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Use of Recombinant Antigens of *Borrelia burgdorferi* in Serologic Tests for Diagnosis of Lyme Borreliosis

LOUIS A. MAGNARELLI,[1]* EROL FIKRIG,[2] STEVEN J. PADULA,[3]
JOHN F. ANDERSON,[1] AND RICHARD A. FLAVELL[4]

Department of Entomology, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504[1]; Section of Rheumatology,[2] Immunobiology and Howard Hughes Medical Institute,[4] Yale University School of Medicine, New Haven, Connecticut 06510; and Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030[3]

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Recombinant antigens of outer surface proteins (Osps) OspA, OspB, OspC, OspE, and OspF of *Borrelia burgdorferi* sensu stricto and of p41-G, an antigenic region of flagellin of this spirochete, were tested with human sera in class-specific and polyvalent enzyme-linked immunosorbent assays (ELISAs). In analyses for immunoglobulin M (IgM) antibodies, 18 (85.7%) of 21 serum samples from persons who had been diagnosed as having Lyme borreliosis on the basis of the presence of erythema migrans reacted positively in ELISAs with one or more Osp antigens or the p41-G antigen. Eleven serum samples contained antibodies to OspC antigen, and of these, six also reacted to the p41-G antigen and to one or more of the other recombinant antigens. The remaining five serum samples reacted solely to OspC ($n = 4$) or to OspC plus OspA and OspE without reactivity to p41-G ($n = 1$). In analyses for IgG antibodies, seropositivity was comparable to that of IgM analyses and was marked by predominant reactivity to p41-G, OspC, and OspF. Similarly, all 21 serum samples were positive in polyvalent and class-specific ELISAs with whole-cell *B. burgdorferi*. Minor cross-reactivity was noted when sera from persons who had syphilis, periodontitis or other oral infections, or rheumatoid arthritis were tested with OspC, OspE, OspF, and p41-G. With relatively high degrees of specificity, ELISAs with recombinant antigens, particularly OspC and p41-G, can help to confirm *B. burgdorferi* infections.

Despite the limitations of laboratory analyses for Lyme borreliosis, detection of antibodies to *Borrelia burgdorferi* sensu lato in serum or cerebrospinal fluid remains diagnostically important. In recent years, Western blot (immunoblot) analyses have been relied on more heavily to provide evidence of *B. burgdorferi* infections in persons who present with cardiac, neurologic, or arthritic disorders. There has been a steady accumulation of information on the patterns of reactivity of serum antibodies to the key immunodominant proteins of *B. burgdorferi*, such as those with molecular masses of 23, 31, 34, 39, 41, and 93 kDa (1, 4, 5, 11, 13, 14, 22, 25–28). However, immunoblotting procedures are laborious, increase costs for laboratory diagnosis, and can be technically problematic if there is comigration of multiple proteins to the same area (4) or if key banding patterns are lacking.

Enzyme-linked immunosorbent assays (ELISAs) continue to be the primary screening methods for detecting antibodies to *B. burgdorferi*. These tests, often used in conjunction with Western blot analyses, have disadvantages. False-positive and false-negative results have been reported for ELISAs containing whole-cell antigen (18, 20, 24, 26). Moreover, these methods can be insensitive during the initial 3 weeks of infection (24). Subunit or recombinant outer surface protein (Osp) antigens of *B. burgdorferi* can be used in an ELISA (17, 19, 22) and, in some instances, have improved the performance of these serologic tests. Immunoglobulin M (IgM) antibody reactivity to OspC, in particular, shows promise for increasing assay sensitivity and specificity for sera obtained during early Lyme disease (22). The present study was conducted with different human serum samples to determine if recombinant Osp antigen is suitable for the detection of class-specific antibodies to *B. burgdorferi* by ELISAs. The main objective was to determine if the use of recombinant antigens, particularly OspC, improved the detection of IgM antibodies during the initial weeks of Lyme borreliosis.

* Corresponding author. Mailing address: Department of Entomology, The Connecticut Agricultural Experiment Station, P.O. Box 1106, New Haven, CT 06504-1106. Phone: (203) 789-7241. Fax: (203) 789-7232.

MATERIALS AND METHODS

Study groups. Five study groups were selected for analyses. Sera from 21 persons who had expanding skin lesions (erythema migrans) and antibodies to *B. burgdorferi*, as determined by class-specific or polyvalent ELISAs (15, 19), were from Connecticut. On the basis of clinical information provided by physicians, the serum samples were obtained between 1 and 5 weeks after the onset of illness. The second group consisted of 32 serum samples from Connecticut residents who were strongly suspected of having Lyme borreliosis. Erythema migrans and clinical evidence of syphilis were not reported, but patients lived in tick-infested areas of Connecticut and presented with fever and arthralgias and sometimes had unexplained neurologic disorders during the summer or early fall. Blood samples were obtained prior to antibiotic therapy within 5 weeks after the onset of illnesses. An additional study group consisted of serum samples from 30 persons who had secondary or latent syphilis and antibodies to *Treponema pallidum* in Venereal Disease Research Laboratory and fluorescent-antibody adsorption tests (titers of 1:64 or greater). The latter test was performed as described previously (16, 20) and also was used to screen sera from the second study group (i.e., those suspected of having Lyme borreliosis without erythema migrans) for antibodies to *T. pallidum*. In another study group, eight serum samples from persons who had acute necrotizing ulcerative gingivitis or periodontitis were included to check for possible cross-reactivity with treponemal antibody. Finally, seven serum samples from patients who had rheumatoid arthritis and rheumatoid factor IgM antibody were tested with recombinant antigens of *B. burgdorferi*. The sources of all serum samples, including 30 serum samples from healthy subjects (negative controls), and serologic reactivities with whole-cell *B. burgdorferi* in polyvalent or class-specific ELISAs have been reported previously (15–20).

Antigen preparations. Whole-cell antigen and six recombinant antigens of *B. burgdorferi* (OspA, OspB, OspC, OspE, OspF, and p41-G) were used in comparative analyses. Whole-cell *B. burgdorferi* (strain 2591) was derived from lab-

TABLE 1. Reactivity of human sera to whole cells and recombinant antigens of *B. burgdorferi* in class-specific ELISA for IgM antibodies

Study groups	Total no. of serum samples tested	No. of serum samples positive to:						
		Whole cells[a]	OspA	OspB	OspC	OspE	OspF	p41-G
EM, antibodies[b]	21	21	6	0	11	8	1	9
No EM[c]	32	8	4	1	1	3	1	1
Syphilis[d]	30	19	0	0	0	1	2	5
Oral infections[e]	8	1	0	0	1	0	1	0
Rheumatoid arthritis[f]	7	2	0	0	0	1	0	0
Normal controls[g]	30	0	0	0	0	0	0	0

[a] Results for some sera were published earlier (15, 16, 19) and are included here for comparison. Whole cells and human sera were tested with peroxidase-labeled anti-human IgM (μ chain specific) to detect IgM antibodies.
[b] EM, erythema migrans; positivity for antibodies to *B. burgdorferi* was also found by a standard polyvalent ELISA.
[c] Suspected Lyme borreliosis but no erythema migrans and no antibodies to whole-cell *B. burgdorferi* in a polyvalent ELISA.
[d] Secondary or latent syphilis with homologous antibodies to *T. pallidum*.
[e] Acute necrotizing ulcerative gingivitis or periodontitis with antibodies to oral treponemes (20) but no histories of Lyme borreliosis.
[f] Diagnosed rheumatoid arthritis but no clinical signs of *B. burgdorferi*.
[g] Negative controls (no evidence of spirochetoses).

oratory cultures of BSK II medium at the Connecticut Agricultural Experiment Station. The spirochetes had been passed hundreds of times after isolation from a white-footed mouse (*Peromyscus leucopus*) caught in East Haddam, Conn. (2). Methods of preparing and standardizing this antigen for use in an ELISA have been described elsewhere (15, 19). The recombinant antigens were cloned and expressed as fusion proteins in *Escherichia coli* at the University of Connecticut (22, 23) or at Yale University (6, 7, 12, 19, 21). The gene encoding OspC was cloned from *B. burgdorferi* 2591. The remaining recombinant antigens were produced at Yale University from *B. burgdorferi* N40 and, like OspC (23 kDa), consisted of purified glutathione transferase fusion proteins; p41-G is a fragment of flagellin (41 kDa) comprising amino acids 197 to 241, while OspA, OspB, OspE, and OspF have molecular masses ranging from 19.2 to 34 kDa. Affinity-purified glutathione transferase was included as a control in an ELISA to check for false-positive reactions.

Serologic tests. Polyvalent and class-specific ELISAs (15) were used to quantitate the concentrations in serum of antibodies to washed whole cells or purified recombinant antigens of *B. burgdorferi*. It was necessary to conduct further tests with negative human serum samples to determine cutoff values for positive results in analyses for IgM and IgG antibodies when ELISAs contained the recombinant antigens. Net optical density (OD) values were computed for serum dilutions of 1:160, 1:320, and 1:640 or greater by analyzing 14 to 30 negative serum samples. Cutoff values were determined by performing statistical analyses (three standard deviations plus the mean) of the net absorbance values for the respective data sets. In analyses for IgM antibodies with OspA or OspB antigens, net OD values of 0.45, 0.27, and 0.20 and 0.37, 0.28, and 0.20 were considered positive for the respective serum dilutions. Net OD values for tests with OspE antigen were lower (0.20, 0.11, and 0.10, respectively). Similar critical regions were calculated for analyses with OspC (0.09, 0.04, and 0.04, respectively) and OspF (0.05, 0.04, and 0.04, respectively). Respective cutoff values for tests with p41-G were 0.08, 0.04, and 0.04. In each case, a commercially available protein assay (Bio-Rad, Richmond, Calif.) was used to determine the protein concentrations of stock antigens. Concentrations of 5 μg of protein per ml were the most suitable for the optimal reactivity of recombinant antigens with known positive human sera, regardless of analyses for IgM or IgG antibodies. In the latter, critical regions for positive results varied from those calculated for analyses of IgM antibodies. The net respective OD values were highest when OspB (0.40, 0.26, and 0.26), OspE (0.38, 0.24, and 0.14), p41-G (0.37, 0.23, and 0.14), OspC (0.30, 0.19, and 0.19), and OspA (0.29, 0.18, and 0.18) were used in the assays. Lower cutoff values were calculated for ELISAs with OspF antigen (0.10, 0.05, and 0.04, respectively). Previous work on the reproducibility of ELISA results demonstrated twofold variability in antibody titers (15). In the present study, 12 serum samples from healthy subjects reacted weakly with one or more recombinant antigens at a serum dilution of 1:160. Therefore, in an effort to obtain conservative assay results, serum dilutions of 1:320 or greater were used to assess seropositivity in all analyses.

When test sera were analyzed, each plate contained the same positive and negative serum controls as well as controls for phosphate-buffered saline solutions and peroxidase-labeled antibodies to ensure assay standardization and to check for false-positive reactions. Positive human sera were from persons who were diagnosed as having erythema migrans and other signs and symptoms of Lyme borreliosis. These serum samples had been analyzed by Western blot analysis, which showed bands verifying reactivity to one or more of the specific antigens included in the study. In addition, murine monoclonal antibodies (H5332, H6831, and H9724), kindly provided by A. G. Barbour of the University of Texas (San Antonio), were used in an ELISA with a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) to verify the antigen reactivities of OspA, OspB, and the flagellin of *B. burgdorferi*. Similarly, a monoclonal antibody to OspC was kindly provided by B. Wilske (University of Munich, Munich, Germany) for similar controls.

RESULTS

In analyses for IgM antibodies, the use of recombinant antigens, particularly OspC and p41-G, confirmed the results of polyvalent and class-specific ELISAs with the whole-cell antigen of *B. burgdorferi*. In analyses for total immunoglobulins or class-specific antibodies to whole cells, all 21 serum samples obtained from persons who had erythema migrans were positive. In analyses for class-specific IgM antibodies to recombinant antigens, 18 (85.7%) of 21 serum specimens showed reactivity to one or more of the Osp or p41-G antigens (Table 1). Eleven serum samples contained antibodies to OspC antigen, and of these, six serum samples also reacted to the p41-G antigen (of a total of nine p41-G-positive serum samples) or to one or more of the other recombinant antigens. The remaining five serum samples reacted solely to OspC ($n = 4$) or to OspC plus OspA and OspE antigens ($n = 1$) without showing antibodies to p41-G. In analyses of the 10 serum samples without antibodies to OspC, 3 contained antibodies to p41-G. There were four positive serum samples in this group that had antibodies to one or more Osp antigens but showed no reactivity with the OspC or p41-G antigen. The remaining three serum samples were negative for all recombinant antigens. Although reactivity with OspE was less frequent than seropositivity with OspC or p41-G, the immune response to the former was more prevalent than that to OspA, OspB, or OspF. In analyses of 32 serum samples from persons who had no histories of having expanding skin lesions but who were strongly suspected of having Lyme borreliosis, eight serum specimens had IgM antibodies (titers of 1:320 to 1:640) to one or more of the recombinant antigens. Twenty-four serum samples were negative for IgM antibodies in a class-specific ELISA with whole-cell antigen, and all 32 specimens lacked antibodies to *T. pallidum*. Analyses of an additional 45 serum samples from persons who had syphilis, oral infections, or rheumatoid arthritis revealed infrequent reactivity to OspC ($n = 1$), OspE ($n = 2$), OspF ($n = 3$), or p41-G ($n = 5$) antigens at serum dilutions of 1:320 to 1:1,280. The highest titer was recorded for a syphilitic serum sample. The 30 serum samples from healthy subjects were negative in all tests.

Seropositivity to recombinant antigens in analyses for IgG antibodies (Table 2) was comparable to the results of IgM antibody tests. In the group with erythema migrans, 11 of 21 serum specimens reacted with the p41-G antigen in ELISA,

TABLE 2. Reactivity of human sera to whole cells and recombinant antigens of *B. burgdorferi* in a class-specific ELISA for IgG antibodies

Study groups[a]	Total no. of serum samples tested	No. of serum samples positive to:						
		Whole cells	OspA	OspB	OspC	OspE	OspF	p41-G
EM, antibodies[b]	21	21	0	0	9	1	7	11
No EM[c]	32	5	1	0	4	1	0	8
Syphilis	30	28	0	0	2	0	0	4
Oral infections	8	0	0	0	1	0	0	0
Rheum. arthritis	7	1	0	0	0	0	0	1
Normal controls	30	0	0	0	0	0	0	0

[a] See Table 1, footnotes *b* to *g*, for explanations of the study groups.
[b] All sera also were positive in polyvalent ELISA for IgG antibodies to whole-cell *B. burgdorferi* (15, 16, 19).
[c] No IgG antibodies to whole-cell *B. burgdorferi* were detected in a polyvalent ELISA, and no antibodies to *T. pallidum* were present.

while 9 and 7 samples contained antibodies to OspC and OspF, respectively. There was seropositivity to p41-G, with less frequent reactivity to Osp antigens or whole-cell *B. burgdorferi* in the study groups lacking erythema migrans. Five of 32 serum samples from persons suspected of having Lyme borreliosis (without expanding skin lesions) had IgG antibodies to *B. burgdorferi* in a class-specific ELISA with whole cells. However, none of these sera were positive in a polyvalent ELISA with whole-cell antigen or in tests for antibodies to *T. pallidum*. There was minor cross-reactivity in the remaining study groups when sera were tested by a class-specific ELISA. As in the analyses for IgM antibodies, the sera from healthy subjects were negative.

Geometric means and ranges of antibody titers were compared for results of class-specific and polyvalent analyses with recombinant and whole-cell antigens. In general, geometric mean values and titration endpoints for sera obtained from patients who had erythema migrans were highest in a polyvalent assay for total antibodies ($\bar{x}$ = 2,712; titer range, 1:320 to 1:20,480). In class-specific ELISAs with whole-cell antigen, geometric means for IgM and IgG antibody titers were 1,613 and 4,200, respectively. In analyses with recombinant antigens in a class-specific ELISA for IgM antibodies, relatively higher geometric means and titration endpoints were recorded when OspC and p41-G antigens were used (Table 3). Similarly, relatively high geometric means and titers were noted in analyses for IgG antibodies when p41-G, OspC, and OspF antigens were coated onto polystyrene plates.

TABLE 3. Geometric means and ranges of antibody titers to recombinant antigens of *B. burgdorferi* in class-specific ELISAs for sera obtained from persons who had erythema migrans

Antigen[a]	$\bar{x}$ (range) antibody titers[b]	
	IgM antibodies	IgG antibodies
OspA	87 (320–640)	49 (5,120)
OspB	40	46 (1,280)
OspC	381 (320–20,480)	143 (320–5,120)
OspE	98 (320–640)	45 (640)
OspF	55 (320–640)	180 (640–40,960)
p41-G	226 (320–10,240)	339 (320–10,240)

[a] Class-specific peroxidase-labeled anti-human IgM (μ chain specific) or anti-human IgG (γ chain specific) antibodies were used with antigens and sera.
[b] Geometric means (a value of 40, the average titer for negative sera, was used for each negative sample in analyses). Single reciprocal titers or ranges of reciprocal antibody titers are listed.

DISCUSSION

Recombinant antigens of *B. burgdorferi*, particularly OspC and p41-G, were useful in class-specific ELISAs in detecting serum antibodies to this bacterium. These results confirm and extend earlier findings (1, 8–10, 19, 22, 27) and indicate that IgM antibodies to these proteins are frequently produced during the early weeks of *B. burgdorferi* infection. In recent work (10), an ELISA with recombinant OspC antigen was significantly more sensitive for the laboratory diagnosis of early Lyme borreliosis than an ELISA with whole cells or by immunoblotting procedures. With adequate sensitivity and an apparently high degree of specificity, recombinant OspC and p41-G antigens are desirable reagents for inclusion in the future development of more accurate ELISA procedures. Antibodies to OspE and OspF are also produced by persons who have Lyme borreliosis (21), but the prevalence of seropositivity was lower than that recorded for results with OspC and p41-G. Detection of IgM antibodies to OspE or IgG antibodies to OspF, with concomitant seropositivity to p41-G or OspC antigens, strengthens serodiagnoses for early Lyme borreliosis because of the relatively high degrees of specificity of these antigens. Finally, the lower degrees of prevalence of seropositivity for ELISAs with OspA or OspB recombinant antigens reaffirm that serum antibodies to these proteins occur infrequently. There is sometimes an absence of antibodies to these antigens during progressing Lyme borreliosis even when there is an expansion in the immune response (4, 5). Inclusion of these antigens in an ELISA or Western blot analysis, however, is still useful because of the high degrees of specificity of positive reactions.

There was minor cross-reactivity in analyses for IgM antibodies when the recombinant antigens were tested. Infrequent false positivity was previously reported for an ELISA with p41-G antigen (19). Net OD values for false-positive results associated with recombinant Osp antigens were usually at or close to the cutoff values calculated for serum dilutions of 1:320. Nonetheless, compared with a polyvalent or class-specific ELISA with whole cells, the use of highly specific recombinant antigens in this assay allowed for a more accurate interpretation of test results. A mixture of key antigens will ultimately be required in seroanalyses, whether they be ELISAs or immunoblots, to compensate for potentially lower levels of assay sensitivity because of variable host immune responses and the absence of specific antibodies to certain proteins. Purified preparations of polypeptides with molecular masses of 39 and 93 kDa were unavailable for the present study. Results of immunoblot analyses, however, indicate that antibodies to the p39 antigen are frequently produced during early Lyme borreliosis (1, 5, 14). Like OspC and p41-G anti-

gens, p39 and p93 antigens appear to be good serologic markers for Lyme borreliosis. Therefore, these antigens should be considered along with OspC, OspE, OspF, and p41-G in future studies of the serodiagnosis of Lyme borreliosis.

In the absence of erythema migrans, it is often difficult to correlate nonspecific signs and symptoms of disease with serologic test results. Even when clinical signs are compatible with those of Lyme borreliosis, such as physician-diagnosed erythema migrans, results of ELISA or Western blot analyses can be negative (3, 26). Seronegativity for clinically diagnosed Lyme borreliosis is well-documented (5, 24, 26). Delayed host immune response and the effects of antibiotic treatment in aborting or curtailing antibody production during early Lyme borreliosis are thought to be important factors (26) contributing to false-negative test results. In the present study, physicians had strongly suspected Lyme borreliosis (in the absence of erythema migrans) for 32 persons who lived in tick-infested areas of Connecticut. The results of polyvalent and class-specific ELISAs with whole-cell *B. burgdorferi* were negative in the majority of cases. There was likewise infrequent serologic reactivity to confirm exposure to *B. burgdorferi* when recombinant antigens were included in class-specific assays. We suspect that presumptive diagnoses for Lyme borreliosis were probably incorrect in many instances. The eight serum samples from this study group, which were negative in the polyvalent ELISA with whole cells but which were positive in a class-specific ELISA with whole cells, had low concentrations (titers of 1:320 to 1:640) of IgM antibody. When whole-cell antigen is used, many polypeptides and other components of *B. burgdorferi*, which are not immunologically recognized, can bind to polystyrene plates. If homologous antibody reactions do not occur or are reduced because of insufficient amounts of exposed key immunodominant antigens, there can be a corresponding loss of assay sensitivity. Therefore, when there are low concentrations of IgM antibodies, the use of purified preparations of highly specific immunodominant antigens, such as OspC and p41-G, might improve assay sensitivity when one is testing sera obtained during the initial weeks of *B. burgdorferi* infections.

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