme disease in their differential diagnosis (rheumatoid arthritis) or that produce antibodies that can be potentially cross-reactive with anti-*B. burgdorferi* antibodies (syphilis and systemic lupus erythematosus). Sera from healthy individuals from an area of endemicity were used as a negative control. The most specific peptides were B31-25, IP90-25, and B31-17, which detected the lowest number of cross-reactive samples (Table 5 and Table 6). The IP90-17 peptide was less specific than the other three peptides, detecting the highest percentages of cross-reactive antibodies. This peptide was 2.7 times as likely to detect cross-reactive antibodies as B31-25 and 2.3 times as likely to detect cross-reactive antibodies as B31-17 and IP90-25. The lack of specificity of this peptide against all cross-reactivity panels indicates that it is not appropriate for development of an assay to be used in the United States.

In summary (Table 6), we conclude that the IR6 peptides based on the *B. burgdorferi* sensu stricto sequence (B31) showed the highest sensitivity and specificity to detect Lyme disease antibodies and that OR determinations for comparisons between the B31-25 and -17 peptides are equivalent (B31-17 versus B31-25, OR = ~1.5 against one panel of early Lyme disease; and B31-25 versus B31- 17, OR = ~1.3 against a second panel of early Lyme disease).

To finalize this study, we compared the sensitivities of the four peptides to two Lyme disease panels obtained from Europe: a panel from clinically defined Lyme disease Austrian patients and a second panel comprising sera from patients with

TABLE 4. Comparison between the four IR6 peptides for sensitivity

Panel	No. of serum samples	No. of positive results (%)[a]			
		B31-25	IP90-25	B31-17	IP90-17
Acute, single EM	42	19 (45.2)*†	15 (35.7)*	16 (38.0)†	15 (35.7)
Acute disseminated, multiple EM	5	3 (60.0)	3 (60.0)	3 (60.0)	3 (60.0)
Acute, neurologic	6	5 (83.3)	5 (83.3)	5 (83.3)	5 (83.3)
Late Lyme disease	15	9 (60.0)‡	7 (46.7)‡	9 (60.0)	9 (60.0)
Lyme disease reinfected	5	5 (100)	5 (100)	5 (100)	5 (100)
Total	73	41 (56)	35 (48)	38 (52)	37 (51)

[a] *, OR = 1.479; †, OR = 1.292; ‡, OR = 1.683.

TABLE 6. Comparison of the sensitivities and specificities of the four IR6 peptides

IR6 peptide	% Sensitivity[a]	% Specificity[b]
B31-25	56	96.2
IP90-25	48	95.4
B31-17	52	95.4
IP90-17	51	90.1

[a] Values were determined with results from a total of 73 serum samples.
[b] Values were determined with results from a total of 131 serum samples.

TABLE 7. Comparison between the four IR6 peptides using European serum panels

Panel	No. of serum samples	No. of positive samples (%)[a]			
		B31-25	IP90-25	B31-17	IP90-17
Austrian	30	11 (37)*	9 (30)*	10 (33)	8 (27)
Portuguese	100	17 (17)†	22 (22)†	8 (8)	13 (13)

[a] *, OR = 1.291; †, OR = 2.492.

signs and symptoms suggestive of Lyme disease from Portuguese patients (Table 7). We found that the B31-25 peptide was only ~1.3 times as likely to be more sensitive at detecting *B. burgdorferi* sensu lato antibodies from patients in the Austrian panel than the IP90-25 peptide. In contrast, we found that the IP90-25 peptide was ~2.5 times as likely to be more sensitive at detecting anti-*B. burgdorferi* sensu lato antibodies from patients in the Portuguese panel than B31-25.

DISCUSSION

The C6 Lyme ELISA is taking on increasing importance because of its superior characteristics compared to whole-*B. burgdorferi*-based assays (1, 6, 8). The findings described in this study are noteworthy for three reasons: we have more precisely defined the human IR6 epitope to a shorter sequence that can lead to future improvement of IR6-based assays; the serum panels chosen can shape overall sensitivity of the assay; and, as importantly, we have demonstrated that the IR6 sequences from different genospecies are not fully interchangeable.

Although the IR6-25 peptide was mapped as a single antigenic determinant in humans (7), we observed that this relatively conserved region is somewhat long for a single epitope and we embarked on a series of experiments to remap this region. Rather than using overlapping peptides to determine the minimum human active epitope(s) of this peptide, we employed a finely detailed mapping strategy by serially deleting amino acids from the natural sequence matrix. We used a human serum panel obtained from patients with culture-confirmed early Lyme disease as our source of anti-*B. burgdorferi* antibodies. We first shortened the 25-residue sequence of IR6 from the C terminus and found that the efficiency to bind antibodies dropped sharply only after the deletion of the 9th C-terminal residue. Then returning to the sequence of IR6 shortened by eight residues at the C terminus, we began deleting residues from the N terminus. Deletion of even the first N-terminal residue caused a decline in antibody detection. Thus, we concluded that the critical immunodiagnostic epitope of the 25-residue IR6 sequence is confined within a 17-residue segment beginning with the N-terminal sequence whose conservation is essential to maintain antigenicity.

This finding, that the epitope encompasses the N terminus of the peptide, does not support what was previously predicted about the IR6 B-cell epitope. Liang et al. hypothesized that the key portion of the human epitope of IR6 was located in the central area of the peptide (7), the core of the epitope, and the key to the tertiary structure of the peptide being a predicted alpha helix comprised of an 11-mer sequence [AA(I or M)(A or V)LRGMAKD]. That hypothesis proposed that the alpha helix anchored the flanking amino acids, that the full 25-residue peptide was required, and that the epitope was most likely discontinuous. We demonstrate that neither the alpha helix nor the full-length peptide is required for the maintenance of the epitope. It also appears that the epitope is more linear than originally hypothesized.

The wide use and acceptance of the C6 assay for detection of anti-*B. burgdorferi* antibodies (1, 3, 16, 17) and the original finding that the six invariable regions of the VlsE protein are conserved among strains and genospecies of the *B. burgdorferi* sensu lato complex (5) led to the suggestion that the IR6 regions of any of the genospecies of *B. burgdorferi* are interchangeable in their ability to bind antibody. We found this not to be the case. Comparing four peptides based on IR6 sequences from *B. burgdorferi* and *B. garinii*, we found differences in sensitivities using the acute, single EM Lyme disease and the late-Lyme disease panels. These are the Lyme disease groups that would benefit the most from a sensitive diagnostic assay, especially the early Lyme disease group. In addition, we found that both peptides based on the *B. burgdorferi* sensu stricto sequence (B31) are more sensitive than *B. garinii* IP90, the sequence used to develop the C6 assay (8), in these panel groups. This increase in sensitivity is due to the use of the IR6 sequence from *B. burgdorferi* sensu stricto, the genospecies that infects humans in North America. Furthermore, we observed that there was no difference in sensitivities between the peptides in detecting *B. burgdorferi* antibodies in the other three panels, defined by clear clinical manifestations of Lyme disease. Most importantly, we observed significant differences in sensitivities between the panels (60 to 100% for the most sensitive peptide), indicating that overall sensitivity of the assay can be affected by the choice of the Lyme disease panel used. These data indicate that in studies of serologic assays, the key factor in determining true test sensitivity is made up of the clinical characteristics of the patients from whom the serum was obtained.

The C6 assay has become the assay of choice both in the United States and Europe, either alone (3, 6, 8, 9, 14, 17) or in combination with other assays (1, 4, 12). Sensitivity of this assay in Europe varies between 91% in a study in The Netherlands against a panel of sera from clinically defined Lyme patients confirmed by immunoblotting (16) and 33% in Italy against a panel of sera from clinically defined Lyme patients with EM confirmed by culture (10). In our study, we found that peptides based in two different *Borrelia* genospecies detected antibodies to this spirochete with significant differences in sensitivity between two panels of Lyme disease obtained from Europe. The most sensitive peptide for the American panel, based on the *B. burgdorferi* B31 sequence, showed a trend toward increased sensitivity for the Austrian panel. The fact

that the peptide based on the *B. garinii* sequence was undoubtedly the most sensitive in detecting antibodies in the Portuguese panel of our seroprevalence study shows a degree of genospecificity in the detection of *B. burgdorferi* sensu lato antibodies, given that in Europe, *B. garinii* and *Borrelia afzelii* are the most frequently cultured pathogenic spirochetes from Lyme disease patients. Recently, IR6 peptides from the three pathogenic *Borrelia* species (*B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*) were analyzed against serum panels from patients with European and American origins, and it was found that *B. burgdorferi* sensu stricto peptide antibodies correlated well with *B. afzelii* peptide antibodies and that *B. garinii* IR6 peptide antibodies were discordant with *B. afzelii* and with *B. burgdorferi* peptide antibodies (15). These data correlate with our findings that *B. burgdorferi* sensu stricto peptide antibodies were detected in the serum panel obtained from Austria (where *B. afzelli* is most frequent), while *B. garinii* peptide antibodies were found in the serum panel obtained from Portugal (where the pathogenic *Borrelia* spp. have not yet been defined). In combination, data from both studies indicate that the IR6 assay that is based on a single genospecies of *Borrelia* may not be suitable for use as a universal diagnostic assay for Lyme disease. In addition, the data suggest that *B. garinii* might be one of the predominant species of *B. burgdorferii* sensu lato that infects humans in Portugal.

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