Cross-Reactivity of Nonspecific Treponemal Antibody in Serologic Tests for Lyme Disease

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Serum samples obtained from 59 persons who had acute necrotizing ulcerative gingivitis, periodontitis, syphilis, or Lyme disease were tested against *Treponema phagedenis* biotype Reiter, *Treponema denticola*, *Treponema vincentii*, and *Treponema scoliodontum* by indirect fluorescent-antibody staining methods. Although there were positive reactions for sera representing each of these study groups and for 20 (13%) of 156 samples collected from the general population (premarital screening for syphilis), titration endpoints were relatively low (≤1:256). Serum samples from 18 persons who had gingivitis or periodontitis but no history of Lyme borreliosis were tested by enzyme-linked immunosorbent assay for antibodies to *Borrelia burgdorferi*. Of these, five (28%) had immunoglobulin M antibody and four (22%) contained immunoglobulin G antibodies to this spirochete. Adsorption with either sorbent commercially prepared from *T. phagedenis* biotype Reiter or with washed, whole cells of *T. phagedenis* biotype Reiter reduced cross-reactivity in the enzyme-linked immunosorbent assay for Lyme borreliosis.

Indirect fluorescent-antibody staining methods and enzyme-linked immunosorbent assay (ELISA) are frequently relied upon to confirm Lyme borreliosis infections. These antibody tests are particularly useful when the clinical presentation is unclear. In an effort to improve the sensitivity of the ELISA, investigators have fractionated *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis, and have coated purified flagellin or flagellin-enriched subunits of this spirochete to the solid phase (6, 9). Flagellin of *B. burgdorferi* has an approximate molecular mass of 41,000 daltons and shares epitopes with similar polypeptides extracted from other *Borrelia* spp. and from *Treponema* spp. (14). Antigenic interrelationships among the endoflagella of treponemes are well documented (5). Although sera from patients with Lyme borreliosis sometimes show reactivity in fluorescent treponemal antibody-absorption tests for syphilis (3, 10, 16, 20), *Treponema pallidum* infections can be excluded serologically with the Venereal Disease Research Laboratory (VDRL) or rapid plasma reagin card tests (20).

Several species of *Treponema* have been isolated from humans. Aside from *T. pallidum* and other spirochetes that have been extensively investigated, such as *Treponema phagedenis* biotype Reiter and *Treponema denticola*, the following organisms have been observed microscopically in or isolated from subgingival plaques removed from areas associated with periodontitis: *Treponema vincentii*, *Treponema socranskii*, and *Treponema pectinovorum* (12, 13, 18, 19, 21). There is a strong association between *T. denticola* and periodontal disease (23), but it has not been conclusively shown that this spirochete causes periodontitis. Patients with periodontal disease have elevated levels of serum antibodies to *T. denticola* and other oral spirochetes (11, 17). The presence of these antibodies could potentially cross-react with *B. burgdorferi* and confuse the interpretation of serologic test results for Lyme borreliosis. Accord-

ingly, it was important to determine whether antibody to either *T. phagedenis* biotype Reiter or oral treponemes reacts with *B. burgdorferi* and whether the use of adsorption procedures could reduce the number of false-positive results in assays for Lyme borreliosis.

MATERIALS AND METHODS

Study groups. Serum samples were obtained from persons who were clinically diagnosed as having periodontitis ($n = 7$) or acute necrotizing ulcerative gingivitis ($n = 11$), syphilis ($n = 29$), or Lyme borreliosis ($n = 12$). Members of the last group had erythema migrans and neurological or arthritic disorders. Sera were also collected from an additional 156 individuals who were being tested for syphilis in compliance with a premarital screening program in Connecticut. Persons in this group were between the ages of 20 and 50, and there were no histories of spirochetoses. The fifth study group consisted of five persons who had no known spirochetal infections and who lacked antibodies to *B. burgdorferi*.

Subjects with histories of oral infections were diagnosed in Texas or Missouri. In Texas, *Fusobacterium nucleatum* or *Bacteroides gingivitis* were isolated from two patients. Information on the presence of treponemes and severity of infection was unavailable. In Missouri, a person with periodontitis and six individuals with acute necrotizing ulcerative gingivitis had treponemes in plaques. In all cases, the dates of the onset of periodontal infection were unknown. Serum samples were collected during various stages of infection.

Serologic tests. The materials and methods used to detect immunoglobulin M (IgM) and IgG antibodies by ELISA have been reported previously (15). An indirect fluorescent-antibody staining procedure also was used to screen human sera against *T. phagedenis* biotype Reiter (hereafter referred to as *T. phagedenis*), *T. denticola*, *T. vincentii*, and *T. scoliodontum*. These treponemes were cultured in spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% normal rabbit serum. After 5 to 6 days of growth, culture tubes (volume, 8 ml) were centrifuged at

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TABLE 1. Reactivity of human sera to *Treponema* spirochetes by indirect fluorescent-antibody staining methods[a]

Study group	T. phagedenis		T. denticola		T. vincentii		T. scoliodontum	
	Total tested	No. (%) positive	Total tested	No. (%) positive	Total tested	No. (%) positive	Total tested	No. (%) positive
Normal	5	0	5	0	5	0	5	0
Syphilis	23	17 (74)	17	14 (82)	29	28 (97)	23	12 (52)
Oral infections[b]	18	4 (22)	14	3 (21)	18	6 (33)	18	3 (17)
Lyme borreliosis[c]	12	12 (100)	12	10 (83)	6	3 (50)	12	5 (42)
Unknown[d]	30	0	156	20 (13)	30	0	26	0

[a] Insufficient quantities of sera available for complete testing against all treponemes.
[b] Acute necrotizing ulcerative gingivitis or periodontitis.
[c] Serologically positive in ELISA (titers of ≥1:1,280).
[d] Routine premarital testing of general population performed by the VDRL method.

35,000 × g for at least 20 min. Concentrated spirochetes taken from the pellet were placed on glass microscope slides, allowed to dry overnight at 37°C, and fixed in cold acetone for 10 min before analyses. Serum dilutions of ≥1:64 were tested, and after washing, the slides were overlaid with a polyvalent, fluorescein isothiocyanate-labeled goat anti-human globulin (GIBCO Laboratories, Grand Island, N.Y.) diluted 1:50 in phosphate-buffered saline solution. A rabbit anti-*T. pallidum* antiserum, obtained from the Centers for Disease Control in Atlanta, Ga., was used to verify the reactivity of treponemal antigens. Negative serum controls were also included.

Adsorption procedures. In attempts to remove cross-reactive antibodies to *Treponema* spirochetes and to increase the specificity of the ELISA for Lyme borreliosis, *T. phagedenis* sorbent (Beckman Instruments, Inc., Fullerton, Calif., or Roach Laboratories, Loganville, Ga.) was applied. A 1:2 dilution of sorbent was mixed in equal volumes (60 μl) with serum samples in microdilution plates. Adsorbed and untreated (control) preparations were held at room temperature (23 ± 3°C) for 90 min and analyzed in a standardized fluorescent-treponemal antibody-absorption test (25). The higher concentration of sorbent was used because indirect fluorescent-antibody titers to *T. denticola* and *T. vincentii* exceeded 1:256 for 44 serum samples. Separate trials for adsorption were conducted by treating sera with washed, whole cells of *T. phagedenis*. Culture tubes containing this treponeme were centrifuged after 6 days of growth at 35,000 × g for 45 min. Pellets were washed twice in 8 ml of phosphate-buffered saline solution (pH 7.2), and spirochetes were suspended in 3 ml of phosphate-buffered saline solution containing 0.01% aqueous thimerosal. Protein concentrations were determined by using a commercial microassay (Bio-Rad Laboratories, Richmond, Calif.). Following standardization at an optimal concentration of 45 μg of protein per ml, these spirochete preparations were used to adsorb test sera. Sixty microliters of freshly prepared spirochetes was added to an equal volume of serum in microdilution plates. Treated and unadsorbed (control) samples were held under the same conditions as the sorbent-treated samples and were subsequently tested by ELISA.

Western blot (immunoblot) analyses. Immunoblotting techniques were applied to determine whether antibodies in serum samples from persons who had gingivitis or periodontitis reacted to surface and subsurface proteins of *B. burgdorferi*. The procedures for these tests have been reported elsewhere (1). A polyvalent, horseradish peroxidase-labeled goat anti-human immunoglobulin (Tago Inc., Burlingame, Calif.) was diluted 1:500 in Tris-buffered saline. Biotinylated sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards (Bio-Rad) were used to mark reference points.

RESULTS

Serum samples obtained from persons who had syphilis, acute necrotizing ulcerative gingivitis, periodontitis, or Lyme borreliosis reacted positively by indirect fluorescent-antibody staining to one or more of the *Treponema* species tested (Table 1). Persons screened with the VDRL test for syphilis (premarital group) contained antibodies to *T. denticola*, but there were no detectable antibodies to *T. phagedenis*, *T. vincentii*, and *T. scoliodontum* at a serum dilution of 1:64. The prevalence of seropositivity varied. As expected, the samples from syphilitic patients with homologous antibody to *T. pallidum* cross-reacted strongly with the other treponemes. Serum samples from persons who had Lyme borreliosis also frequently reacted to treponemes (≥42%), particularly when *T. phagedenis* and *T. denticola* were included as antigens. By contrast, the number of positive serum samples for groups of individuals who had gingivitis or periodontitis or who were screened for syphilis was markedly lower (16 to 32%). Sera from persons in the control group were negative.

In a frequency distribution of titration endpoints for 137 positive results (Table 2), an antibody titer of 1:256 was recorded most often (38%). The remaining titers, recorded less frequently, were 1:128 (31%), 1:64 (30%), 1:512 (0.7%), and 1:2,048 (0.7%). In tests with *T. phagedenis*, *T. vincentii*, and *T. scoliodontum*, antibody titers ranged between 1:64 and 1:256. Titration endpoints of 1:512 and 1:2,048 were recorded when sera from two syphilitic patients (one identified from the premarital population group) were screened against *T. denticola*.

Serum samples obtained from patients who had gingivitis or periodontitis but no history of Lyme borreliosis were tested for antibodies to *B. burgdorferi* by ELISA. Of the 18 specimens analyzed, 5 (28%) had detectable amounts of IgM antibody and 4 (22%) contained IgG antibodies to this spirochete. Titers of IgM antibody were elevated (1:2,560) for two samples (Table 3). In tests for IgG antibody, titration endpoints ranged from 1:160 to 1:1,280.

The use of sorbent or fresh preparations of *T. phagedenis* reduced cross-reactivity in the ELISA for IgM and IgG antibodies to *B. burgdorferi*. Adsorption with a commercially prepared reagent resulted in a significant decrease in titers of IgM or IgG antibodies for three serum samples from persons with periodontitis (no. 1 to 3) and samples from all three syphilitic patients (no. 8 to 10). Treatment with washed, whole cells of *T. phagedenis* eliminated all cross-reactivity in the ELISA for IgM antibody, regardless of the source of the serum samples, and resulted in an eightfold decrease in IgG antibody titers for four serum samples from persons who had periodontitis and for two samples from

TABLE 2. Frequency distributions of antibody titers to treponemes by indirect fluorescent-antibody staining methods

Study group	No. of sera with indicated titer of reciprocal antibody to:													
	T. phagedenis			T. denticola					T. vincentii			T. scoliodontum		
	64	128	256	64	128	256	512	2,048	64	128	256	64	128	256
Syphilis[a]	7	6	4	1	2	10	1		8	10	10	3	5	4
Oral infections	4				3					2	4	2	1	
Lyme borreliosis	7	3	2	1	2	7			1	1	1	3	2	
Unknown				4	5	10		1[a]						
Total	18	9	6	6	12	27	1	1	9	13	15	8	8	4

[a] Serologically confirmed with the VDRL and fluorescent treponemal antibody-absorption methods.

syphilitic patients. Regardless of whether antibody titers were high or low, application of sorbent or fresh preparations of T. phagedenis to 10 serum samples from patients who had Lyme borreliosis normally reduced IgM and IgG antibody titers by twofold (within normal test variability).

Western blot analysis was conducted to determine which surface or subsurface proteins of B. burgdorferi were recognized immunologically. Of the 11 serum samples listed in Table 3, 10 had antibody to flagellin; serum sample 7 was nonreactive. With the exception of serum sample 9, which had another distinct band to a protein with a molecular mass of about 25,000 daltons, there were no detectable antibodies to other proteins of B. burgdorferi.

To further assess serum reactivity in the ELISA for Lyme disease, samples from persons who were being screened for syphilis (premarital group) by the VDRL method were tested along with the normal serum samples for IgM and IgG antibodies to B. burgdorferi. Of 156 persons, 3 (2%) had IgM antibody to this spirochete at titers of 1:640 to 1:2,560. Tests for IgG antibody were negative. Results for the five normal serum specimens remained negative in assays for both classes of immunoglobulins.

DISCUSSION

Antibodies produced to treponemes that are indigenous to humans cross-reacted in assays for Lyme borreliosis. How-

TABLE 3. Reactivity of 11 positive serum samples to B. burgdorferi in ELISA before and after adsorption with T. phagedenis

Patient no.	Diagnosis[a]	Titer of antibody[b] to B. burgdorferi			
		Before adsorption		After adsorption[c]	
		IgM	IgG	IgM	IgG
1	Periodontitis	2,560	640	Neg	Neg
2	Periodontitis	Neg	160	Neg	Neg
3	Periodontitis	Neg	640	Neg	Neg
4	Periodontitis	320	Neg	320	Neg
5	ANUG	320	Neg	320	Neg
6	ANUG	2,560	1,280	640	1,280
7	ANUG	320	Neg	640	Neg
8	Syphilis	320	40,960	Neg	5,120
9	Syphilis	Neg	2,560	Neg	Neg
10	Syphilis	320	10,240	320	1,280
11	PS	320	Neg	160	Neg

[a] ANUG, Acute necrotizing ulcerative gingivitis; PS, premarital screening group (no known spirochetal disease and nonreactive by the VDRL method).
[b] Reciprocal titers. Neg, Negative at serum dilutions of ≤1:160.
[c] Sera were adsorbed with 1:2 dilution of T. phagedenis biotype Reiter sorbent.

ever, antibody concentrations were relatively low. Aside from cross-reactivity to T. denticola (13%), there were no antibodies detected to T. phagedenis, T. vincentii, or T. scoliodontum in the premarital population. Therefore, if cutoff values for positive test results are properly determined, the number of false-positive reactions due to nonspecific treponemal antibody should be minimal.

Adsorption procedures worked effectively in removing cross-reactive antibodies in most serum samples. The sorbent used in the standard fluorescent treponemal antibody-absorption test for syphilis or application of washed preparations of whole-cell T. phagedenis may aid in serologic analyses for Lyme borreliosis. On the basis of the present study, washed, whole cells are preferred. Cross-reactivity with T. denticola occurred, and although preparations of this spirochete were not used as a sorbent, treatment of sera with either whole cells or extracts containing flagellin may increase the specificity of assays for Lyme borreliosis. Adsorption procedures have been used in the Federal Republic of Germany (26) and Sweden (2) in attempts to increase the specificity of serologic tests for Lyme borreliosis. With continued interests in the use of purified flagellin or flagellin-enriched fractions of B. burgdorferi as an antigen in ELISAs, some consideration should be given to possible false-positive results. Flagellar proteins are recognized during the early stage of Lyme borreliosis (9), but because Borrelia species share common antigens with treponemal components (4), including flagellin, nonspecific antibody can cause false-positive test results. The use of sorbent or the development of a more specific antigen for an ELISA (e.g., flagellin without the cross-reactive epitopes shared with Treponema species) might further improve serologic diagnoses of Lyme borreliosis.

Aside from flagellin, there are additional proteins of B. burgdorferi that are common to other bacteria. Polypeptides of an approximate molecular mass of 60,000 daltons are common to a wide range of bacteria (8). When sonicated whole cells of B. burgdorferi are coated in the solid phase of the ELISA, preadsorption of serum with Escherichia coli is reported to have improved test specificity without significantly reducing assay sensitivity (7). Based on an ELISA study of serum samples from pediatric rheumatology patients, 20% of the results were equivocal or false-positive when the unadsorbed samples were tested at dilutions of <1:200. Cross-reactivity was attributed to elevated levels of immune complexes or high concentrations of antinuclear antibody or both. Adsorption with E. coli, however, reduced nonspecific binding to B. burgdorferi antigens and allowed serum dilutions as low as 1:40 to be analyzed. This advancement may facilitate the detection of antibodies during early

Lyme borreliosis, a time when antibody tests tend to be negative (22).

In addition to adsorption techniques, proper evaluations of normal control sera to determine cutoff values for positive test results can minimize the problems associated with nonspecific antibody reactivity. The number of serum samples tested should be large enough to represent the general population. Moreover, clinical data should be provided to rule out the occurrence of Lyme borreliosis and other pathogenic spirochetal infections and even prior exposure to *Ixodes dammini*, a tick vector of *B. burgdorferi*. Subclinical infections of *B. burgdorferi* have been reported (24), albeit at a low prevalence (<10%). High antibody titers have been reported for some persons who have not had the clinical manifestations of Lyme borreliosis. Therefore, with continued expansion of the geographic ranges of ticks in the *Ixodes ricinus* complex and further amplification of *B. burgdorferi* in established foci, the selection of representative normal sera for assay cutoff evaluations becomes particularly important. Once established, reference collections of normal serum samples and positive serum controls should be shared with those who are developing new antibody tests and those who are currently conducting serologic assays for Lyme borreliosis. Standardization of antibody tests for this disease remains in high priority.

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