DIAGN MICROBIOL INFECT DIS
1993;16:43–51

Identification of Species-Specific, Non-Cross-Reactive Proteins of *Borrelia burgdorferi*

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The low specificity of diagnostic tests for Lyme disease is due to the fact that Borrelia burgdorferi *possesses many antigenic proteins that are cross-reactive with other spirochetes and bacteria. The low sensitivity is a result of high (≥1:100) dilutions used for patient sera during testing to eliminate nonspecific cross-reactivity. The present study was conducted to identify species-specific non-cross-reactive protein(s) of* B. burgdorferi *that might be used as antigen(s) in serologic tests. Whole-cell sonicates of* B. burgdorferi *were tested against pooled sera from patients with symptoms, signs, and serologic features diagnostic of Lyme disease (LD), rheumatoid arthritis, infectious mononucleosis, systemic lupus erythematosus, Rocky Mountain spotted fever, secondary syphilis, and from healthy individuals. Different LD pools were also tested against whole-cell sonicates of* Treponema pallidum, Treponema phagedenis, Leptospira interrogans, *and* Escherichia coli. *Comparison among patterns obtained by each serum pool revealed that IgM antibodies to species-specific 39-, 23-, and 22-kD proteins and IgG antibodies to 34- and 31-kD proteins were present only in the patients with LD and absent from patients with rheumatoid arthritis, infectious mononucleosis, systemic lupus erythematosus, Rocky Mountain spotted fever, secondary syphilis, and healthy individuals pools. These results suggest that 39-, 23-, and 22-kD proteins may be used in an IgM immunoassay for diagnosis of LD.*

INTRODUCTION

Lyme disease (LD), the most prevalent arthropod-borne infection in the United States [Centers for Disease Control (CDC), 1989a], is a multisystem inflammatory disorder caused by the tick-borne spirochete *Borrelia burgdorferi* (Burgdorfer et al., 1982; Johnson et al., 1984; Steere et al., 1983b). It has a broad clinical spectrum involving the skin (Steere, 1989; Steere et al., 1983a), joints (Steere et al., 1977 and 1987), peripheral and central nervous systems (Halperin et al., 1987; Reik et al., 1979), and the heart (Reznick et al., 1986; Steere et al., 1980). Between 1982 and 1988, the CDC (Atlanta, GA) received 13,825 reports of cases of Lyme borreliosis from all 50 of the United States. Lyme borreliosis also seems to have a global distribution; it has been reported in Europe (Schmid, 1985), China (Ai et al., 1988), the former Soviet Union (Dekonenko et al., 1988), Canada (CDC, 1989b), Africa (Habergerger et al., 1989; Stanek et al., 1986), Japan (Kawabata et al., 1987), and from a restricted focus in Australia (Stewart et al., 1982).

Although a clinical staging system for this chronic infection has been proposed, the stages are not always clear-cut. Manifestations from different stages can overlap, or involvement of a single organ system can be the sole manifestation of the disease (Garcia-Monco et al., 1990). This state of affairs has caused confusion among LD and other illnesses characterized by neurologic or arthritic manifestations. In such situations, physicians have attempted to use serologic tests to rule out LD in the differential diagnosis. For example, detection of IgG antibodies to *B. burgdorferi* has been used to distinguish LD, in the later

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Received 23 January 1992; revised and accepted 22 July 1992.

0732-8893/93/$5.00

stage of infection, from aseptic meningitis, multiple sclerosis, rheumatoid arthritis, and juvenile rheumatoid arthritis (Craft el al., 1984). Many potential problems exist with the currently available serologic tests for Lyme borreliosis. These problems have led to both false-positive and false-negative results (Schwartz et al., 1989). The following vignette is typical of the problems encountered. Because several studies appeared to demonstrate a 41-kD flagellar protein (flagellin) of *B. burgdorferi* that generated the earliest specific antibody response in infected humans (Barbour et al., 1983; Coleman and Benach, 1987; Grodzicki and Steere, 1988), several investigators focused on using this protein to increase the sensitivity of serologic tests (Hansen and Asbrink, 1989; Hensen et al., 1988). There proved to be, however, two potential problems with the specificity of the flagellar protein. First, flagella of other *Borrelia* species (Barbour et al., 1986), and probably *Treponema pallidum* subsp. *pallidum* Nichols strain (Blanco et al., 1988; Coleman and Benach, 1989), share epitopes common to the flagella of *B. burgdorferi*. Second, studies screening normal human sera by Western blot analysis have demonstrated antibodies to a 41-kD protein that appears to be flagellin (Barbour et al., 1983; Coleman and Benach, 1987; Craft et al., 1986; Nadal et al., 1989). In the study reported here, we make a consistent observation that *Treponema phagedenis* biotype Reiter also contains a 41-kD protein that reacted on Western blots with sera from patients with LD. Because of these circumstances, there is an increasing demand for more sensitive and specific serologic tests for the diagnosis of LD. At the present time, the serologic tests commercially available use whole-cell sonicates of *B. burgdorferi* as the antigen, introducing many cross-reactive nonspecies-specific proteins, thereby decreasing the specificity of the serologic assays. The assays also use highly diluted ($\geq$1:100) serum samples (to decrease nonspecific antigen–antibody binding), thereby potentially losing species-specific antibodies that may exist in low concentrations.

In an effort to develop a better diagnostic method for Lyme borreliosis, the present study was conducted to identify unique antigenic proteins of *B. burgdorferi* that may lead to the development of more sensitive and specific serologic tests for LD.

MATERIALS AND METHODS

Bacterial Strains

Borrelia burgdorferi isolated from an *Ixodes dammini* nymph taken from an ovenbird from the St. Croix River in Minnesota was originally obtained from Dr. Russell Johnson, University of Minnesota (Minneapolis), and grown in modified Barbour–Stoener–Kelly II medium (Barbour, 1984) without gelatin at 33°C for 14 days. *Treponema pallidum* Nichols strain (a generous gift of Dr. David L. Cox, CDC, and Dr. Michael V. Norgard, University of Texas Southwestern Medical School) was propagated by intratesticular passage in male New Zealand white rabbits. *Treponema phagedenis* biotype Reiter (originally a gift of Elizabeth F. Hunter, CDC) was grown in National Institutes of Health (NIH)–thioglycolate broth (Difco, Detroit, MI) with 10% inactivated rabbit serum (Pel-Freez Biologicals, Rogers, AK) at 37°C for 5 days. *Leptospira interrogans* serotype *pomona* (also gift of Dr. David L. Cox) was grown in *Leptospira* medium (Difco) supplemented with 10% Bacto Leptospira Enrichment EMJH (Difco) at room temperature in the dark for 14 days. All spirochetes were collected by centrifugation at 12,000 *g* at 4°C for 20 min and washed twice with 0.01 M phosphate-buffered saline (PBS) pH 7.4. *Escherichia coli* ATCC 25922 was cultured on 5% sheep blood agar and washed once in PBS.

Serologic Test Kits

Commercially available test kits were used to perform the serologic diagnostic tests. Lyme disease testing was performed using the IgG/IgM FASTLYME test kit (3M Diagnostic Systems, Santa Clara, CA). Rocky Mountain spotted fever (RMSF) was tested for by using *Rickettsia rickettsii* latex agglutination system (Integrated Diagnostic, Baltimore, MD). The SERATEST latex kit (Seradyn, Indianapolis, IN) was used to test for rheumatoid arthritis (RA). Infectious mononucleosis (IM) testing was performed using MONOLERT latex kit (manufactured by Ortho and distributed by Johnson and Johnson, Raritan, NJ). Antinuclear antibodies were detected by using QUANTAFLUOR test kit (Kalestad, Austin, TX). Finally, microhemagglutination for *T. pallidium* was performed using a test kit manufactured by Ames and distributed by Miles (Elkhardt, IN).

Patient Sera

Individual patient sera with historical and clinical presentations of LD, RA, IM, systemic lupus erythematosus (SLE), RMSF, and secondary syphilis (SY) were tested using commercial test kits to confirm the diagnosis. The individual sera (4–14 samples) with similar clinical and serologic findings were then pooled. Pooled sera from patients with the established diagnosis of LD (pools 1–4), RA (pool 12), IM (pool 11), SLE (pool 10), RMSF (pool 9), and SY (pool 8) were used to perform the immunoblot analysis. Pool 13 (negative control) was from healthy individuals without history or physical findings diagnostic of any of the above diseases and with negative routine serologic tests. Two pools were from patients presenting with symptoms of fatigue (pool 5), fa-

tigue and rash (pool 6), and erythema chronicum migrans (pool 7), in which the diagnosis of LD had been considered. Table 1 summarizes the characteristics of all of the pools.

Serum Absorption with Selected Organisms

Following the method of E.F. Hunter (Hunter et al., 1986), all pools of sera were sequentially absorbed by *E. coli*, *L. interoogans*, *T. phagedenis*, and *T. pallidum*. First, *E. coli* was suspended in the pooled patient sera to a concentration of approximately 3×10^9/ml. The suspension was incubated at 37°C for 1 hr, then overnight at 2°–8°C. Cells were removed by centrifugation, and an aliquot of the absorbed sera was removed. The rest of the sera was further absorbed in a stepwise manner by *L. interrogans*, *T. phagedenis*, and *T. pallidum* (in the given order here), and at each step aliquots were removed and stored at −20°C.

Preparation of Samples for SDS–PAGE

All chemicals were purchased from Sigma (St. Louis) unless indicated otherwise. Bacterial samples were suspended in PBS (1 g wet cells/2 ml PBS, pH 7.4) and sonicated as previously described (Altaie and Cox, 1991) with the addition of 20 μg/ml leupeptin along with 50 μg/ml DNase and RNase. Aliquots were then frozen at −20°C until used.

SDS–PAGE

Sixty microliters of each sonicated bacterial sample was thawed and mixed with 30 μl of SDS buffer [0.2 M Tris (hydroxymethyl) aminomethane buffer, pH 6.8, containing 3% SDS, 30% glycerol, and 0.002% bromophenol blue]. After addition of 9 μl mercaptoethanol (10%), samples were heated at 100°C for 5 min, and 30 μl (containing ~100 μg protein) per lane was loaded onto 10% polyacrylamide, 0.1% SDS slab gels. Prestained molecular weight standards (Bethesda Research Laboratories, Life Technologies) were run along with the samples in each run. As previously described (Sayahtaheri-Altaie and Cox, 1991), electrophoresis was performed overnight until the dye front was 1 cm from the bottom of the gel.

Immunoblot Analysis

Immunoblot analysis was performed as previously described (Sayahtaheri and Cox, 1990). Briefly, proteins were transferred to nitrocellulose membranes

TABLE 1 Summary of the Characteristics of Pooled Patient Sera

Pool No./ No. of Patients per Pool	Tick Bite	Geographic Area for LD	Symptoms and Signs	Serologic Test Results						Diagnosis	Treatment for LD
				LD	RMSF	RF	IM	ANA	MHA-TP		
1/4	Yes	Endemic	ECM, arthritis (positive control)	+	−	−	−	−	−	LD[a]	Yes
2/4	Yes	Endemic	ECM, neurologic disease	+	−	−	−	−	−	LD[a]	Yes
3/6	Yes	Endemic	ECM, neurologic disease	+	−	+	−	−	−	LD[a]	Yes
4/10	Yes	Endemic	ECM, malaise and fatigue	+	−	+	+	−	+	LD[a]	Yes
5/14	Yes	Endemic	Fatigue	−	−	−	−	−	−	LD?	No
6/12	Yes	Endemic	Atypical rash and fatigue	−	−	−	−	−	−	LD?	No
7/14	Yes	Endemic	ECM	−	−	−	+	−	−	LD?	Yes
8/9	No	NA	Condylomata	−	−	−	+	−	+	SY	No
9/2	Yes	NA	Macular rash	−	+	−	−	−	−	RMSF	No
10/8	No	NA	Malar rash	−	−	−	−	+	−	SLE	No
11/14	No	NA	Pharyngitis and fatigue	−	−	+	+	−	−	IM	No
12/5	No	NA	Arthritis	−	−	+	−	−	−	RA	No
13/7	No	Nonendemic	None (negative control)	−	−	−	−	−	−	None	No

[a]Based on the Centers for Disease Control criteria for definitive Lyme disease.
LD, Lyme disease; RMSF, Rocky Mountain spotted fever; RF, rheumatoid factor; IM, infectious mononucleosis, heterophile antibodies; ANA, antinuclear antibodies; MHA-TP, microhemagglutination—*T. pallidum*; ECM, erythema chronicum migrans; NA, not applicable; SY, secondary syphilis; SLE, systemic lupus erythematosis; RA, rheumatoid arthritis; +, positive; and −, negative.

(0.2-μm pore size) by using a ramping procedure for a total of 3 hr and 15 min. The nitrocellulose membranes bearing transferred bacterial proteins were probed with selected patient pooled sera at a 1:25 dilution; antigenic protein bands were visualized using horseradish peroxidase conjugated goat anti-human IgG or IgM at a 1:3000 dilution. The hydrogen peroxide substrate used was 4-chloronaphthol.

RESULTS

Tables 2 and 3 list the molecular weights of the antigenic proteins of *B. burgdorferi* as demonstrated by IgG and IgM immunoperoxidase staining using pooled sera from groups of patients defined by geographic area, history, diagnosis, and non-Western-blot serologic test results. Pool 1, from patients with LD (based on criteria set by the CDC) and no history of any of the other listed conditions, served as the positive control. This pool demonstrated 22 IgG bands and six IgM bands. Proteins with apparent molecular weights (MWs) of 18, 26, 28, 66 and 176 kD were detected with both IgM and IgG antibodies (Tables 2 and 3). Pool 13 from healthy individuals without history or serologic findings of any of the previously listed diseases was the negative control. This pool showed 11 IgG and no IgM bands (Tables 2 and 3).

When the immunoblot banding patterns of positive and negative control sera were compared with the patterns in well-characterized non-LD patient sera, proteins of *B. burgdorferi* showed extensive cross-reactivity with the antibodies produced against proteins of other organisms, especially *T. pallidum*, *Rickettsia rickettsii*, and Epstein–Barr virus (Tables 2 and 3), (that is, syphilitic patient sera had IgG antibodies that bound to *B. burgdorferi* proteins with approximate MWs of 15*, 22, 23, 24, 26*, 28*, 30*, 41*, 58, 66*, 70*, 100*, 104*, 176*, and 200 kD. Sera from patients with RMSF contained IgG antibodies that bound to *B. burgdorferi* proteins with approximate MWs of 24, 26, 28, 31, 51, 55, 58, 66*, 70*, 75*, 100, 104, and 176* kD. Also, sera from patients with IM possessed IgG antibodies that bound to *B. burgdorferi* proteins with approximate MWs of 26*, 28*, 31, and 75 kD. (The proteins marked with an asterisk also bound to IgM antibodies present in the corresponding patient sera). In addition to the proteins just mentioned, IgM antibodies, present in sera from patients with IM, also bound to proteins of *B. burgdorferi* with approximate MWs of 15, 18, 30, 41, 55, 58, 66, 70, 104, and 176 kD. Antibodies produced in autoimmune diseases, such as SLE and RA, were noted to bind nonspecifically to the proteins of *B. burgdorferi* (Tables 2 and 3).

The cross-reactive IgG antibodies in the negative control pool sera (detected in the immunoblot) are probably due to exposure to the oral treponemes or to remote infections or colonization with *E. coli* or related enteric Gram-negative bacterial species. To investigate this possibility, the degree of antigenic similarity among the proteins of *B. burgdorferi* and the proteins of *T. pallidum*, *T. phagedenis*, *L. interrogans*, and *E. coli* was determined. The positive control (pool 1) was used to probe proteins of the listed organisms on the electroblots. *Treponema pallidum* showed 10 cross-reactive proteins with the same MWs as proteins of *B. burgdorferi* detected by IgG antibodies present in the LD positive serum pool. Three of these proteins (with approximate MWs of 58, 66, and 176 kD were detected by IgM as well as IgG

TABLE 2 Proteins of *Borrelia burgdorferi* Detected by IgG Antibodies Present in Patient Pool Sera

Pool No.	Molecular Weight (kD)																					
	15	18	22	23	24	26	28	30	31	34	39	41	51	55	58	66	70	75	100	104	176	200
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+			+	+	+	+	+	+		+	+			+	+	+	+	+	+	+	+
3	+		+		+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
4	+		+	+	+	+	+	+	+		+	+		+	+	+	+	+		+	+	+
5	+		+			+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	
6	+				+	+	+	+	+	+				+	+	+	+	+	+	+	+	+
7	+		+		+	+	+	+	+		+	+	+	+	+	+	+		+	+	+	
8	+		+	+	+	+	+	+				+			+	+	+		+	+	+	+
9					+	+	+		+				+	+	+	+	+	+	+	+	+	
10	+	+	+		+	+	+	+										+				
11						+	+		+									+				
12	+	+			+	+	+							+	+	+	+	+		+	+	
13	+	+			+	+	+				+	+	+	+	+	+						

+, Protein bands detected by IgG antibodies in each pool.

TABLE 3 Proteins of *Borrelia burgdorferi* Detected by IgM Antibodies Present in Patient Pool Sera

Pool No.	Molecular Weight (kD)																					
	15	18	22	23	24	26	28	30	31	34	39	41	51	55	58	66	70	75	100	104	176	200
1		+				+	+									+	+				+	
2	+	+		+		+	+									+	+				+	
3	+					+	+								+	+	+		+	+	+	
4		+	+	+		+	+	+	+		+	+		+	+	+	+	+		+	+	
5						+									+	+	+				+	
6						+											+	+			+	
7	+					+	+	+			+	+		+	+	+	+		+	+	+	
8	+					+	+	+				+				+	+		+	+	+	
9																+	+	+			+	
10		+				+	+	+	+			+			+	+	+	+			+	
11	+	+				+	+	+				+		+	+	+	+			+	+	
12	+					+	+	+				+				+	+	+			+	
13																						

+, Protein bands detected by IgM antibodies in each pool.

antibodies (Figure 1, lane 1; and Table 4). *Treponema phagedenis* demonstrated seven cross-reactive proteins in the IgG immunoblot; the 66-kD protein was also present in the IgM immunoblot (Figure 1, lane 3; and Table 4). *Leptospira interrogans* demonstrated 12 proteins that reacted with the IgG antibodies present in the LD-positive pool; three of these proteins (with approximate MWs of 15, 18, and 66 kD) were also detected by IgM antibodies (Figure 1, lane 4; and Table 4). Finally, *E. coli* demonstrated 14 cross-reactive proteins (with MWs similar to *B. burgdorferi* proteins) detected by the IgG antibodies present in the positive pool. Four of these (18, 31, 66, and 70 kD) proteins were also detected by IgM antibodies (Figure 1, lane 4; and Table 4).

To determine whether any of these cross-reactivities could be absorbed by *E. coli*, *L. interrogans*, *T. phagedenis*, or *T. pallidum*, all patient pools were sequentially absorbed using these organisms in the sequence just listed. The immunoblot patterns of *B. burgdorferi* using the LD-positive control, after sequential absorption, are shown in Figure 2 and Table 4. Only *T. phagedenis* removed antibodies to a 75-kD protein (Figure 2, lanes 3 and 4).

DISCUSSION

Western blot analysis has been used in several studies (Barbour et al., 1983; Dattwyler et al., 1988; Fister et al., 1989; Hyde et al., 1989; Lane et al., 1990; Mensi et al., 1990) to confirm the presence or absence of LD. Antibodies to the 17-, 25 (Osp c)-, 31 (Osp A)-, 34 (Osp B)-, 41 (flagellin)-, 55-, 58-, and 66 (common antigen)-kD proteins are believed to be indicative of the presence of LD; however, in different studies,

TABLE 4 Cross-Reactive Antibodies (IgG) Between *Borrelia burgdorferi* and Selected Organisms

Organism	Molecular Weight (kD)																	
	15	18	26	28	30	31	34	39	41	51	55	58	66	70	75	100	104	176
Escherichia coli		+[a]	+	+	+	+[a]	+		+	+	+	+	+[a]	+[a]		+	+	
Leptospira interrogans serotype *pomona*	+[a]	+[a]		+	+	+	+	+	+	+	+	+	+[a]					
Treponema phagedenis biotype Reiter	+							+	+	+			+[a]		+[b]			+
Treponema pallidum susp. *pallidum*				+	+			+	+	+	+	+[a]	+[a]			+		+[a]

[a]Cross-reactive IgM antibodies in addition to the IgG antibodies.
[b]Antibodies that were absorbed by *T. phagedenis* biotype Reiter.
+, Cross-reactive IgG antibodies present in the Lyme-disease-positive control pool.

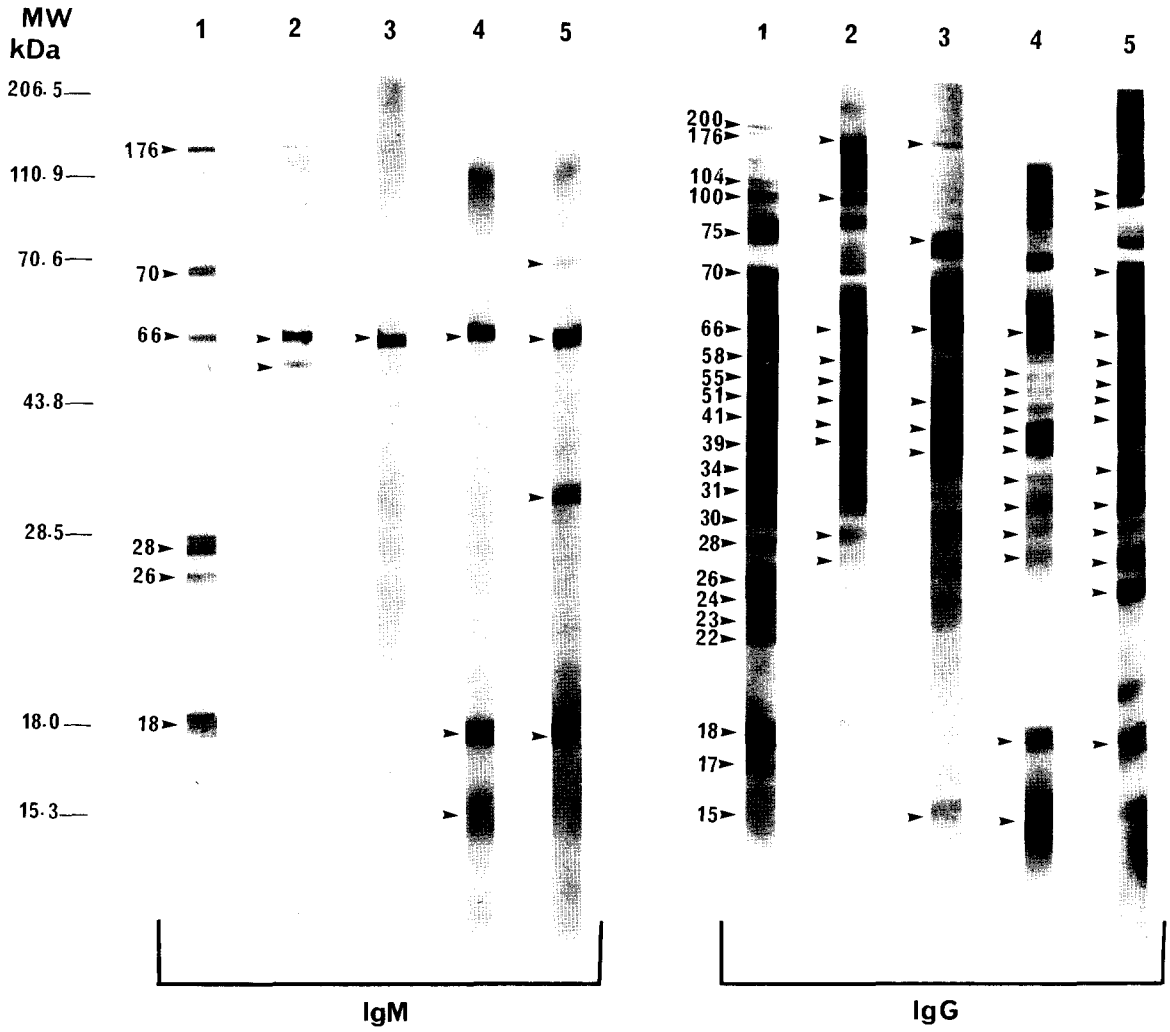

FIGURE 1 Cross-reactive proteins of *Borrelia burgdorferi*. The extent of cross-reactivity of proteins of *B. burgdorferi* (*lane 1*) with *Treponema pallidum* subsp. *pallidum* Nichols strain (*lane 2*); *Treponema phagedenis* biotype Reiter (*lane 3*); *Leptospira interrogans* serotype *pomona* (*lane 4*); and *Escherichia coli* (*lane 5*) are demonstrated. Whole-cell sonicates of the organisms were first separated on 10% SDS–PAGE gels and then transferred to nitrocellulose membranes. The cross-reactive bands were visualized using pool 1 (positive control) at a 1:25 dilution and horseradish peroxidase-conjugated goat antihuman IgG and IgM antibodies at a 1:3000 dilution. Molecular weight (*MW*) markers in kilo-Daltons (*kDa*) are on the left.

the criteria for calling a Western blot positive have varied. One reason for this variation is cross-reactivity of putatively specific *B. burgdorferi* antigens.

In this study, IgG antibodies to proteins with the same (or similar) MWs as "species specific" antigens were detected in sera from both healthy individuals and patients with various diseases (SY, RMSF, SLE, IM, or RA). In contrast to IgG antibodies, cross-reacting IgM antibodies could be detected in patients with either SY, SLE, IM, RA, or RMSF but not in healthy individuals. We also demonstrated that IgG and IgM antibodies present in patients with LD recognize not only several proteins of other spirochetes, but also of *E. coli*, implicating shared epitopes among all these organisms.

This extensive cross-reactivity can be explained as follows:

1. The antibodies present in patients with autoimmune conditions (SLE and RA) may have resulted from spontaneous or background immunoglobulin synthesis (Benner et al., 1981) or from polyclonal B-cell stimulation (Barbour et al., 1983); similar polyclonal stimulation has been demonstrated in IM (Straus and Smith, 1986).
2. The cross-reactive antibodies may have resulted from the idiotype–anti-idiotype regulation of antibody formation through exposure to the oral treponemes (Jerne, 1974).

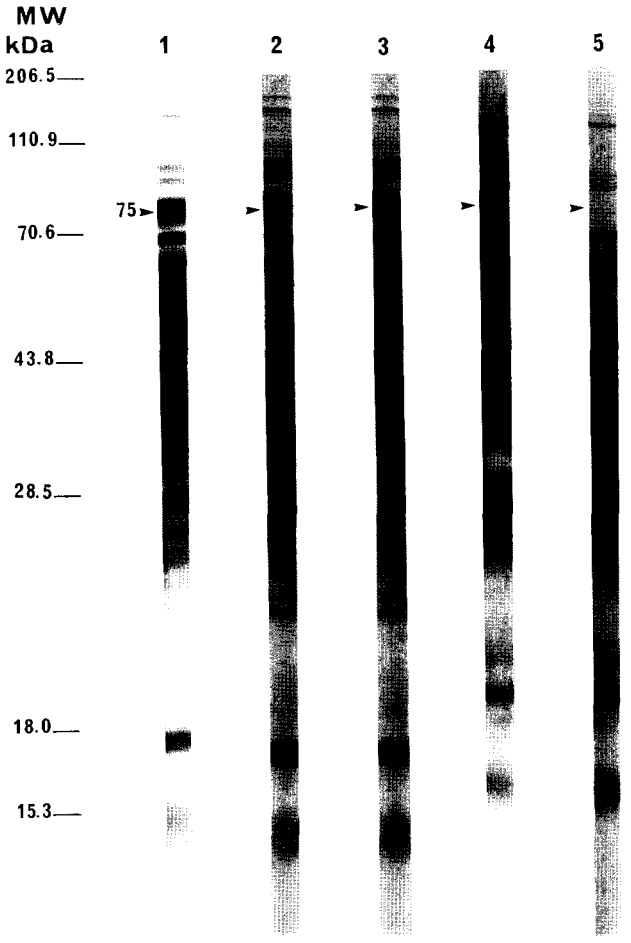

FIGURE 2 Immunoblot patterns of the Lyme disease-positive control (pool 1) after sequential absorption with *Escherichia coli* (*lane 2*), *Leptospira interrogans* (*lane 3*), *Treponema phagedenis* (*lane 4*), and *Treponema pallidum* (*lane 5*). The molecular weight markers (*MW*) in kilo-Daltons (*kDa*) are on the *left*. *Lane 1* is the immunoblot pattern of unabsorbed pool 1.

3. They may have resulted from colonization or remote infection with *E. coli* or other related species.

Sequential absorption using *E. coli*, *L. interrogans*, *T. phagedenis*, and *T. pallidum*, in this order, resulted in absorption of antibodies to only one polypeptide (a 75-kD protein). *Treponema phagedenis* was the single organism that specifically absorbed the cross-reactive antibodies to the 75-kD protein. This could be due to the absorption procedure utilized in this study, which used whole organisms and did not allow extensive exposure of the antigenic epitopes to the antibodies.

IgM antibodies to the 22-, 23-, and 39-kD proteins were present only in the sera from patients with LD. These antibodies did not cross-react with proteins of *E. coli*, *L. interrogans*, *T. phagedenis*, or *T. pallidum* of Western blots. The situation with IgG antibodies is more complex. Even though we demonstrated that *E. coli*, and *L. interrogans* have 31- and 34-kD proteins that cross-reacted with IgG antibodies to proteins of *B. burgdorferi* (with the same MWs), we did not detect the same bands in patients with RA, IM, SLE, RMSF, SY, or in healthy individuals.

Further studies are needed to determine the potential of these five species-specific antigenic proteins of *B. burgdorferi* to be used as antigen(s) for detection of human antibodies in serologic diagnostic tests. Populations of individuals with LD must be studied to determine which antigenic component(s) of *B. burgdorferi* is(are) recognized most frequently by the host's immune system during each stage of the infection. It may also be worthwhile to investigate the exact cellular location of these proteins. If they are determined to be located on the surface of *B. burgdorferi*, then they are candidates for development of direct antigen detection techniques (and, possibly, vaccine). Such studies are being conducted in our laboratory, and, in the meantime, we propose these proteins as candidates for specific detection of antibodies to *B. burgdorferi* on Western blots.

The authors gratefully acknowledge Quyn Vantu, MT(ASCP), State Health Laboratories, Richmond, VA; and Dr. Thomas Quan, Chief, Diagnostic and Reference Section, CDC, Fort Collins, CO, for help in obtaining the patient samples used in this study. The authors also thank Mrs. Paula Peters for her careful preparation of the manuscript.

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