Western Blotting in the Serodiagnosis of Lyme Disease

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There are currently no accepted criteria for positive Western blots in Lyme disease. In a retrospective analysis of 225 case and control subjects, the best discriminatory ability of test criteria was obtained by requiring at least 2 of the 8 most common IgM bands in early disease (18, 21, 28, 37, 41, 45, 58, and 93 kDa) and by requiring at least 5 of the 10 most frequent IgG bands after the first weeks of infection (18, 21, 28, 30, 39, 41, 45, 58, 66, and 93 kDa). When these definitions were tested in a prospective study of all 237 patients seen in a diagnostic Lyme disease clinic during a 1-year period and in 74 patients with erythema migrans or summer flu-like illnesses, the IgM blot in early disease had a sensitivity of 32% and a specificity of 100%; the IgG blot after the first weeks of infection had a sensitivity of 83% and a specificity of 95%. Among patients with indeterminate IgG responses by ELISA, 6 of 9 patients with active Lyme disease had positive blots compared with 2 of 34 patients with other illnesses ($P < .001$). Thus, Western blotting can be used to increase the specificity of serologic testing in Lyme disease.

Lyme disease or Lyme borreliosis is a multisystem infection caused by the tickborne spirochete *Borrelia burgdorferi* [1]. The illness often begins with localized infection of the skin, erythema migrans [2], followed within days to weeks by dissemination of the spirochete to many organs, particularly to other skin sites, the nervous system [3], or joints [4]. Months to years later, usually after periods of latent infection, patients may develop persistent arthritis [4], chronic neurologic involvement [5], or acrodermatitis chronica atrophicans [6].

Because culture or visualization of *B. burgdorferi* from patient specimens has been difficult [7], diagnosis has depended on recognition of a characteristic clinical picture with serologic confirmation. Serologic tests currently available for use in this disorder include ELISA [8–14], indirect immunofluorescence assay (IFA) [8–10], and Western blotting or immunoblotting [15–23]. Antigen preparations for these tests include sonicated spirochetes [8–11, 15–23] or partially purified [12, 13, 15] or recombinant proteins [14].

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Informed consent was obtained from patients or their parents, and human experimentation guidelines of the US Department of Health and Human Services were followed.

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Serodiagnosis with each of these methods has been com cated by the cross-reactivity of certain spirochetal polyp tides with other antigens [24–26], the delay in the deve ment of the humoral immune response [7, 27, 28], dampening effect of early antibiotic therapy on this respo [11, 29], and the variability of the response in different tients [15, 28]. Furthermore, in a small subset of pati with late Lyme disease who are incompletely treated w antibiotics during the first several weeks of infection, the moral immune response to *B. burgdorferi* may be abor but a cellular immune response to the spirochete may usu be demonstrated in these patients by the T cell prolifera assay [30, 31]. With each of these methods, lack of standa ization has caused significant interlaboratory variation in results [32, 33], which has led to considerable diagno confusion.

The specific immune response in Lyme disease devel gradually over a period of months to years to ≥10 spiroch polypeptides [28]. These antigens include the 31-kDa ou surface protein (Osp) A, the 34-kDa OspB [34, 35], the kDa OspC (Wilske B, Max von Pettenkofer Institut, Muni personal communication), the 41-kDa flagellar prot which is similar to the flagellar antigens of other spiroche [24], and the 58-, 66-, and 74-kDa heat-shock proteins t have homologies with the 60-kDa groEL and the 70-k DnaK heat-shock protein families of *E. coli* [25, 26]. T functions of other prominent antigens, including those at 28, 30, 37, 39, 45, and 93 kDa, are not yet clear.

There are currently no accepted criteria for positive W ern blots in Lyme disease. The purpose of the current stu was to develop such criteria in a retrospective analysis patients with various manifestations of Lyme disease, to termine the sensitivity and specificity of these criteria in p spective studies of patients with early or late manifestatio of the disorder, and to compare the results obtained ELISA and Western blotting.

Methods

Study patients. For the retrospective study, frozen samples stored at −70°C were selected from our serum bank, in alphabetical order, from the first 25 patients each with erythema migrans, meningitis, arthritis, or encephalopathy or polyneuropathy due to Lyme disease. Patients with erythema migrans were classified as having localized skin infection or disseminated infection according to clinical criteria [36]. The patients with erythema migrans, meningitis, or arthritis had not received prior antibiotic therapy, whereas half of the patients with encephalopathy or polyneuropathy had previously been given antibiotics. For comparison, sera were tested from 25 patients who had participated in an influenza vaccination program (an acute infectious disease antigen), 15 patients with multiple sclerosis and 10 with amyotrophic lateral sclerosis (neurologic diseases), 15 patients with rheumatoid arthritis and 10 with systemic lupus erythematosus (rheumatologic diseases), 25 patients with chronic fatigue syndrome (often misdiagnosed as Lyme disease), and 25 patients with secondary or latent syphilis (another spirochetal infection).

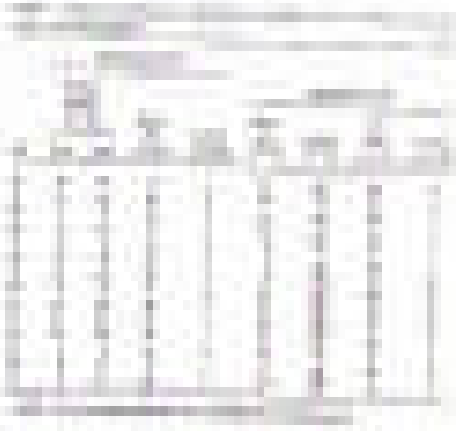
In the prospective study, the sera were tested from all 237 patients evaluated in our weekly diagnostic Lyme disease clinic from July 1990 through June 1991. By clinical criteria, these patients were categorized as having active Lyme arthritis or neuroborreliosis, inactive Lyme disease, or other illnesses. Active Lyme arthritis was defined as brief attacks of oligoarticular arthritis in a few large joints, not caused by other known types of arthritis, in a person from an area endemic for Lyme disease. These patients were required to have objective evidence of joint inflammation at the time of evaluation. Active neuroborreliosis (meningitis, chronic encephalopathy, or polyneuropathy) was diagnosed in patients with meningeal signs, memory impairment, or sensory abnormalities accompanied by a cerebrospinal fluid (CSF) pleocytosis, increased CSF protein, or electromyographic evidence of an axonal polyneuropathy, not caused by other known diseases, in a person from a Lyme disease–endemic area [31]. These patients often had a history of erythema migrans, but this clinical marker was not required for diagnosis. After clinical categorization, all 237 patients were tested for serum antibodies to *B. burgdorferi* by ELISA and Western blotting. To determine the sensitivity and specificity of these tests, results in the 54 patients who met clinical criteria for Lyme disease were compared with those in the 183 patients who did not meet these criteria. If patients met clinical criteria for Lyme disease but were seronegative by ELISA, their cellular immune response to borrelial antigens was determined by the T cell proliferative assay, as previously described [31].

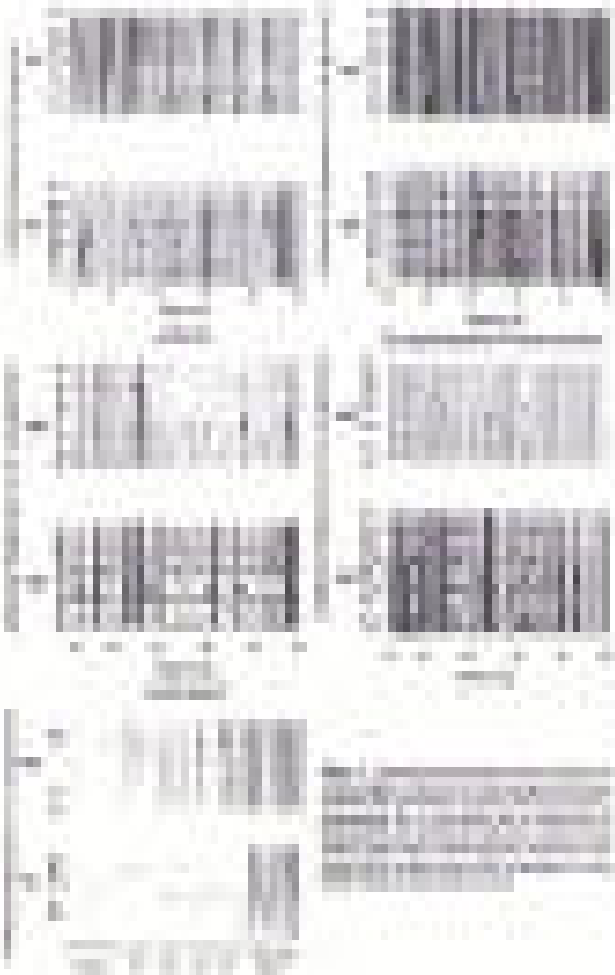
Since patients with early Lyme disease are not commonly seen in this clinic, acute and convalescent sera were tested from all 57 patients with erythema migrans entered into a multicenter antibiotic treatment study in 1989 [36] and from the patients with summer flu-like illnesses evaluated for entry into that same study who were not thought to have Lyme disease. Erythema migrans was defined as an expanding annular skin lesions ≥5 cm in diameter, usually with central clearing and a bright red outer border. Diagnosis was based on observation of this rash by the study physicians.

ELISA methods. The IgG antibody response to *B. burgdorferi* was determined by indirect ELISA, and the specific IgM response was determined by an antibody capture ELISA with modifications of previously described methods [8, 11]. For IgG determinations, 96-well microtitration plates (Immulon I; Dynatech Laboratories, West Chester, PA) were coated with 25 μL of supernatant from sonicated *B. burgdorferi* G39/40 overnight at 4°C. After being washed three times with 0.05% Tween 20 in PBS and again between each step, the plates were blocked with 5% nonfat dried milk in PBS and 0.05% Tween 20 (M-PBS, pH 7.6), incubated with 50 μL of patient sera (1:400 in M-PBS) and with alkaline phosphatase–conjugated goat anti-human IgG (1:1000 in M-PBS; Tago, Burlingame, CA) in each instance for 45 min at 37°C. After a wash with PBS without Tween 20, the substrate, 1 mg/mL *p*-nitrophenyl phosphate (PNPP) with 1 mM $ZnCl_2$, was added. For IgM determinations, 96-well plates (Immulon 2; Dynatech) were coated with goat anti-human IgM (1:1000 in 50 m*M* carbonate, pH 9.6; Tago) overnight at 4°C. The plates were blocked with M-PBS followed by the sequential addition (50 μL) and incubation (45 min at 37°C) of the following reagents diluted in M-PBS buffer: patient serum (1:400), normal control serum (1:100), sonicated *B. burgdorferi* antigen (25 μg/mL), rabbit anti–*B. burgdorferi* antibody (1:1000), alkaline phosphatase–conjugated goat anti-rabbit IgG (1:1000; Tago); then the substrate, 1 mg/mL PNPP with 25 μM $ZnCl_2$, was added.

The cutoff optical density readings (405 nm) were 3 SD (IgG) or 5 SD (IgM) above the mean optical density of 8 normal control samples included on the same plate. These samples were representative of 50 previously tested normal control samples. To calculate an antibody titer, we adjusted the value of each unknown sample with a standard curve made from dilutions of the same known positive serum included on the same plate. This patient had had Lyme disease for 3 years at the time the sample was taken; the manifestations of his illness included erythema migrans, meningitis, facial palsy, atrioventricular nodal block, and intermittent attacks of oligoarticular arthritis. For IgG determinations, a titer of ≥1:800 was defined as positive, 1:200–1:400 as indeterminant, and ≤1:100 as negative. For IgM determinations, ≥1:200 was defined as positive, 1:100 as indeterminant, and <1:100 as negative.

Immunoblotting methods. SDS-PAGE was done using a miniblot system (Bio-Rad Laboratories, Richmond, CA). Supernatant from sonicated *B. burgdorferi* G39/40 (50 μg for IgG blots, 100 μg for IgM blots) was electrophoresed on a 10% acrylamide gel (10-cm plate; 0.75-mm gel thickness; acrylamide/bis-acrylamide ratio, 30:1) at 20°C and 175 V. Gel proteins were transferred to nitrocellulose paper at 4°C and 100 V for 1 h. The paper was placed in TRIS-buffered saline (TBS: 20 m*M* TRIS, 500 m*M* NaCl, pH 7.6) and 0.1% Tween 20 for 10 min and then cut into 2-mm strips. After being washed three times between each step with 0.1% Tween 20 in TBS, the strips were blocked in 5% nonfat dried milk in TBS and 0.1% Tween 20 (M-TBS) and then incubated with patient sera (1:250 in M-TBS) and with alkaline phosphatase–conjugated goat antibody to human IgM or IgG (1:3000 in M-TBS; Tago), all for 1 h at 20°C. Substrate consisting of 1 mL of 70% *N,N*-dimethylformamide (DMF) with 30 mg of nitroblue tetrazolium chloride and 1 mL of DMF with 15 mg of 5-bromo-4-chloro-3-indolyl phosphate mixed in 100 mL of carbonate buffer (100 m*M* NaHC





10 most frequent IgG bands after the first weeks of infection. The IgM criteria should be used with caution after the initial weeks of infection; most patients have an IgG response to the spirochete by that time. We believe that our prospective study provides a rigorous test of sensitivity and specificity because the comparison patients had illnesses that are commonly confused with Lyme disease, and a number of them had previously had positive serologic tests in other laboratories.

The IgG blot in the patients with Lyme disease for at least several weeks was not 100% specific primarily because 4 patients with early neurologic involvement still had only IgM responses to *B. burgdorferi* and 2 patients with late neurologic disease who had been incompletely treated with antibiotics for erythema migrans were seronegative. The IgG blot in the comparison patients was not 100% specific because 7 patients had serologic evidence of exposure to *B. burgdorferi* but clinical pictures of other illnesses. In addition, patients with past Lyme disease usually remain seropositive for years, even after treatment with antibiotics. If patients with inactive Lyme disease or asymptomatic infection have other illnesses, particularly with joint or neurologic symptoms, positive serologic tests for *B. burgdorferi* may cause diagnostic confusion. This may be a greater problem in Europe where the frequency of subclinical infection has been reported to be higher than in the United States [39]. Furthermore, our definitions may not be suitable for European patients because the immune response may be more restricted there [18, 20, 40].

Several technical problems of Western blotting with sonicated *B. burgdorferi* should be stressed. The molecular weight of the same protein may be somewhat different depending on the strain of the spirochete or the conditions of the assay, multiple proteins may comigrate to the same area, the number of bands apparent in the blot is influenced by the concentration of reagents, and the results of Western blotting are observer-dependent. Care must be taken in reading the correct molecular weights of the bands, and faint bands, which we discounted, may pose interpretation difficulties. Video densitometry may help with this problem [20], but it is not suitable for reading miniblots, the method used here, or for reading bands that are close together, a common problem in Lyme disease. Although the use of recombinant borrelial proteins may improve specificity, sensitivity may not be as good.

In this study, the results obtained by ELISA and Western blotting were concordant in patients with clearly positive or negative tests, and Western blotting was of no additional value in these patients. However, among patients who had indeterminate responses by ELISA, Western blots were helpful in identifying false-positive results. We conclude that Western blotting can be used to increase the specificity of serologic testing in Lyme disease.

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