Surface Immunofluorescence Assay for Diagnosis of Lyme Disease

ROBERTO CEVENINI,[1]* VITTORIO SAMBRI,[1] FRANCESCA MASSARIA,[1]
ROBERTO FRANCHINI,[1] ANTONIETTA D'ANTUONO,[2]
GIUSEPPE BORDA,[2] AND MASSIMO NEGOSANTI[2]

*Institute of Microbiology[1] and Institute of Dermatology,[2] Policlinico S. Orsola,
University of Bologna, 9 Via Massarenti, 40138, Bologna, Italy*

A surface immunofluorescence assay (SIFA) was analyzed and compared with a conventional indirect immunofluorescence assay (IFA) and whole-cell enzyme-linked immunosorbent assay (ELISA) for detecting immunoglobulin G (IgG) antibodies to *Borrelia burgdorferi* in sera from patients with Lyme disease. Fifty-five patients with syphilis and 33 patients with rheumatoid arthritis were used as disease controls. The sensitivity of the SIFA was low during the acute phase of Lyme disease (sera from seven of nine patients presenting with erythema chronicum migrans were negative during the first 2 months of illness); later, seroconversion was observed in all patients at various times during convalescence. Sera from five patients with complicated Lyme disease were strongly positive. SIFA was found to be highly specific, since sera from all patients with secondary or latent syphilis and patients with rheumatoid arthritis did not react in the test. Strong cross-reactivity occurred when these sera were tested in conventional IFA and ELISA; sera from 38 (69%) patients with syphilis were positive by IFA and sera from 51 (93%) patients were positive by ELISA, whereas 7 (21%) and 12 (36%) of the serum samples from patients with rheumatoid arthritis were positive by IFA and ELISA, respectively. Immunoblot analysis of SIFA-positive sera showed that the 31- and 34-kDa outer surface proteins (proteins A and B, respectively) of *B. burgdorferi* were the major reactive antigens involved in the test. The results support a role for SIFA in the investigation of complicated Lyme disease as well as in the differentiation of Lyme disease from other diseases associated with *B. burgdorferi* cross-reactive antibodies.

Lyme disease is a tick-transmitted infection caused by the spirochete *Borrelia burgdorferi* (9). The diagnosis of Lyme disease is mainly clinical. However, when clinical records are incomplete, serological tests may be required to identify the cause of infection, since Lyme borreliosis shares many symptoms and signs with other established diseases (31). Indirect immunofluorescence assay (IFA) staining of whole *Borrelia* cells and enzyme-linked immunosorbent assay (ELISA) are used to detect antibodies to *B. burgdorferi* (28). However, false-positive reactions are well documented in the serodiagnosis of Lyme disease (24), at least in part because of the cross-reactive antigens that are present on the flagella of *B. burgdorferi*, other *Borrelia* spp., and *Treponema* spp (1). A 60-kDa antigen of *B. burgdorferi* is also believed to be the common antigen which has cross-reactive epitopes with analogous antigens produced by many other bacteria (17). In addition, serological tests for Lyme borreliosis also have been shown to be inadequate in separating Lyme borreliosis from rheumatoid arthritis (21).

To improve the specificity of diagnostic tests for Lyme disease, whole *B. burgdorferi* cells have been replaced either with fractions of spirochetes in the ELISA (23) or the sera have been previously absorbed with heterologous cross-reactive antigens in the IFA (25). In this study we evaluated the possibility of using immunoglobulin G (IgG) antibodies directed against the surface antigens of living *B. burgdorferi* cells in a surface immunofluorescence assay (SIFA) for the diagnosis of Lyme disease.

MATERIALS AND METHODS

Study groups. Serum samples were obtained from subjects who were clinically and serologically diagnosed as having Lyme borreliosis (*n* = 14) or syphilis (*n* = 55). In addition, 33 serum samples from patients with rheumatoid arthritis were studied.

Lyme disease patients. Nine patients had a history of tick bite and erythema chronicum migrans (ECM), whereas five patients presented with one of the late manifestations of the disease: arthritis (four patients) and neuritis (one patient). A serum sample was obtained from the patients at the time of clinical diagnosis. Serum samples were similarly obtained after 2 to 3 and 6 to 12 months. Two additional serum samples were collected from two patients. All patients gave informed consent for the serial bleedings. All patients received antibiotic therapy. The duration of oral administration of doxycycline or amoxicillin was usually up to 3 weeks for patients with ECM and 1 month for patients with Lyme arthritis. Treatment for neuritis was with ceftriaxone given intravenously for 4 weeks.

Bacterial strains and culture conditions. *B. burgdorferi* IRS and *B. hermsii* HS-1 were originally obtained from M. A. Lovett, University of California, Los Angeles. Borreliae were maintained in Barbour-Stoenner-Kelly (BSK-II) medium (2) at 34°C in a standard air incubator and were subcultured every 5 days, as described previously (12).

SIFA. Immunoglobulin binding to *Borrelia* cell surfaces was assessed by using fluorescein-conjugated rabbit anti-human IgG (Dako, Copenhagen, Denmark). Strain IRS cells that were grown for 3 days in BSK-II medium were collected from the upper part of the culture. One-milliliter amounts of

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* Corresponding author.

growing spirochetes (1.0×10^7 organisms per ml, as detected by counting in a Petroff-Hausser chamber) were dispensed into sterile Eppendorf vials. Serial dilutions (1:50, 1:100, 1:200, 1:400) of heat-inactivated (56°C for 1 h) serum samples were obtained by adding to each test vial 20, 10, 5, and 2.5 μl of serum, respectively, by using a high-precision pipette. Bacterial suspensions were incubated at 37°C for 30 min, pelleted by centrifugation at 8,200 × g for 30 min at 28°C, and washed three times in Veronal buffer solution (VBS). The pelleted bacteria were suspended in fluorescein-labeled antiserum at a 1:30 dilution in VBS containing 1% bovine serum albumin. After incubation at 37°C for 45 min, the stained cells were washed twice with VBS and were resuspended in 200 μl of VBS, and then 10 μl of suspension was spotted onto a microscope slide and observed under UV light at ×400 magnification. Absolutely comparable results were obtained more easily when living borreliae treated with serum samples and washed as described above were spotted (10 μl) onto microscope slides, air dried, fixed for 10 min with cold acetone, and treated with fluorescein conjugate. After preliminary comparative experiments, this procedure was used throughout the study. When requested, SIFA experiments were performed with fluorescein-conjugated rabbit anti-mouse immunoglobulins to detect mouse immunoglobulins present in immune ascites obtained by immunizing BALB/c mouse either with whole B. burgdorferi IRS or B. hermsii HS-1 cells (see below).

IFA. In the conventional IFA, the spirochetes were grown in BSK-II medium for 5 to 7 days, and the cells were harvested by centrifugation (8,200 × g) for 30 min. The organisms were washed in phosphate-buffered saline (PBS) two times and diluted to approximately 200 bacteria per dry high-power field (magnification, ×400). A total of 10 to 20 μl of the suspensions was added to each well of microscope slides, air dried, and fixed in acetone for 5 min. Serial twofold dilutions of test sera or mouse immune ascites were made in PBS. A total of 10 to 15 μl of each dilution was placed onto each antigen-coated well, and the slides were incubated in a humidified chamber at 37°C for 30 min and were then washed three times in PBS. Fluorescein-conjugated rabbit anti-human IgG or anti-mouse immunoglobulins diluted 1:30 in PBS were then added, as above described. A commercially available IFA (DMD, Gailingen, Germany) was used to validate our in-house procedure. The results were absolutely comparable as far as the 1:2 dilution.

ELISA. Sonicated whole spirochetes were prepared by the method of Craft et al. (14). Briefly, B. burgdorferi IRS grown in BSK-II medium for 5 to 7 days was centrifuged at 10,000 × g for 20 min, washed with PBS, and then sonicated on ice. The indirect ELISA was done by standard methods as described previously (10). Ninety-six-well microtitration plates (Dynatech Laboratories, Guernsey, United Kingdom) were coated with 200 μl of a 0.015-mg/ml solution of spirochetal protein. After washing, 200-μl aliquots of serum samples diluted 1:200 were added to the wells in triplicate; this was followed by incubation with peroxidase-conjugated rabbit antibody to human IgG (Dako) diluted 1:500. Thereafter, 100 μl of citric acid-phosphate buffer (pH 5.6) containing 40 mg of 1,2-phenylendiamine dihydrochloride (Merck, Darmstadt, Germany) was added to each well. The enzymatic reaction was stopped after 15 min by adding 100 μl of 2 N NH_2SO_4 to each well. The results were read at 492 nm by using a Dynatech micro-ELISA autoreader. A test was considered positive if the absorbance was ≥0.4 optical density units, which was the mean plus 3 standard deviations

of the absorbances of sera from 245 subjects who had no known history of Lyme disease.

Production of mouse polyclonal antibodies. Mouse immune ascitic fluid containing polyclonal antibodies was obtained by immunizing BALB/c mice with B. hermsii HS-1 or B. burgdorferi IRS whole cells by a previously described technique (13). Briefly, 0.8-ml volumes of immunogen (0.05 mg of protein) emulsified 1:9 (vol/vol) with complete Freund's adjuvant were injected intraperitoneally into 8- to 12-week-old mice on days 0, 7, 14, and 21. On day 6, 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma, St. Louis, Mo.) was injected intraperitoneally. Mice were monitored daily for the appearance of ascites. Ascitic fluid samples were collected on day 30 by peritoneal paracentesis by using a sterile 21-gauge needle.

SDS-PAGE and Western immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20) by using a 12.5% acrylamide gel as described previously (11). The Western blot (immunoblot) procedure of Towbin et al. (33) was performed as described previously (11). Briefly, after electrophoretic transfer, the blots were incubated for 12 h at room temperature with human sera or mouse immune ascites diluted 1:100 and 1:1,000, respectively, in PBS containing 0.05% (vol/vol) Tween 20. Antigen-antibody complexes were detected with peroxidase-labeled rabbit antiserum against human IgG or mouse immunoglobulins (DAKO, Copenhagen, Denmark) diluted 1:500 in PBS and by using 40 mg of benzidine (Fluka, Buchs, Switzerland) dissolved in 4 ml of acetone–96 ml of PBS–100 μl of hydrogen peroxide from a 33% stock solution as the enzyme substrate.

FTA-ABS. The fluorescent treponemal antibody absorption test (FTA-ABS) was performed with a commercial kit in accordance with the manufacturer's directions (Behring AG, Marburg/Lahn, Germany). FTA-ABS was done with multiwell Treponema pallidum antigen substrate slide. For specific staining by conjugates, controls were the reference diluents of Behring-phosphate buffer alone and sorbent (standardized extract of Treponema phagedenis biotype Reiter) alone, which gave no distinct fluorescence, and the following control specimens (the expected relative fluorescences are given in parentheses): reactive control diluted 1:5 with PBS (4+), reactive control diluted 1:5 with sorbent (3+), a specific positive control diluted with PBS (2+) and with sorbent (−). Test specimens were diluted 1:5 in sorbent before incubation with antigen on the slide.

RESULTS

Preliminary observations from our laboratory showed that immune ascitic fluids from mice immunized with whole B. hermsii cells were negative when tested by surface immunofluorescence with living B. burgdorferi spirochetes, whereas the same samples were highly positive (titers range, 2,048 to 4,096) when tested by conventional IFA, as was expected. Immunoblot analysis (Fig. 1) of immune ascites showed that the cross-reactive antigens in the IFA were the 39-kDa protein of B. hermsii (32), the 41-kDa protein of B. burgdorferi (3), and a 60-kDa antigen (17) which are known to be not surface exposed (30). These results prompted us to evaluate whether the SIFA performed with living B. burgdorferi cells could detect antibody against only species-specific epitopes when sera from patients with Lyme disease and controls were tested. Antibody levels detected by IFA, ELISA, and SIFA in sera from 14 patients with Lyme disease are
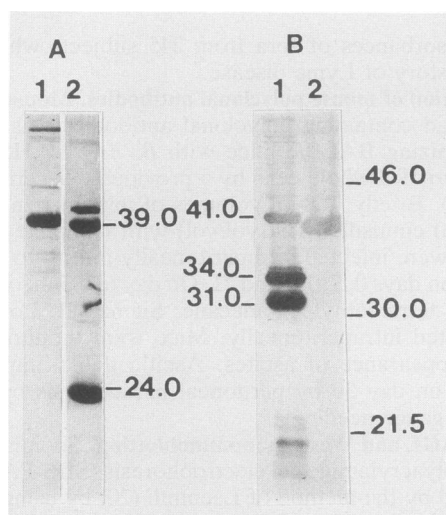
FIG. 1. Cross-reactivities of *B. burgdorferi* and *B. hermsii* studied by immunoblotting. Immune ascitic fluid obtained by immunizing BALB/c mice with *B. hermsii* HS-1 (A) and *B. burgdorferi* IRS (B) were tested with homologous (A, lane 2; B, lane 1) and heterologous (A, lane 1; B, lane 2) antigen preparations. Note that *B. hermsii* immune ascites recognized the 41-kDa protein (flagellin) and two other proteins of *B. burgdorferi* with higher molecular masses. None of the known surface-exposed proteins of the Lyme spirochete was recognized. Molecular masses (10^3) of the internal standard are shown on the right, and the apparent molecular masses of individual *B. burgdorferi* (B, lane 1) and *B. hermsii* (A, lane 2) proteins are shown between the two panels.

reported in Table 1. The SIFA (Fig. 2) was negative, with only two exceptions, for sera obtained during the acute phase of the disease (i.e., during the ECM stage) and often for sera obtained during the following 3- to 6-month period; after that, SIFA became positive for sera from all patients with ECM. Sera from patients who entered the study with a diagnosis of Lyme arthritis or neuritis were positive throughout the study period, showing stable high titers.

To evaluate the specificity of the test, sera from 55 patients with secondary or latent syphilis that were reac-

tive in the FTA-ABS (19 serum samples were 4+; 17 serum samples were 3+; 13 serum samples were 2+; 6 serum samples were 1+) as well as in the microhemagglutination test for *T. pallidum* and in the Venereal Disease Research Laboratory assay (data not shown) and 33 serum samples from patients with rheumatoid arthritis were tested for *B. burgdorferi* antibody by IFA, ELISA, and SIFA. All sera were negative by SIFA, whereas various degrees of cross-reactivity were found by IFA and ELISA. Of the 55 serum samples from patients with syphilis, 38 (69%) and 51 (93%) reacted by IFA and ELISA, respectively. Of the 33 serum samples from patients with rheumatoid arthritis, 7 (21%) and 12 (36%) reacted by IFA and ELISA, respectively.

Western blotting analysis showed that sera from patients with Lyme disease became strongly positive by SIFA when antibodies against the 31-kDa protein and, often, the 34-kDa protein were detectable. Antibodies against few other proteins with molecular masses that varied between 14 and 22 kDa were sometimes detectable (Fig. 3). On the contrary, sera from patients with syphilis or rheumatoid arthritis that were reactive with *B. burgdorferi* antigens by IFA or ELISA did not recognize the 31- or 34-kDa proteins (Fig. 3D and E).

DISCUSSION

So far, the criteria for the diagnosis of Lyme borreliosis have not been clearly defined and may depend on subjective clinical evaluations and unreliable laboratory tests. This is because Lyme disease tends to mimic several immune disorders and inflammatory diseases. Therefore, there is a demand for improved serological tests for diagnosis of the disease. IFA (28), whole-cell ELISA (7, 14, 28), and immunoblotting (16) have been used as serological tests for the diagnosis of Lyme borreliosis. ELISA has been also performed with *B. burgdorferi* cellular fractions (18, 23), and IFA has been performed with preabsorption of sera, owing to problems of cross-reactivity, since *B. burgdorferi* shares common antigens with borreliae that cause relapsing fever (22, 24) and with treponemal components (22, 24–27), including flagellin. Aside from flagellin, there are additional pro-

TABLE 1. IgG antibody response to *B. burgdorferi* in 14 patients with Lyme disease determined by IFA, ELISA, and SIFA[a]

| Patient no. | Disease stage | IgG antibody response by the indicated method at the following times (mo): | | | | | | | | |
| | | 0–2 | | | 3–6 | | | 7–12 | | |
		IFA	ELISA	SIFA	IFA	ELISA	SIFA	IFA	ELISA	SIFA
1	ECM	N[b]	N	N	256	1.1	N	512	1.1	100
2	ECM	N	0.7	N	N	1.5	N	512	1.5	100
3	ECM	N	N	N	256	1.4	N	256	1.4	200
4	ECM	256	1.2	50	256	1.3	50	1,024	1.5	200
5	ECM	N	1.3	N	256	1.5	50	2,048	1.5	200
6	ECM	N	N	N	N	0.9	N	1,024	1.2	200
7	ECM	256	1.1	50	528	1.4	50	1,024	1.5	200
8	ECM	N	N	N	256	1.2	N	1,024	1.4	100
9	ECM	N	0.8	N	256	1.5	N	512	1.5	200
10	Arthritis	2,048	1.5	400	1,024	1.4	400	2,048	1.4	400
11	Arthritis	1,024	1.5	400	2,048	1.4	400	2,048	1.5	400
12	Arthritis	2,048	1.4	400	2,048	1.5	400	1,024	1.4	400
13	Arthritis	1,024	1.3	400	1,024	1.2	400	1,024	1.1	400
14	Neuritis	4,096	1.5	400	4,096	1.5	>400	4,096	1.5	>400

[a] Sera were collected from patients at various times after the onset of illness for up to 12 months. A positive IFA result was defined as a titer of ≥256. A positive ELISA result was defined as optical density of ≥0.4 optical density units. A positive SIFA result was defined as a titer of ≥1:50.
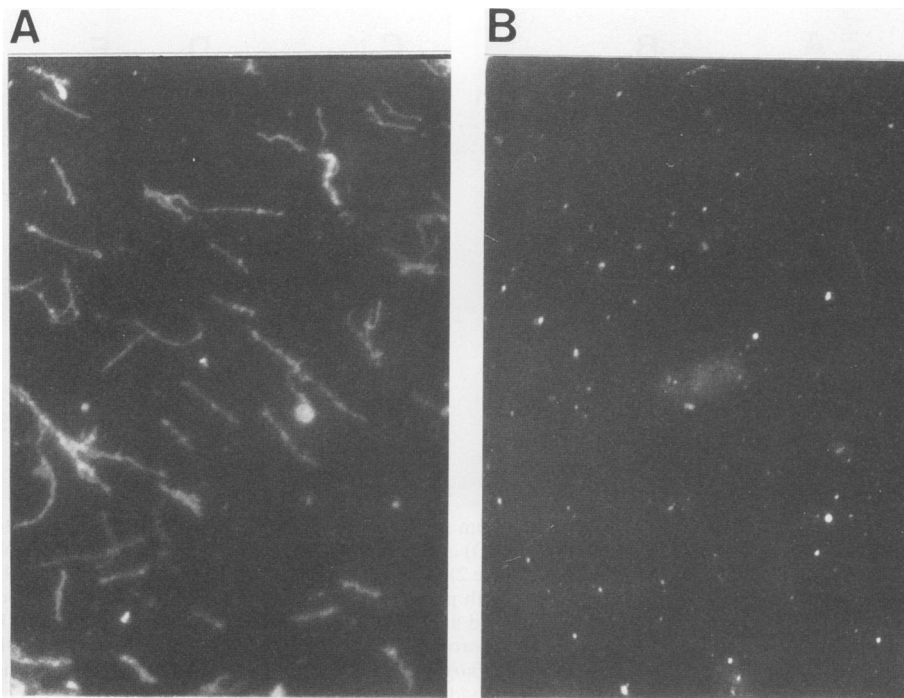[b] N, negative.

FIG. 2. Photomicrograph of *B. burgdorferi* IRS stained by SIFA. Living bacteria were incubated with *B. burgdorferi*-positive (A) and -negative (B) human serum samples. *B. burgdorferi* cells were collected from the upper part of the culture (see text), where the presence of cellular debris is minimal. Spots of fluorescent but amorphous material were sometimes observed. The point fluorescence, which might be due, at least in part, to endocellular antigens that are accessible to antibodies in fragmented bacteria, did not interfere with interpretation of the test. Magnification, ×630.

teins of *B. burgdorferi* that are common to other bacteria (17). In this study, the sensitivity of the SIFA determined in patients with clinically and serologically diagnosed Lyme disease was shown to be low during the acute phase of the illness, since 78% of serum samples from patients with ECM were negative during the first 2 months of illness. However, all patients seroconverted during the convalescent phase (3 to 12 months from the time of disease onset). Serum samples from patients with complicated Lyme disease presenting with arthritis or neuritis were always positive.

The specificity of the SIFA was also analyzed by using sera from patients with syphilis (14, 22, 24, 26, 27) or rheumatoid arthritis (21), since these sera are known to be highly (syphilis) or moderately (rheumatoid arthritis) cross-reactive in the conventional IFA and ELISA for *B. burgdorferi*. The SIFA was nonreactive with all sera from patients with syphilis and rheumatoid arthritis. In contrast, IFA and ELISA demonstrated high degrees of cross-reactivity with sera from patients with syphilis (69 and 93%, respectively) and rheumatoid arthritis (21 and 36%, respectively), as was expected.

The results of this study show that the surface-exposed antigens of *B. burgdorferi* are highly specific for detection of Lyme disease. In addition, data obtained by using mouse immune ascites also suggested that cross-reactive antigens are not present on the surfaces of *B. burgdorferi* and the spirochete that causes relapsing fever, *B. hermsii*. Since sera from patients with relapsing fever were not available, we could not confirm this observation in human infections. In our study, all serum samples that were positive by SIFA also

reacted with the 31-kDa (6) and often with 34-kDa (5) outer surface proteins (proteins A and B, respectively) of *B. burgdorferi* IRS by the immunoblotting technique, suggesting that these proteins are major antigens involved in SIFA. A few other low-molecular-mass proteins (14, 20, and 22 kDa) previously shown to be surface exposed (19, 29) were recognized, which was at variance with the results obtained with SIFA-positive sera.

It is known that surface-exposed immunodominant antigens of OspA and OspB appear to be antigenically variable when they are studied by using monoclonal antibodies (4, 5, 34). However, a recent study by Dorward et al. (15) has shown that, unlike several monoclonal antibodies against the OspA and OspB proteins that fail to bind to some strains, polyclonal antibody directed against intact vesicles of *B. burgdorferi* reacted primarily with OspA and OspB from all strains of *B. burgdorferi*, suggesting that polyclonal IgG antibodies bind to multiple epitopes on recognized proteins. This observation seems to be in agreement with our results, showing that all patients with Lyme borreliosis reacted with *B. burgdorferi* IRS at any time of the disease. The specificity of SIFA is also in agreement with previously presented results, showing that expression of surface proteins A and B is apparently unique to *B. burgdorferi* (4, 6, 8, 15).

In summary, the results indicate that SIFA, because of its specificity, may be useful in the serodiagnosis of complicated Lyme disease and helps to confirm an infection when *B. burgdorferi* cross-reactive antibodies are present in sera from patients with other diseases.
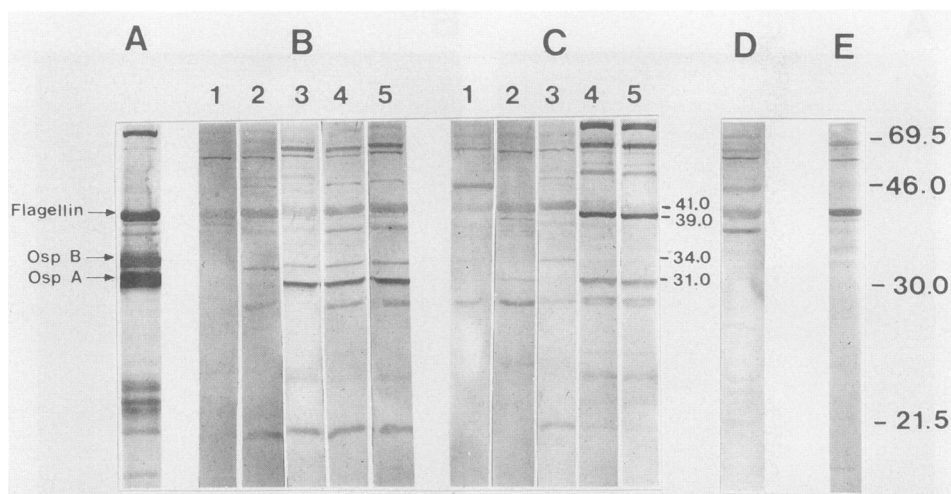
FIG. 3. *B. burgdorferi* Western blot reactivities of five serial serum samples from two patients (B and C) with Lyme disease, presenting with ECM and one serum sample each from patients with syphilis (D) and rheumatoid arthritis (E). Specimens were obtained from patients with Lyme disease early in the course of disease (lane 1) and at 2 (lane 2), 3 (lane 3), 6 (lane 4), and 12 (lane 5) months from the onset of ECM. Note that the OspA protein was clearly recognized by sera from both patients (B and C), whereas OspB was clearly detected only by sera from patient B. Serum from patient C (lanes 4 and 5) also recognized a 39-kDa protein. The serum from a patient with syphilis (D) reacted with flagellin and a few other proteins but not with the OspA or OspB protein of *B. burgdorferi*. Serum from a patient with rheumatoid arthritis (E) reacted principally with the 41-kDa protein. The reactivity of *B. burgdorferi* IRS immune mouse ascitic fluid with homologous antigen (a control) is shown in panel A. The apparent molecular masses of individual *B. burgdorferi* proteins are shown between panels C and D, and the positions of the molecular mass markers (10^3) are indicated on the right.

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