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Comparison of Whole-Cell Antibodies and an Antigenic Flagellar Epitope of *Borrelia burgdorferi* in Serologic Tests for Diagnosis of Lyme Borreliosis

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A recombinant protein (p41-G) of an antigenic region of flagellin was used in a standard and amplified enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *Borrelia burgdorferi*, the causative agent of Lyme borreliosis. Comparable sensitivities (88 to 94%) were noted when sera from 17 persons who had erythema migrans and antibodies to whole-cell *B. burgdorferi* were tested against the p41-G antigen. In tests of a second study group of 36 persons who had erythema migrans but no detectable antibodies to whole-cell *B. burgdorferi*, 3 (8%) were positive when the p41-G antigen was used. Assay specificity likewise increased when the p41-G fragment was included in an ELISA with human sera containing treponemal antibodies. Recombinant flagellar proteins of *B. burgdorferi*, such as the p41-G antigen, can be used in an ELISA and may help confirm Lyme borreliosis during early stages of infection and improve specificity.

With greater public awareness of Lyme borreliosis and the knowledge that this widely distributed disease (35, 37, 38) can be difficult to clinically diagnose, there has been a strong reliance on laboratory testing. Progress has been made on improving antibody detection assays (3, 15, 16, 23, 24). Moreover, newly developed immune electron microscopy and polymerase chain reaction (PCR) techniques (11, 13, 26–31) can detect subunits or whole cells of the causative agent, *Borrelia burgdorferi*, in tissues or bodily fluids of ticks and mammals. However, these antigen or DNA detection procedures are experimental and need further scrutiny before they can be routinely used to help diagnose Lyme borreliosis. Therefore, laboratory testing for this disease mainly consists of detecting serum immunoglobulin M (IgM) or IgG antibodies by indirect fluorescent antibody staining, enzyme-linked immunosorbent assay (ELISA), and immunoblotting methods (10, 14, 18–20, 22, 23, 32). Unfortunately, there can be inconclusive or misleading results when there is relatively low assay sensitivity or poor specificity (25, 34), particularly if blood samples are obtained from patients within 3 weeks after the onset of illness or if treponemal infections go undetected.

To improve assay sensitivity, various preparations of flagellin, a highly immunodominant antigen of *B. burgdorferi* that is recognized during early and later stages of disease (6, 7, 9, 14), have been used in ELISA and Western blot (immunoblot) analysis (15–18, 20, 33). Recombinant antigens also have been tested in these assays (4, 8, 12). Recently, purified selected fragments of flagellin, generated by PCR and expressed as fusion proteins in *Escherichia coli*, reacted with sera collected from persons who were in early or late stages of Lyme borreliosis. Since these affinity-purified antigens proved useful in an ELISA and improved the sensitivity and specificity of Western blot analysis (4, 12), further independent testing was appropriate. The present study was conducted with different groups of sera to evaluate a recombinant fragment (p41-G) of flagellin in a standard and amplified ELISA, developed and performed in a separate laboratory, and to compare the sensitivities and specificities of these assays with those of an ELISA containing whole-cell *B. burgdorferi*. The main objective was to determine whether the use of the p41-G antigen could improve an ELISA for serum analysis during the early weeks of infection.

MATERIALS AND METHODS

Study groups. Serum samples used previously (22–24) were selected for analyses. Six study groups were chosen. Persons who had erythema migrans were divided into groups with (n = 17) or without (n = 36) antibodies to *B. burgdorferi*, as determined by a polyvalent, standard ELISA with whole-cell antigen coated to the solid phase. These persons lived in tick-infested areas of Connecticut. Blood samples were collected within 10 weeks after the onset of illness. The third group (n = 13) consisted of persons who had acute necrotizing ulcerative gingivitis or periodontitis and antibodies to oral treponemes but no histories of Lyme borreliosis or syphilis. Clinical data and serologic test results have been reported elsewhere (25). Sera from another 20 patients, who were diagnosed as having secondary or latent syphilis, were included as an additional group to further assess the specificity of an ELISA with the recombinant p41-G antigen. These serum specimens contained antibodies to *Treponema pallidum* in standardized fluorescent antibody adsorption (titers, ≥1:64) and Venereal Disease Research Laboratory (titers, ≥1:16) tests. In addition, six serum samples from patients with systemic lupus erythematosus (n = 3) or connective tissue disease (n = 3) were included for specificity analyses. There was no history of spirochetal infections for these patients or in 40 healthy persons in a sixth study group. All serum specimens were stored at −60°C at The

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Connecticut Agricultural Experiment Station prior to analyses.

Antigen preparations. Washed whole cells of *B. burgdorferi* (strain 2591) were used as previously reported (22, 24). Details on the amplification of the 41-kDa flagellar gene by PCR methods, isolation of the PCR product, purification and digestion of DNA, subcloning, and production of fusion proteins have been described previously (4, 12). On the basis of immunoblotting analyses of human sera with various flagellin fragments and epitope mapping, an immunodominant domain comprising amino acids 197 to 241 was identified as being strongly reactive. Intense serologic reactions with homologous antibodies to *B. burgdorferi* were noted for overlapping fragments E, F, and G. The antigen selected for the present study was fragment G (p41-G), which included amino acids 197 to 273. This fragment was chosen for the present evaluations because of encouraging results in preliminary serologic testing (4, 12) and because there was minimal sequence similarity to flagellin of *T. pallidum*.

Serologic tests. ELISAs (22, 23) were used to quantitate concentrations of antibodies to whole cells or the recombinant p41-G fragment of *B. burgdorferi*. For the persons who had erythema migrans, human sera were collected during or shortly after the presentation of these skin lesions, a time when IgM and IgG antibodies can be present. Previous work (38) showed that the latter first appear in detectable amounts at about 4 to 6 weeks after the onset of illness and can concomitantly occur with IgM antibodies. Therefore, polyvalent horseradish peroxidase-labeled goat anti-human IgG antibodies (heavy and light chain specific) were used in standard and biotin-streptavidin-amplified ELISAs (23) to detect antibodies in both classes. To confirm the presence of IgM antibodies in human sera, a class-specific ELISA (22) was employed.

Preliminary tests were conducted to standardize antigens and to determine cutoff values for ELISA results when the p41-G fragment was used. A commercially available protein assay (Bio-Rad, Richmond, Calif.) was used to determine protein concentrations of stock antigens. By utilizing positive control sera for Lyme borreliosis, antigen concentrations of 3 and 5 μg of protein per ml were most suitable for whole cells and the recombinant p41-G fragment, respectively. Dilutions of antigens and sera were screened by checkerboard titrations to determine optimal working concentrations. Forty negative human serum specimens from 40 persons who had no histories of spirochetal infections were analyzed with the p41-G fragment to determine cutoff values for positive results. In polyvalent ELISA, net optical density (OD) values of 0.13, 0.12, and 0.10 were considered positive for serum dilutions of 1:160, 1:320, and ≥1:640, respectively. These values were considerably lower than those computed in the ELISA containing whole-cell *B. burgdorferi* (net OD = 0.35, 0.24, and 0.17). In the amplified, polyvalent ELISA with the p41-G antigen, the same cutoff values computed for the standard ELISA with this recombinant antigen were acceptable. A net OD of 0.08 was the cutoff value for IgM analyses, regardless of the antigen coated to the solid phase. All critical regions for positive results were calculated by analyses of 40 negative control serum specimens. The mean OD reading for each serum dilution was added to three standard deviations of that respective set of data.

During routine analyses of test sera, each plate contained the same positive and negative serum controls and phosphate-buffered saline solution controls for each serum dilution tested to ensure standardization and to monitor nonspecific background readings. In tests with the recombinant p41-G antigen, affinity-purified glutathione *S*-transferase was included as an additional control to check for false-positive reactions. This was required because p41-G was purified as a glutathione transferase fusion protein. New lots of commercially prepared conjugated antibodies and other reagents were standardized before regular use.

Amplified ELISA. Affinity-purified biotin-labeled goat anti-human IgG antibodies (heavy and light chain specific) and horseradish peroxidase-labeled streptavidin were included in an ELISA as described earlier (23) to determine whether assay sensitivity could be enhanced. Human sera from each study group were analyzed in parallel tests with whole cells or p41-G antigens in standard and amplified ELISAs.

Adsorption. Removal of nonspecific treponemal antibodies from human sera was required before the amplified ELISA was used. A 1:10 dilution of commercially prepared sorbent of *Treponema phagedenis* biotype Reiter (SciMedx Corp., Denville, N.J.) was mixed with an equal volume of diluted human serum and incubated (23). Preliminary tests were conducted to determine whether preadsorption of sera with *E. coli* extracts was necessary. Forty negative control serum specimens, presumably containing antibodies to *E. coli*, did not react in ELISAs with the recombinant p41-G antigen or to the glutathione transferase controls at the cutoff values established. Also, earlier studies with the p41-G fragment (4) revealed minimal sequence similarities to flagellins of other bacteria, including *E. coli*. For these reasons, sera were not preadsorbed with an *E. coli* extract.

RESULTS

Serum samples from persons who had erythema migrans and antibodies to whole-cell *B. burgdorferi*, as determined by a standard ELISA with a polyvalent conjugate, also reacted positively in an ELISA with the p41-G antigen. Comparable sensitivity was noted (Table 1). In addition, the ELISA with the p41-G antigen detected antibodies in 3 of 36 serum specimens from patients who had expanding skin lesions but no detectable antibodies to whole-cell *B. burgdorferi*. These three positive serum samples were collected 3 to 6 weeks after the onset of illness. In analyses of the sera with antibodies to treponemes, there was more than a twofold decline in the number of false-positive reactions in an ELISA with the p41-G antigen, compared with results of an ELISA with whole-cell *B. burgdorferi*.

Preadsorbed sera reacted similarly to whole cells and recombinant antigens in an amplified ELISA (Table 1). Results closely parallel those noted for analyses of untreated sera. The numbers of positive reactions for the two study groups with erythema migrans and for the persons who had oral infections were nearly equal, regardless of the antigen or assay method used. Increased specificity, however, was noted when the p41-G antigen was used in analyses of syphilitic sera. The lowest number of false-positive reactions ($n = 4$) for this group was recorded when sera were treated with *T. phagedenis* sorbent and tested by an amplified ELISA with the p41-G antigen.

Geometric mean antibody titers and the ranges of titration endpoints recorded were generally higher for sera tested by an ELISA with whole-cell *B. burgdorferi* (Table 2) than for those analyzed with the p41-G fragment. For example, the geometric mean antibody titers for unadsorbed sera collected from persons who had erythema migrans and *B. burgdorferi* antibody were more than twofold greater (but not statistically significant by Student's t test; $t = 1.32$, $P = 0.05$) than those obtained with the p41-G antigen. Reactivity

TABLE 1. Reactivity of human sera to whole cells and affinity-purified flagellin fragment (p41-G) of *B. burgdorferi* in standard and biotin-streptavidin-amplified ELISA

Study group	Total no. of serum specimens tested	No. (%) of serum specimens positive by:			
		Unadsorbed standard ELISA with:		Biotin-streptavidin-amplified ELISA[a]	
		Whole cells	p41-G	Whole cells	p41-G
EM, antibodies[b]	17	17 (100)	15 (88)	17 (100)	16 (94)
EM, no antibodies[b]	36	0	3 (8)	3 (8)	3 (8)
Oral infections[c]	13	5 (39)	2 (15)	5 (39)	2 (15)
Syphilis[d]	20	13 (65)	6 (30)	14 (70)	4 (20)
Control[e]	40	0	0	0	0

[a] Sera were preadsorbed with a 1:10 dilution of *T. phagedenis* biotype Reiter.

[b] Erythema migrans (EM) with or without total immunoglobulins to *B. burgdorferi* in the standard, polyvalent ELISA.

[c] Acute necrotizing ulcerative gingivitis or periodontitis with antibodies to oral treponemes but no histories of Lyme borreliosis. Results for the standard, polyvalent ELISA were published earlier (25) and are shown here for comparison.

[d] Homologous antibodies to *T. pallidum* in Venereal Disease Research Laboratory and fluorescent antibody adsorption tests were cross-reactive in the standard, polyvalent ELISA for Lyme borreliosis, but there was no clinical evidence of Lyme borreliosis.

[e] No histories of spirochetoses (negative controls). Note that all sera from patients with systemic lupus erythematosus or mixed connective tissue disease were nonreactive in an ELISA with whole cells or the p41-G antigen.

of these sera in an ELISA with the recombinant antigen, however, remained strong. Also, analysis of unadsorbed sera from patients with oral infections or syphilis demonstrated geometric mean titers markedly greater with whole-cell *B. burgdorferi* than with p41-G antigen. Differences in geometric means for syphilitic sera were highly significant ($t = 4.13$, $P = 0.01$), whereas the nearly threefold difference noted for the study group with oral infections was not statistically significant ($t = 1.62$, $P = 0.05$).

To verify the presence of IgM antibody, a subset of unadsorbed sera were retested by an ELISA including whole cells or the p41-G recombinant antigen. Results of both assays (Table 3) indicate comparable sensitivities when sera from serologically confirmed cases of Lyme borreliosis were analyzed. Similar to results of an ELISA with polyvalent, peroxidase-conjugated antibodies, sera from persons who had expanding skin lesions but no detectable antibodies to whole-cell *B. burgdorferi* were positive when tested against the p41-G antigen. As before, there were far fewer false-positive reactions ($n = 4$) when 27 serum specimens from syphilitic patients or persons with oral infections were tested against the recombinant p41-G antigen.

DISCUSSION

The recombinant p41-G antigen in an ELISA is suitable for serologic confirmation of Lyme borreliosis during early weeks of infection. This supports and extends earlier work (4, 12). Compared with results of an ELISA with whole-cell *B. burgdorferi*, there was comparable sensitivity and increased specificity. Use of the p41-G fragment improved assay sensitivity for the group of sera obtained from persons who had erythema migrans but lacked antibodies to whole-cell *B. burgdorferi*. Moreover, the lower OD readings for negative serum controls in the polyvalent ELISA with the p41-G antigen clearly reflect improved specificity and assay performance. However, false-positive reactions, due to treponemal antibodies, still occurred, albeit less frequently than in an ELISA with whole-cell *B. burgdorferi*. In analyses of amino acid sequences of *B. burgdorferi* and *T. pallidum* flagellins (4), a short segment of homology for amino acids 211 to 216 (four of six being identical) was identified. Whether this contributed to cross-reactivity is unknown. Nonetheless, if clinical data or further analysis of sera by a Venereal Disease Research Laboratory test could be used to reliably separate syphilis from Lyme borreliosis, the specificity problems noted here would be greatly minimized.

Detection of IgM antibody during early weeks of *B. burgdorferi* infection is difficult, regardless of the antigen or assay method used. Concentrations of IgM antibodies in serum are usually much lower than those of IgG antibodies 6 weeks or later after the onset of illness (10, 22, 34, 38). In the absence of antibiotic therapy, IgM antibody is slow to rise and normally is not detected until at least 3 to 6 weeks after onset of illness. Therefore, the time when a blood sample is obtained from a patient is a critical factor. The number of false-negative reactions can be reduced if blood samples are collected during periods of peak IgM antibody titer and rising IgG antibody concentrations. Clinical data are needed to help determine such sampling times.

During the later stages of Lyme borreliosis, marked by carditis, neuritis, or arthritis, IgM antibody can be absent or at much lower concentrations than IgG antibodies. The sensitivity of currently used assays for IgG antibodies is notably higher than the sensitivity of those designed to detect IgM antibodies because with disseminated infection and progressing disease, there is an expansion in humoral response to multiple surface and flagellar antigens of *B. burgdorferi* (6, 9, 37). This broad immune response consists

TABLE 2. Geometric means and ranges of antibody titers to whole cells and flagellin fragment (p41-G) of *B. burgdorferi* in standard and biotin-streptavidin-amplified ELISAs[a]

Study group[b]	$\bar{x}^c$ (range) of antibody titers in:			
	Unadsorbed standard ELISA with:		Biotin-streptavidin-amplified ELISA with:	
	Whole cells	p41-G	Whole cells	p41-G
EM, antibodies	2,560 (160–40,960)	1,180 (640–10,240)	3,270 (640–40,960)	1,280 (160–5120)
EM, no antibodies	40	48 (160–640)	40	49 (320–640)
Oral infections	152 (160–5120)	58 (320–640)	209 (640–20,480)	61 (640)
Syphilis	380 (640–2,560)	67 (160–320)	663 (640–10,240)	65 (160–1,280)

[a] Polyvalent peroxidase-labeled anti-human IgGs (heavy and light chain specific) were used to detect IgM and IgG antibodies in sera.

[b] See Table 1, footnotes *b* to *d*, for explanations.

[c] Geometric means (a value of 40, the average titer for negative sera, was used for each negative sample in analysis).

TABLE 3. Reactivity of human sera to whole cells and affinity-purified flagellin fragment (p41-G) of *B. burgdorferi* in a standard ELISA for IgM antibodies[a]

Study group[b]	Total no. of serum specimens tested	Whole-cell results			p41-G results		
		No. (%) of positive specimens	Antibody titer		No. (%) of positive specimens	Antibody titer	
			$\bar{x}^c$	Range		$\bar{x}^c$	Range
EM, antibodies	9	8 (89)	871	160–10,240	7 (77.8)	470	320–10,240
EM, no antibodies	10	1 (10)	49	320	4 (40)	90	160–320
Oral infections	9	7 (78)	160	160–1,280	2 (22.2)	87	320–640
Syphilis	18	11 (61)	127	160–640	2 (11.1)	52	320
Controls	40	0			0		

[a] Peroxidase-labeled goat anti-human IgM (μ-chain-specific) antibodies used.
[b] See Table 1, footnotes *b* to *e*, for explanations.
[c] Geometric mean (a value of 40, the average titer for negative sera, was used for each negative sample in analyses).

mainly of IgG antibodies directed to flagellin (41-kDa polypeptide) and other key antigens, such as those with molecular masses of about 31, 34, 39, 83, and 110 kDa. These proteins are thought to be highly immunogenic and more specific than flagellin (1, 2, 5, 21, 36).

The use of key subunit antigens, including recombinant proteins, appears to be an acceptable option for a class-specific or polyvalent ELISA. Removal of irrelevant proteins (i.e., those not immunologically recognized) from material coated to the solid phase of ELISA would allow the most important antigens to bind to the polystyrene plates. This eliminates competition for binding sites, optimizes serum antibody reactivity to coated antigen, and reduces nonspecific antibody reactivity. Comparable assay sensitivity and greater specificity were achieved earlier by using mixtures of purified subunit antigens (24). The addition of the p41-G fragment to this or other highly specific combinations of subunit antigens should further improve assay performance.

An assortment of immunologic methods has been presented here and elsewhere to improve serologic diagnosis of Lyme borreliosis. Despite these advances, false-negative and false-positive reactions still occur, albeit less frequently when purified preparations of antigens and supportive serologic testing are employed. Therefore, clinical data must still take priority when diagnoses are made. Epidemiological and serological findings can best be used as supportive evidence of *B. burgdorferi* infection.

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