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Serodiagnosis of Lyme Borreliosis by Western Immunoblot: Reactivity of Various Significant Antibodies against *Borrelia burgdorferi*

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The significance of various antibodies against *Borrelia burgdorferi* was studied by Western blot (immunoblot) by using 578 human serum samples. The proteins regularly detected by using samples from patients with Lyme borreliosis were those with bands with molecular masses of 94, 83, 75, 66, 60, 55, 46, 41, 39, 34, 31, 29, 22, and 17 kDa. The detectable frequencies of most of these proteins appeared to be significantly different between the sera from patients with Lyme borreliosis and those from normal control individuals as well as from the group with syphilis. The 39-kDa protein band recognized by polyvalent antibody was found to be the most significant marker for Lyme borreliosis. Furthermore, an anti-39-kDa immunoglobulin M response was detected in the samples from patients with early-stage Lyme borreliosis. Results from the use of monoclonal antibodies and patient sera revealed that the 39- and 41-kDa proteins may be structurally related but are immunologically distinct antigens. The significance of antibody reactivities to the 41-, 94-, 22-, 31-, and 34-kDa protein bands is also discussed.

Lyme borreliosis is a multisystemic disorder caused by the spirochete *Borrelia burgdorferi* (24). Erythema migrans (EM), an expanding skin lesion that often develops at the site of an *Ixodes dammini* tick bite, is the best clinical marker for Lyme borreliosis (24), but it is not always observed; therefore, Lyme borreliosis does not always present a consistently clear clinical picture. Serodiagnostic tests are important tools and are widely used for identifying the disease in ambiguous clinical situations. The most commonly used serological assays are the indirect immunofluorescent assay (IFA) and the indirect enzyme-linked immunosorbent assay (ELISA) (6, 12, 25). The Western blot (immunoblot) is also used to characterize the antibody response in patients with Lyme borreliosis, as well as to confirm the IFA and ELISA results. The Western blot can provide more detailed information on different antibody response patterns to various antigens of *B. burgdorferi* and may be more sensitive and specific than either IFA or ELISA (5, 10, 15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell lysates of *B. burgdorferi* has identified at least 29 components, 11 or 12 of which are recognized by Western blot by using sera from patients with Lyme borreliosis (10, 29). Among those polypeptides recognized are the 31- and 34-kDa outer surface proteins (OspA and OspB, respectively) (2, 3), the 41-kDa flagellin protein (1), the 83-kDa major extracellular protein (7), the 21/22-kDa protoplasmic cylinder protein (2, 22, 27), the 60-kDa common antigen (11), and the 60- to 72-kDa heat shock proteins (4). On the basis of the early observations of the Western blot procedure, a guideline was established stating that at least two to five bands need to be detected for a patient serum specimen to be considered positive for Lyme borreliosis by Western blot (8, 10). Recently, a 39-kDa protein has been identified as the most immunodominant component with no cross-reactivity to serum samples containing the syphilis spirochete or normal control serum samples (23). Another study (29) found the 39-kDa protein to be nonsignificant; rather, a 94-kDa protein appeared to be the best marker for Western blot in the diagnosis of stage III Lyme borreliosis. An insufficient demographic variety of samples and controls used, different methods, or an unclear separation of the 39- and 41-kDa proteins in some immunoblots may have created these discrepant conclusions.

In this report, we describe the results of further analysis on the significant immunological features of various antibodies against *B. burgdorferi* from 578 human serum samples by using the FASTLYME Western blot test (U.S. Food and Drug Administration 510(k) approval pending). The possible implications of some antigens and antibodies in the diagnosis and pathogenesis of Lyme borreliosis are discussed.

MATERIALS AND METHODS

Antigen preparation. All strains of *B. burgdorferi* used in this study were provided by the Bioscience Laboratory at the 3M Center, St. Paul, Minn. For preparation of the Western blot strips, whole cells of spirochetes B31 (ATCC 35210) suspended in sterile saline were sonicated, and the final protein concentration was adjusted to about 0.7 mg/ml, which was measured by a bovine serum albumin microassay (Pierce, Rockford, Ill.). Storage was at 4°C with 0.01% sodium azide for 1 to 7 days. The following 10 additional strains of *B. burgdorferi*, which were obtained from different geographical areas, were used for a comparison study: ACA-1 (Sweden), IPS (Russia), CTI (United States), German tick (Germany), Pacificus (United States), 297 (United States), VEERY (United States), PBi (Germany), G25 (Germany), and DVI (Germany).

Monoclonal antibodies. Several monoclonal antibodies were used in this study. H9724, which is specific for flagellin proteins of all tested *Borrelia* species (1), was provided by A. Barbour (The University of Texas Health Science Center, San Antonio, Tx.); monoclonal antibody 158, which was prepared by immunizing mice with the purified flagellar

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protein and which was specific for the 41-kDa protein, was provided by B. Luft (State University of New York at Stony Brook); and additional monoclonal antibodies designated LYB008, LYM039, and LYM065, which reacted with the OspA, OspB, and flagellar proteins of *B. burgdorferi*, respectively, were provided by the Bioscience Laboratory at the 3M Center (13). Goat anti-mouse immunoglobulin G (IgG) antibody alkaline-phosphatase conjugate was purchased from Boehringer, Mannheim, Germany.

Serum specimens. A total of 578 serum samples from the patients and controls used in this study were divided into three groups. (i) One hundred eighty-six serum samples from patients with Lyme borreliosis were collected from several major endemic areas, including Wisconsin, Minnesota, and the northeastern United States. All of the patients had a history of Lyme borreliosis, as confirmed by a diagnosis of EM and/or other clinical manifestations, as well as a positive serological result tested by the IgG-IgM fluorescence ELISA FASTLYME test (17). An additional 16 serum samples were collected from patients with early Lyme borreliosis who had a history of EM and whose test results for specific IgG were negative or indicated a low titer but who tested positive or were borderline by the FASTLYME ELISA specific for IgM (see below). For most of these 16 patients, the serum samples were collected during EM or within 6 weeks from the onset of illness. (ii) Three hundred twenty serum samples from healthy in-house employees as well as from healthy blood donors in California, Wisconsin, and Minnesota without clinical evidence of Lyme borreliosis were collected and were used as normal controls. All sera were negative in screening tests performed by the aforementioned IgG-IgM fluorescence ELISA. (iii) Fifty-six syphilitic serum samples were collected from several clinical laboratories and serum banks.

Rabbit and goat antisera. The rabbit antisera, which were provided by the Bioscience Laboratory at the 3M Center, were derived from three rabbits that were chronically immunized over a 2-year period (every 2 months) with 10^9 whole *B. burgdorferi* organisms in saline. The antisera were collected and pooled prior to the fractionation into IgG by protein A affinity chromatography. The antisera (total IgG range, 3 to 7 mg/ml) were supplied in 0.01 M phosphate-buffered saline. The goat antisera for *B. burgdorferi* were purchased from KPL (Gaithersburg, Md.).

SDS-PAGE. The majority of materials used for electrophoresis were purchased from Hoefer Scientific Instruments, San Francisco, Calif., unless stated otherwise. Sonicated antigen was dissolved in lysing buffer (Integrated Separation Systems, Hyde Park, Mass.), heated for 5 min in boiling water, and subjected to SDS-PAGE (12.5% polyacrylamide gel) by using the system of Laemmli (16). A prestained low-range-molecular-mass standard (Bio-Rad, Richmond, Calif.) was run with each gel. Electrophoresis was carried out with a constant current of 25 mA at room temperature until the marker dye front migrated down to an optimized point in the gel. Proteins separated by the gel were then transferred onto nitrocellulose sheets by using the system of Towbin et al. (26). The nitrocellulose sheets were subsequently blocked with 5% fat-free milk for 2 h at room temperature and then cut into 3-mm-wide strips each containing about 2.5 μg of protein. Two strips from each gel were used for quality control; one was stained with amido black, to assess the efficiency of transfer; the other was used to react with a reference serum (a pool of samples from patients with a history of EM and a positive IFA titer [>1:512] that contained the antibodies against the 39- and 41-kDa and other antigens of *B. burgdorferi*). If this strip did not show a clear separation between the 39- and 41-kDa proteins, the whole blot was considered unqualified and was not used for further study.

Immunoblotting. The strips that qualified for the study were overlaid with sera (diluted 1:100) or monoclonal antibodies and were gently incubated at room temperature for 2 h. After three 5-min washes with a buffer containing 0.1 M Tris–0.002% Tween 20, the strips were incubated for 2 h with a combination of alkaline phosphatase-conjugated goat antibodies to human IgM (diluted 1:5,000; Cappel, West Chester, Pa.) and IgG (diluted 1:1,000; ICN Biomedicals, Costa Mesa, Calif.). After three additional washings, substrate (5-bromo-4-chloro-3-indolylphosphate toluidinium, nitroblue tetrazolium) was added and was allowed to incubate with the strips at room temperature until the color was optimized. The reaction was stopped by adding deionized water, and the reactive band pattern was visually compared with the positive control strip. For the detection of IgM antibody alone, the IgG fraction was removed by the QUIK-SEP IgM/IgG Isolation System II (Isolab Inc., Akron, Ohio). The serum was diluted 1:50.

Fluorescence ELISA. The FASTLYME test for Lyme borreliosis was performed as described previously (17). Briefly, sonicated *B. burgdorferi* was coated on opaque (black) microtitration wells. Fifty microliters of a Lyme reference serum sample or diluted serum samples from patients were incubated in antigen-coated microtitration wells at room temperature for 30 min. After three aspiration and washing cycles with phosphate-buffered saline, alkaline phosphatase-labeled goat anti-human IgG and/or IgM antibodies were added and allowed to incubate for 30 min. After three additional washings, fluorescence substrate, 4-methylumbelliferyl phosphate, was added and the substrate was incubated for another 30 min. The degree of fluorescence emitted by the substrate in each well was then measured with a 3M FluoroFAST 96 fluorometer, reported as fluorescence signal units, and was compared with that of the reference serum. For the IgG-IgM assay, the cutoff was 10% of the reference serum; for the IgG assay, the cutoff was 8%; and for the IgM assay, below 20% was negative, between 20 and 25% was borderline, and above 25% was positive.

Statistics. Frequencies of antibody reactivity to each protein band observed in patients with Lyme borreliosis were compared with those observed in normal controls and syphilis groups by χ^2 analysis. Their degrees of significance and difference were also analyzed by use of the Z score (9). The greater the Z score, the larger the difference between two comparative protein band frequencies.

RESULTS

Spirochetal proteins recognized by human sera and monoclonal antibodies. An immunoblot that reacted to pooled sera from six patients with early or late Lyme borreliosis indicated the recognition of at least 22 protein bands by the human sera. Their positions were referenced to the molecular mass standard and monoclonal antibodies (Fig. 1). Monoclonal antibodies H9724 (diluted 1:1,000) and LYM065 (diluted 1:2,000) recognized both the 41- and 39-kDa proteins, the latter being less predominant (Fig. 1, lanes 5 and 7), whereas monoclonal antibody 158 specifically recognized a single polypeptide at the 41-kDa position, even when the sample was undiluted and incubated overnight (Fig. 1, lane 6). In an immunoblot of 10 different strains of *B. burgdorferi* with the pooled sera from patients with Lyme borreliosis,

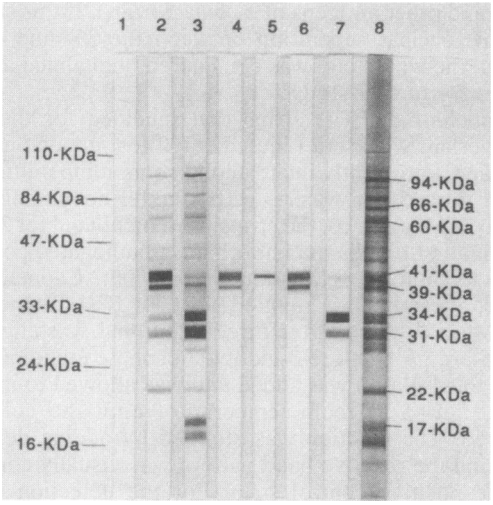

FIG. 1. Immunoblots of *B. burgdorferi* B31 with several monoclonal antibodies, animal antisera, and human patient sera as follows: amino black staining (lane 1), goat antisera (lane 2), rabbit antisera (lane 3), H9724 (lane 4), monoclonal antibody 158 (lane 5), LYM065 (lane 6), LYM008-LYM039 (lane 7), pooled human sera from patients with Lyme borreliosis (lane 8). The migrations of prestained molecular mass standards are shown on the left, and the numbers on the right represent the apparent molecular masses of some characteristic *B. burgdorferi* proteins.

39-, 41-, and 60-kDa proteins were consistently visible in all of the strains, whereas variation was observed in 31-, 34-, and 22-kDa and other protein bands (Fig. 2).

Frequency of each protein bands shown in immunoblots. Protein bands with molecular masses of 94, 83, 75, 66, 60, 55, 46, 41, 39, 34, 31, 29, 22, and 17 kDa were regularly detected by the human sera used in this study. Comparison of the detectable band frequencies between patients and controls is shown in Fig. 3 and Table 1. Representative immunoblots for the different ELISA titer groups are shown in Fig. 4. The statistical analysis showed that the detectable frequencies of the antibody reactivities to these regular protein bands in patients with Lyme borreliosis were all significantly different from those in the normal control group, but not all of them were significantly different from those in the syphilis group. Among those regularly detectable bands, the 41-kDa species was the most frequently seen protein band in all groups, whereas the 39-kDa protein appeared to be the most significant band because it had the highest Z score in a global analysis that compared the three groups. The Z scores for each protein band are given in Table 1.

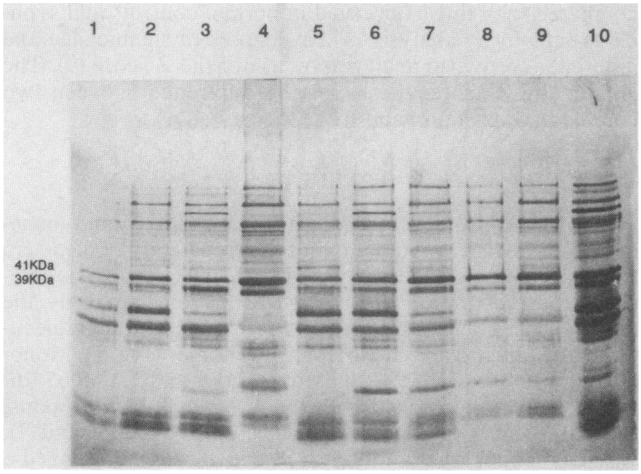

FIG. 2. Antibody reactivities of serum pooled from several patients with Lyme borreliosis to 10 different strains of *B. burgdorferi*. Lanes: 1, ACA-1; 2, IPS; 3, CTI; 4, German tick; 5, Pacificus; 6, 297; 7, VEERY; 8, PBi; 9, G25; 10, DVI.

Correlation between ELISA titer and band frequency. To analyze the correlation between the antibody level detected by ELISA and the frequency of antibody reactivity to each protein band by Western blot, 186 serum samples from patients with Lyme borreliosis were divided into three ELISA titer groups according to the results of the 3M IgG/IgM FASTLYME test (percent of reference serum), as follows: 10 to 20% for a low titer; 21 to 70% for a medium titer; and >70% for a high titer. The results indicate that the higher the titer in the ELISA, the higher the frequency of bands in the Western blot. Interestingly, some low-ELISA-titer samples of sera from patients with Lyme borreliosis displayed multiple bands (Fig. 4, lane 4), while some patient sera with fewer protein bands had medium or high ELISA titers (Fig. 4, lanes 24, 27, and 35). Both the 41- and 39-kDa proteins were usually found to be predominant in the sera of all three ELISA titer groups, with 100% frequency found in the sera of high-titer group. The frequencies of each protein band in the sera of the different ELISA titer groups are given in Table 2.

IgM response in patients with early Lyme borreliosis. Sixteen serum samples from patients with early Lyme borreliosis were tested by IgM Western blot. All of these samples recognized the 41-kDa protein, and about half of them reacted with the 39-, 94-, and 60-kDa components. The IgM antibody reactivity to the 39-kDa protein band in the sera of patients with early Lyme borreliosis appeared to be slightly sharper than that observed in the other three IgG-IgM ELISA titer groups of sera from patients with Lyme borreliosis. Representative immunoblots are shown in Fig. 4, lanes 49 to 57.

DISCUSSION

A visually distinguishable band pattern in our Western blot was achieved by use of a nonradioactive alkaline phosphatase-conjugated goat anti-human IgG-IgM antibody. The protein bands regularly detected by 186 serum samples from patients with Lyme borreliosis were those with the molecular masses of 94, 83, 75, 66, 60, 55, 46, 41, 39, 34, 31, 29, 22, and 17 kDa (Fig. 3). These band frequencies of sera from patients with Lyme borreliosis were all significantly different from those of sera from the normal group, but not all of them were significantly different from those of sera from the syphilis group; syphilis is one of the major cross-reactive diseases for Lyme borreliosis (Table 1).

Among all of the regularly detected bands, the 41-kDa protein always remained as the most frequently detectable band in the sera of every group, including sera of the normal control and syphilis groups. Since the 41-kDa protein was detected by the majority (87.1%) of the sera from patients with Lyme borreliosis, it is reasonable to assume that antibody bound to the 41-kDa protein must contribute to part of the signal detected by ELISA, especially in the early stage of the disease as well as in the low-titer group (Table 2). On the basis of our unpublished observations, it was found that the specific IgG or IgM antibodies in serum samples from

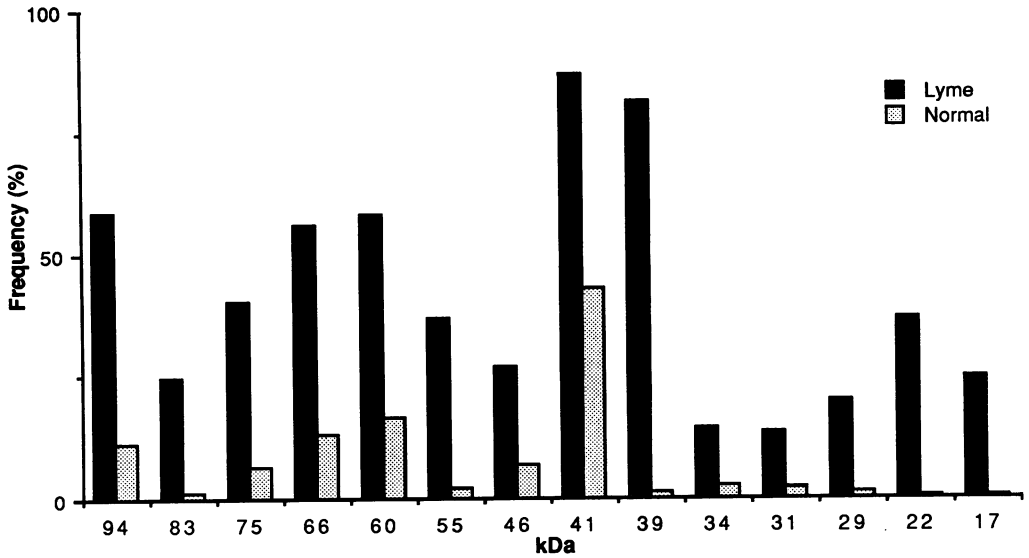

FIG. 3. Comparison of the frequency of antibody reactivity to various *B. burgdorferi* protein bands between 186 patients with Lyme borreliosis and 320 normal controls.

patients with Lyme borreliosis did not always generate identical band patterns. The reactivity of IgM to the 41-kDa protein band was sometimes stronger than that of IgG, suggesting that a lower frequency of the 41-kDa band might occur if only a labeled protein A is used to detect the IgG antibodies (23). Results of this study indicate that the antibody bound to the 41-kDa protein is still a statistically significant marker for Lyme borreliosis, in comparison with the detection of normal and syphilitic sera. Nevertheless, because this protein was also the highest cross-reactive protein band in the normal group (43.1%) and the syphilis group (77.5%), we do not recommend use of the 41-kDa protein as a single purified antigen for ELISA, unless specific epitopes, if any, are identified and isolated from the cross-reactive elements. It seems possible that there could be several antigenic epitopes within the 41-kDa protein molecule and that the epitope(s) recognized by monoclonal antibody 158 might be more specific for the 41-kDa flagellin protein than it is for those recognized by the two other monoclonal antibodies used in this study (Fig. 1, lanes 5 to 7). It is also important that such a high cross-reactivity of the 41-kDa protein in the normal group could also be due to the oral infection with treponemes (18).

From our studies, the antibody reactivity to the 39-kDa protein was found to be the most significant marker for Lyme borreliosis. Its frequency was 81.2% for the Lyme borreliosis group, whereas it seemed to be negligible in the normal control group. Its Z score reached 28.5, the highest among all of the regularly detected proteins bands (Fig. 3 and Table 1). Similar to the observation of Simpson et al. (23), antibody reactivity to the 39-kDa protein appeared in 100% of patients with a high antibody level (Table 2); false

TABLE 1. Comparison of antibody reactivities to various Lyme antigen protein bands in different serum groups

Protein band (kDa)	No. (%) of patient serum samples with reactivity to protein band[a]			*P* value[a]		Z score (total)[b]
	Lyme (n = 186)	Normal (n = 320)	Syphilis (n = 56)	Lyme versus normal patients	Lyme versus syphilitic patients	
94	109 (58.6)	36 (11.3)	6 (10.7)	<0.05	<0.05	17.7
83	46 (24.7)	4 (1.3)	1 (1.8)	<0.05	<0.05	12.3
75	75 (40.3)	21 (6.6)	9 (16.1)	<0.05	<0.05	12.7
66	104 (55.9)	42 (13.1)	21 (37.5)	<0.05	<0.05	12.7
60	108 (58.1)	53 (16.6)	24 (42.9)	<0.05	<0.05	11.7
55	69 (37.1)	7 (2.2)	2 (3.6)	<0.05	<0.05	15.4
46	50 (26.9)	22 (6.9)	9 (16.1)	<0.05	<0.05	7.8
41	162 (87.1)	138 (43.1)	42 (75.0)	<0.05	<0.05	11.9
39	151 (81.2)	4 (1.3)	6 (10.7)	<0.05	<0.05	28.5
34	27 (14.5)	8 (2.5)	7 (12.5)	<0.05	<0.05	5.5
31	25 (13.4)	7 (2.2)	0 (0.0)	<0.05	<0.05	7.9
29	37 (19.9)	4 (1.3)	1 (1.8)	<0.05	<0.05	10.7
22	69 (37.1)	2 (0.6)	0 (0.0)	<0.05	<0.05	16.8
17	46 (24.7)	2 (0.6)	1 (1.8)	<0.05	<0.05	12.7

[a] The *P* value was analyzed by χ^2.
[b] The total Z score is a summation of the Z scores from patients with Lyme borreliosis and normal patients and from patients with Lyme borreliosis and patients with syphilis.

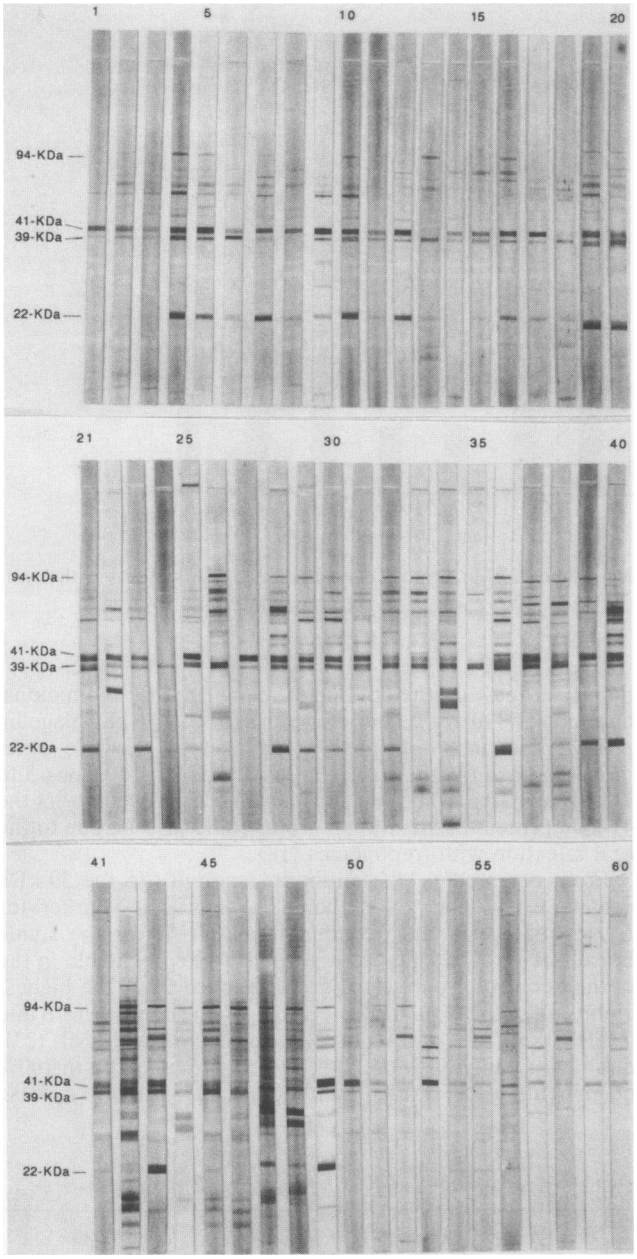

FIG. 4. Representative immunoblots of *B. burgdorferi*. Lanes are numbered along the top in increments of five to correspond with the lane numbers 1 to 60. Except for lanes 49 to 57, all these strips are IgG-IgM blots. Numbers on the left represent the apparent molecular masses of some characteristic *B. burgdorferi* antigens. Lanes 1 to 11, low-ELISA-titer sera from patients with Lyme borreliosis (10 to 20% of reference serum); lanes 12 to 33, medium-ELISA-titer sera from patients with Lyme borreliosis (21 to 70% of reference serum); lanes 34 to 48, high-ELISA-titer sera from patients with Lyme borreliosis (>70% of reference serum); lanes 49 to 57, IgM blot of sera from patients with early Lyme borreliosis; lanes 58 to 60, sera from patients with syphilis.

positivity or cross-reactivity was rare in this group. It has been reported that mice infected with *B. burgdorferi* produce anti-P39 antibodies no later than 7 days postinfection (21). In our study, 8 of 16 patients with early Lyme borreliosis showed anti-39-kDa IgM antibody reactivity (Fig. 4), indicating that the 39-kDa protein may be one of the antigens recognized by the human immune system in the early stages of the disease.

TABLE 2. Frequency of antibody reactivity to various Lyme antigen protein bands in serum samples from patients with Lyme borreliosis with different fluorescent ELISA titers

Protein band (kDa)	No. (%) of protein band with a[a]:			
	Low titer (n = 52)	Medium titer (n = 79)	High titer (n = 55)	Total (n = 186)
94	21 (40.4)	40 (50.6)	48 (87.3)	109
83	5 (9.6)	22 (27.8)	19 (34.5)	46
75	13 (25.0)	26 (32.9)	36 (65.5)	75
66	21 (40.4)	46 (58.2)	37 (67.3)	104
60	25 (48.1)	50 (63.3)	33 (60.0)	108
55	20 (38.5)	27 (34.2)	22 (40.0)	69
46	9 (17.3)	19 (24.1)	22 (40.0)	50
41	39 (75.0)	68 (86.1)	55 (100.0)	162
39	31 (59.6)	65 (82.3)	55 (100.0)	151
34	6 (11.5)	7 (8.9)	14 (25.5)	27
31	5 (9.6)	8 (10.1)	12 (21.8)	25
29	3 (5.8)	12 (15.2)	22 (40.0)	37
22	20 (38.5)	24 (30.4)	25 (45.5)	69
17	5 (9.6)	20 (25.3)	21 (38.2)	46

[a] Low titer, 10 to 20% of reference serum in fluorescence ELISA; medium titer, 21 to 70% of reference serum in fluorescence ELISA; high titer, >70% of reference serum in fluorescence ELISA.

Our results reveal that the 39- and 41-kDa proteins may be structurally related but immunologically distinct antigens. (i) The position of the 41-kDa protein, which was identified as a single band by monoclonal antibody 158, is very close to but distinct from the 39-kDa protein band (Fig. 1). (ii) From the finding of Jiang et al. (14), cross-antigenicity between the OspA and OspB proteins as well as other proteins of *B. burgdorferi* has been demonstrated by monoclonal antibodies and monospecific polyclonal antibodies. Our study shows that two monoclonal antibodies, H9724 and LYM065, against the 41-kDa protein were also able to recognize the 39-kDa protein (Fig. 1, lanes 5 and 7), indicating the possibility that some cross-epitopes are shared by these two polypeptides. (iii) In most cases, both the 41- and the 39-kDa proteins were simultaneously detectable in the sera of patients with Lyme borreliosis. The frequencies of antibody reactivity to these two proteins were similarly predominant in the Lyme disease group, but their prevalency rates were markedly different in both the normal control and the syphilis groups (Table 1 and Fig. 3). This suggests that the 39-kDa protein may be a more specific antigen in *B. burgdorferi* infection and, therefore, should be considered one of the major antigenic components used for the ELISA. (iv) Compared with the other protein bands that were detected, the density of the 41-kDa protein was usually predominant but was not always stronger than that of the 39-kDa band (Fig. 4, lanes 13, 18, 24, 26, 34, 35, 44, and 46). Except in the early stage of the disease, it is unclear when the host antibody response reacts more strongly to the 41-kDa as opposed to the 39-kDa protein or vice versa.

Despite the antigenic variability of *B. burgdorferi* in 22- and 34-kDa and other proteins, as reported previously (28) and confirmed by us (Fig. 2), the 39- and 41-kDa proteins were consistently visualized in B31 as well as in the 10 other strains of *B. burgdorferi* analyzed in our study, suggesting that most strains of the *B. burgdorferi* spirochete may be homogeneous in their reactions to these two proteins. Since the 39-kDa protein is located so close to the 41-kDa protein

on the blot, the significance of the antibody reactivity to the 39-kDa protein has sometimes been overlooked. The function of the antibody against the 39-kDa protein is unknown, but determination of the nature of this protein may lead to a better understanding of the pathogenesis of Lyme borreliosis.

Simpson et al. (23) stated that no cross-reactivity of P39 was found with syphilitic sera; however, we found that 6 of the 56 serum samples from patients with syphilis reacted with the 39-kDa protein band, although the intensity of this band was markedly less than that observed for the samples from patients with Lyme borreliosis (Fig. 4, lanes 58 to 60). This could be explained if the P39 identified by Simpson et al. (23) is not identical to the 39-kDa protein discussed in this study or simply because only five syphilitic serum samples were used in their study. The low frequency of the 39-kDa band in the normal group may be explained by the possibility of a previous or subclinical infection, but it is noteworthy that this protein's cross-reactivity to syphilitic sera was about 10% in this study, indicating that there may be some cross-reactive epitopes to syphilis within the 39-kDa protein, although not to the same degree found in the more cross-reactive 41-kDa protein.

The 94-kDa protein, one of the best markers for stage III Lyme borreliosis, as described by Zoller et al. (29), is another significant protein band that was found in this study. As noticed by other investigators (19), the number of proteins recognized rose from stage I to stage III of Lyme disease, the samples from patients known to have late-stage disease in this study were mainly within the high-ELISA-titer group, in which the frequency of the antibody reactivity to the 94-kDa protein was 87.8%, second only to those of the 39- and 41-kDa proteins. The total Z score of the 94-kDa protein was 17.7, suggesting that antibody reactivity to the 94-kDa protein is also a significant marker for the diagnosis of Lyme borreliosis, particularly in stage III of the disease. The IgM antibody reactivity to this protein also appeared by use of the IgM Western blot in some samples from the patients with an early stage of Lyme borreliosis (Fig. 4).

IgG and IgM immune responses to the 20/22-kDa protein were found in the earlier stages of European *B. burgdorferi* infections, with no cross-reactivity in normal controls (27). The gene encoding this antigen was recently cloned and was suggested to be a useful marker for the diagnosis of Lyme borreliosis (22). In our study, IgG-IgM antibody reactivity to the 22-kDa protein appeared as an immunodominant band in the serum samples from patients with Lyme borreliosis with good specificity (Table 1 and Fig. 4). A high Z score (16.8) for this protein band supports the possibility that it might be yet another very significant marker for Lyme borreliosis.

As indicated by others (29), the antibodies against the 31- and 34-kDa proteins were rarely detected and, consequently, became less significant when compared with other protein bands in this study (Table 1). Furthermore, abundant 31- and 34-kDa proteins were not helpful in increasing the sensitivity of the ELISA (data not shown), since antibodies against these epitopes were seldom seen in our patient population. Some samples from normal or syphilitic groups recognized the 31- or 34-kDa band, but it seems that these two protein bands did not usually show up in tandem except in some patients with the late stage of Lyme borreliosis. Although the detection of antibodies against the 31- and 34-kDa proteins may not be crucial for the diagnosis in terms of sensitivity, these two proteins are very specific markers of late Lyme borreliosis when they are both present.

In conclusion, by use of the well-prepared and stringently controlled Western blot, we will be able to learn more about the true picture of the antibody response in patients with Lyme borreliosis. On the basis of a clear identification of each significant band in the Western blot, and noting its density, related band patterns, and related stages of the disease, it may be possible to establish new interpretation criteria for the diagnosis of Lyme borreliosis by the Western blot procedure. In addition, Western blot might also be used to identify the immunodominant subunits of antigen for the development of more sensitive and specific serological assays, to study the pathogenesis of Lyme borreliosis, and to evaluate the efficiency of a vaccine for Lyme borreliosis in the future.

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