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Adsorption and Biotin-Streptavidin Amplification in Serologic Tests for Diagnosis of Lyme Borreliosis

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Serum samples from persons with Lyme borreliosis, periodontitis, or acute necrotizing ulcerative gingivitis were analyzed by an enzyme-linked immunosorbent assay (ELISA) with and without adsorption and amplification procedures. When biotin and streptavidin reagents were used as an amplification procedure in ELISA without the use of commercially prepared sorbent (*Treponema phagedenis* biotype Reiter), sensitivity increased. Of the 85 serum samples collected from persons with erythema migrans but no detectable antibodies to *Borrelia burgdorferi* by standard ELISA, 17 (20%) were reactive after amplification. Adsorption of serum samples with a 1:10 dilution of *T. phagedenis* biotype Reiter sorbent used in conjunction with amplified ELISA also improved the sensitivity of this method. However, cross-reactivity could not be completely eliminated. An adsorbed-amplified ELISA may be helpful in the diagnosis of Lyme borreliosis in the laboratory, particularly during early weeks of infection, when antibodies to *B. burgdorferi* can be present at a low concentration.

Serologic analyses for Lyme borreliosis are diagnostically important, particularly when the characteristic skin lesion, erythema migrans, is atypical or absent (3, 18, 19, 27, 28). However, the sensitivities of indirect fluorescent-antibody staining methods and enzyme-linked immunosorbent assays (ELISAs) can be low if serum samples are obtained within 3 weeks after the onset of illness (27). With an expansion in the immune response during the later stages of disease (6, 9), however, detection of serum and intrathecal antibodies improves (18, 27, 29, 32). In addition, false-positive reactions can occur if there are high concentrations of antibodies to *Treponema* spirochetes (7, 16, 21, 23, 24).

Recent work has improved the sensitivity and specificity of serologic tests for the diagnosis of Lyme borreliosis. Purified flagellin (with or without biotinylated reagents) or flagellin-enriched fractions of *Borrelia burgdorferi* have been used in ELISAs to more efficiently detect antibodies during early weeks of infection (4, 5, 11, 12, 15). Subunit preparations lacking flagellin reduce cross-reactivity and improve serologic diagnosis during later stages of Lyme borreliosis (20). Moreover, antibody-capture ELISA (3, 14) and use of *Treponema* sorbent (23) apparently enhance sensitivity and specificity, respectively. The use of the biotin-streptavidin complex can also enhance the sensitivity of immunoassays. For example, implementation of this procedure has improved immunoenzyme staining techniques (1, 30, 31) and the detection of antibodies to viral antigens (26). Accordingly, we tested biotin-streptavidin amplification procedures with and without adsorption methods to determine whether the sensitivity and specificity of ELISA for the diagnosis of Lyme borreliosis could be improved. Emphasis was placed on enhancing assay sensitivity for the detection of antibodies in serum produced during early Lyme borreliosis.

MATERIALS AND METHODS

Study groups. Serum samples were obtained from persons with erythema migrans. In some cases, sera contained antibodies to *B. burgdorferi* (n = 40), while in other instances, there was no serologic confirmation (n = 85). Sera were also collected from persons with periodontitis or acute nectrotizing ulcerative gingivitis but no history of Lyme borreliosis (n = 13) or from persons who were being tested for syphilis in a premarital screening program (n = 36). An additional 20 serum samples from 20 persons with no histories of spirochetoses were used as negative controls. Those who presented with expanding skin lesions for Lyme borreliosis lived in Connecticut, and blood samples were collected from these individuals within 10 weeks after the onset of illness. Further details on clinical histories and sources of sera for these patients, persons with oral infections, or those selected from a premarital screening program have been reported previously (18, 19, 23). Following initial analyses by standard ELISA (19, 22), serum samples were stored at −60°C at the Connecticut Agricultural Experiment Station.

Serologic tests. Antibodies were detected and quantitated by a standard or modified ELISA in parallel tests. In the standard assay, whole cells of *B. burgdorferi* were coated to the solid phase, and analyses with polyvalent horseradish peroxidase-labeled goat anti-human immunoglobulin G (IgG) antibodies were performed as described before (22). In the modified amplification assay, affinity-purified biotin-labeled goat anti-human IgG antibodies (heavy and light chain specific) were diluted to 1:7,000 in phosphate-buffered saline solution (containing 5.0% horse serum and dextran sulfate) and were added to plates (60 μl per well). After 1 h of incubation at 37°C and removal of unbound biotinylated antibodies by washing, horseradish peroxidase-labeled streptavidin was diluted to 1:10,000 in the phosphate-buffered saline diluent and added (60 μl per well) to the test plates. Following incubation for 1 h and washing, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) substrate was added, and plates were incubated for another hour. Net optical density values of 0.25, 0.21, and 0.17 were considered positive for the respective serum dilutions of 1:160, 1:320, and ≥1:640 in the amplified ELISA. Two of these values were lower than those computed in the standard ELISA (0.35, 0.24, and 0.17, respectively). Significant optical density values (i.e., critical regions) for positive results were determined by analyzing 20 negative serum samples from 20

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TABLE 1. Reactivities of nonadsorbed human sera to whole cells of *B. burgdorferi* by ELISA, with and without biotin-streptavidin

Study group	No. of serum samples tested	Standard ELISA			Amplified ELISA		
		No. (%) positive	Antibody titer		No. (%) positive	Antibody titer	
			Range	$\bar{x}^a$		Range	$\bar{x}^a$
EM, antibodies[b]	40	40 (100)	160–40,980	791	40 (100)	160–40,980	1,298
EM, no antibodies[b]	85	0		40	17 (20)	160–2,560	67
Oral infections[c]	13	5 (39)	160–5,120	152	10 (77)	160–40,960	751
Premarital screening[d]	36	1 (3)	640	43	8 (22)	160–1,280	70
Controls[e]	20	0		40	0		40

[a] Geometric means (a value of 40, which is the average titer for negative sera, was computed for each negative sample in the analysis).
[b] Erythema migrans (EM) with or without detectable antibodies by standard ELISA to *B. burgdorferi* 10 weeks or less after onset of illness.
[c] Acute necrotizing ulcerative gingivitis or periodontitis, antibodies to oral treponemes, and no histories of Lyme borreliosis. Results for standard ELISA were published earlier (23) and are listed here for comparison.
[d] Routine premarital testing for antibodies to *T. pallidum* and no histories of spirochetoses.
[e] No histories of spirochetoses (negative controls).

persons with no history of spirochetal infections and by calculating three standard deviations plus the mean for net optical density readings representing each negative serum dilution analysis. During routine testing, each plate contained positive and negative serum controls. New lots of commercially prepared biotinylated antibodies and other conjugated reagents (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were standardized before regular use.

Adsorption procedures. A commercially prepared sorbent of *Treponema phagedenis* biotype Reiter (SciMedx Corp., Denville, N.J.) was used to remove nonspecific treponemal antibodies in serum before standard and amplified ELISAs were performed. This reagent was chosen for evaluation because it is commercially available and is widely used in diagnostic laboratories. Different sorbent dilutions (1:2, 1:5, and 1:10) were tested in standard and amplified ELISAs to determine an optimal concentration which would increase the specificity and maintain the sensitivity. Equal volumes of diluted sorbent and human sera were mixed in microdilution plates and incubated at 37°C for 1 h. Aliquots (60 μl per well) of diluted treated or untreated sera were then added to the test plates for analyses in standard or amplified ELISA.

RESULTS

Serum samples from patients with erythema migrans or from those with oral infections reacted positively by standard ELISA (Table 1). The geometric mean antibody titer for the former group ($\bar{x}$ = 791) was more than fivefold greater than that for the latter group ($\bar{x}$ = 152). When sera were retested by ELISA with biotin-streptavidin, sensitivity increased. Amplification nearly doubled the geometric mean titer for serologically confirmed cases of Lyme borreliosis ($\bar{x}$ = 1298), while a fivefold increase was noted for the study group that had gingivitis or periodontitis ($\bar{x}$ = 751). Of the 85 serum samples tested from patients with erythema migrans but that lacked detectable antibodies to *B. burgdorferi* by standard ELISA, 17 (20%) were positive after amplification. Improved sensitivity likewise increased the number of false-positive reactions. However, the 20 negative control serum samples remained nonreactive by standard and amplified ELISAs.

Commercially prepared sorbent was added to serum samples in attempts to reduce cross-reactivity. By standard and amplified ELISAs, a 1:10 concentration of sorbent decreased the number of false-positive reactions (Table 2). For example, 6 of the 8 serum samples from the individuals in the premarital screening program that reacted positively by amplified ELISA without adsorption (Table 1) were negative after treatment with sorbent and amplification. Adsorption without amplification, however, also removed homologous antibodies to *B. burgdorferi* and resulted in decreased sensitivity. Adsorption combined with amplification improved the sensitivity, but false-positive reactions caused by antibodies to oral treponemes could not be totally eliminated. Treatment of sera with a 1:2 dilution of sorbent followed by amplified ELISA improved the specificity by removing more nonspecific antibodies to oral treponomes (n = 5 positive), but the sensitivity was substantially reduced. Of the 26 serum samples from patients with erythema migrans and serologically confirmed by standard ELISA without adsorption, 19 (73%) remained positive when the higher concentra-

TABLE 2. Reactivities of adsorbed human sera to whole cells of *B. burgdorferi* by ELISA

Study group[a]	Total no. of serum samples tested[b]	Adsorbed ELISA[c]			Adsorbed[c] and amplified ELISA		
		No. (%) positive	Antibody titer		No. (%) positive	Antibody titer	
			Range	$\bar{x}^d$		Range	$\bar{x}^d$
EM, antibodies	26	12 (46)	640–40,960	266	26 (100)	640–40,960	1,961
EM, no antibodies	22	0		40	8 (36)	160–640	88
Oral infections	13	3 (23)	2,560	104	8 (62)	320–20,480	418
Premarital screening	36	0		40	2 (6)	320–640	46
Controls	20	0		40	0		40

[a] See footnotes *b* through *e* in Table 1 for descriptions of the respective study groups.
[b] Subsets of serum samples from patients with erythema migrans were analyzed in adsorption trials.
[c] SciMedx sorbent (*T. phagedenis* biotype Reiter) was diluted 1:10 in phosphate-buffered saline solution.
[d] Geometric means.

tion of sorbent was used in the amplified ELISA. Similarly, seropositivity for the other group of clinically defined cases of Lyme borreliosis (i.e., initially seronegative but positive in the amplified ELISA after treatment with a 1:10 dilution of sorbent) decreased by 50%. The status of negative control sera was unchanged in all experiments with adsorption and biotin-streptavidin.

In general, antibody titers in the amplified ELISA were higher than those recorded by standard analyses (Table 1). However, in the group with erythema migrans and antibodies, the mean titer of samples in the standard ELISA was not significantly different from the mean titer obtained after amplification (Student's t test, $t = 0.95$, $P = 0.05$) or by the adsorbed-amplified ELISA ($t = 1.15$, $P = 0.05$). Use of sorbent prior to amplification with biotin-streptavidin had a moderate effect on homologous titration endpoints. Fourfold or greater declines in antibody titers were noted for 10 of 26 samples, while twofold or no changes in titration endpoints were recorded for the remaining 16 samples. Twofold changes in antibody titers were within the range of normal test variation.

DISCUSSION

Use of biotin-streptavidin amplification procedures in conjunction with the removal of nonspecific treponemal antibodies can increase the sensitivity of ELISA for the diagnosis of Lyme borreliosis. These modifications may be helpful in the diagnosis of this disease in the laboratory, particularly during the early weeks of infection, when standard antibody test results tend to be negative. Amplification with or without adsorption, however, resulted in false-positive reactions. There are common antigens shared by *Borrelia* and *Treponema* spirochetes (2), and nonspecific antibodies, such as those directed to oral treponemes or *Treponema pallidum*, can react to flagellar components of *B. burgdorferi* (23). In addition, cross-reactivity of antibodies directed to another common antigen with a molecular mass of about 60 kDa (8, 10) also may be enhanced in the amplified ELISA. Therefore, if biotin-streptavidin reagents are used in conventional serologic tests for the diagnosis of Lyme borreliosis, adsorption is required. If a specific antigen (i.e., recombinant proteins) of *B. burgdorferi* becomes available, cross-reactivity problems could be reduced or eliminated and adsorption would be unnecessary.

The sorbent concentration in standard or amplified ELISA is an important factor. If the working reagent is too concentrated, assay sensitivity decreases. A sorbent dilution of 1:10 seemed to be the one most suitable in the present study. However, the ELISA methods currently being used in private laboratories, research institutions, and hospitals are not standardized; assay sensitivities and specificities vary (13, 17, 25). Therefore, experiments should be conducted in each laboratory to determine the optimal concentrations of sorbent for the particular assay being used. Concentrations of nonspecific antibodies in patient sera differ and should be considered when cutoff values are determined for positive results and when adsorption or amplification procedures are implemented.

The prevalence of false positivity, which is caused by Ig's directed to *Escherichia coli* or *Treponema* spirochetes, can be reduced by adsorption (8, 23). Use of washed, whole cells of *T. phagedenis* biotype Reiter decreased treponemal cross-reactivity (23). As observed in the present study, however, antibodies to treponemes sometimes can be difficult to completely remove when commercially prepared sorbents are used. Therefore, clinical findings must be relied on to separate cases of periodontitis, acute necrotizing ulcerative gingivitis, or syphilis from Lyme borreliosis. If further serologic testing is required to diagnose syphilis, Venereal Disease Research Laboratory tests or rapid reagin card tests can be used (6, 24). A specific antigen of *B. burgdorferi*, such as recombinant proteins or mixtures of subunit components, is ultimately needed to develop a highly specific ELISA.

With the potential for false-positive reactions, the adsorbed-amplified ELISA should not be used for the routine screening of patient sera unless clinical histories of the patients are provided. Although there was no history of Lyme borreliosis in the individuals in the premarital screening program group, positive reactions were noted. These results may indicate unknown prior exposure to *B. burgdorferi* or they may be false positives. Currently, clinical findings are not usually shared with the serologists who perform these assays, and reporting of false-positive test results could confuse diagnosis. If patients lack erythema migrans but present with signs and symptoms that are similar to those that develop in patients with Lyme borreliosis, if human cases occur in communities where this disease and tick vectors (*Ixodes dammini*) are endemic, and if standard ELISA results are negative, then adsorbed-amplified ELISA procedures or the use of capture IgM ELISA methods will help to clarify the cause of infection.

Immunoblotting techniques (Western blot analysis) have been used to confirm *B. burgdorferi* infections, particularly when standard ELISA or indirect fluorescent-antibody staining methods fail to detect antibodies to this spirochete. Amplification procedures can be applied to Western blot analysis. However, during the first 3 weeks of *B. burgdorferi* infection, IgM antibody can be slow to rise. When present, IgM antibody is primarily directed to flagellin (4–6, 9), which is a protein with an approximate molecular mass of 41 kDa. Sera containing treponemal antibodies but lacking homologous antibody to *B. burgdorferi* also can react to flagellin by Western blot analysis (23). Therefore, a single band to this antigen may not be specific for Lyme borreliosis. Regardless of whether immunoblotting, the adsorbed-amplified ELISA, or the capture IgM ELISA is chosen for serological diagnosis, a second serum sample should be obtained for analysis when initial results are inconclusive or negative. There is an expansion in the immune response to multiple antigens of *B. burgdorferi* 6 weeks or later after the onset of illness (6, 9, 32). As Lyme borreliosis progresses to later stages, antibody titers usually rise and laboratory confirmation is more easily achieved.

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