European Lyme Borreliosis: 231 Culture-Confirmed Cases Involving Patients with Erythema Migrans

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In 1994, we isolated *Borrelia burgdorferi* sensu lato from 231 patients with erythema migrans who presented to the University Medical Center in Ljubljana, Slovenia. Samples of erythema migrans–affected skin were placed in media to support the growth of *Borrelia* species and evaluated in Ljubljana and Chicago. Patients whose cultures were positive included 132 women and 99 men; 136 of these 231 patients recalled a tick bite. Patients noted a rash an average of 24 days after a bite and presented a mean of 34 days after the bite with erythema migrans (mean diameter, 16 cm). Itching (44%), burning (18%), and pain (11%) were the most common local symptoms. Systemic complaints (40%) included headache, fatigue, malaise, and arthralgia. Other than erythema migrans, findings on physical examination were minimal (<5% had fever, and in <10% local lymph nodes were affected). Serial serological studies using indirect immunofluorescence assay, ELISA, and Western blot methods were performed, and antibodies to *B. burgdorferi* sensu lato were detected in <50% of samples from patients. This is the largest series reported to date of patients with culture-confirmed Lyme borreliosis. It highlights the deficiencies of serological tests in early disease, demonstrates the sensitivity of direct detection methods for evaluation of patients with erythema migrans, and suggests that patients with early Lyme borreliosis in Slovenia may suffer a milder illness than those in the United States.

Lyme borreliosis is a tick-transmitted spirochetal disease endemic to regions in temperate climates throughout much of the globe [1, 2]. Early disease is often characterized by an erythematous exanthem, erythema migrans (EM) [3, 4]. Accompanying the rash may be a variety of local and systemic signs and symptoms [5]. Without treatment, some patients experience progressive disease involving a number of organ systems [6–8].

Much of the information regarding the manifestations of early disease is based upon cases of clinically defined disease [9]. This approach was acceptable in the past because serological response in early disease was inconsistent and cultivation of *Borrelia burgdorferi* sensu lato was often difficult [10, 11].

As experience with early disease evolved, it became apparent that there were difficulties in making a clinical diagnosis of Lyme borreliosis on the basis of a typical cutaneous eruption [12]. In addition, methods of culturing *B. burgdorferi* sensu lato from skin lesions gradually improved [13], while serological methods have continued to be problematic [14]. Recently, it has been recognized that the classification of spirochetes associated with Lyme disease, *B. burgdorferi* sensu lato, comprises three species (*B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*) [15], and it has been postulated that these different species may relate to differences seen in clinical manifestations [16]. Study of a large group of patients with culture-confirmed Lyme borreliosis may lend clarity to some of these issues. We report our clinical and laboratory findings from the largest series yet described of patients with culture-confirmed early Lyme borreliosis.

Materials and Methods

Patients. A total of 467 patients consented to skin biopsy. These patients had presented to the Department of Infectious Diseases at the University Medical Center in Ljubljana, Slovenia, between January and December 1994. Each had a rash compatible with EM. The majority of tick bites occurred during outdoor activity in the area surrounding Ljubljana. This area has been shown to have a ubiquitous tick population with a high rate of infection with *B. burgdorferi* sensu lato [17].

Study site. Slovenia, formerly northern Yugoslovia, has a population of ~2 million people. The country is bordered by Italy, Croatia, Hungary, and Austria. Ljubljana, the capital, lies in the central region of the country.

Demographic information. A standardized questionnaire was completed for each patient by the clinician. Details regarding the patient's recollection of a tick bite, local symptoms, systemic complaints, and previous illness were recorded. A

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physical examination was performed for all patients and abnormal findings were documented.

Biopsy procedure. The skin was prepared with 70% ethanol. An elliptical biopsy specimen (8 × 5 × 3 mm) was obtained from the leading edge of the erythema. The tissue was divided into four equal parts, and two parts were introduced into appropriate media as described below. The other two samples were frozen.

Culture. Skin biopsy specimens were inoculated into one of two media. To increase spirochete recovery, all cultures were performed by two laboratories. Culture specimens remaining in Ljubljana were placed in monobasic potassium phosphate medium [18], and those evaluated at Rush Medical College (Chicago) were placed in BSK II medium [19] modified by the addition of L-cysteine (1 m*M*), dithiothreitol (1 m*M*) [20], rifampin (40 μg/mL), and ciprofloxacin (0.4 μg/mL) [13]. The samples sent to Rush Medical College were held at room temperature until shipping and were received within 1–4 weeks of the biopsy.

Cultures were incubated in both laboratories at 33°C for a minimum of 6 weeks. All original cultures were subcultured at least once by the transferring of 0.5 mL of the original culture medium into fresh medium. The remaining 1.5 mL of original culture medium was retained and reincubated. Cultures were examined every 7–10 days for the presence of characteristic spirochetes. Organisms were identified as *B. burgdorferi* sensu lato by dark-field microscopy.

To determine the effect of prolonged incubation and blind subculturing, we examined 93 cultures that had remained negative after 6 weeks of incubation. These cultures were centrifuged at 1,500 rpm for 10 minutes and subcultured by the transferring of a 0.5-mL aliquot from the base of the tube to 1.5 mL of fresh medium. All 93 negative cultures as well as the subcultured material were reincubated for an additional 4 weeks.

Serological methods. Blood samples were obtained from patients at presentation, at 2 weeks post-presentation, and again at 2 months. Serum samples were stored at −70°C until tested. In Ljubljana, an indirect immunofluorescence assay [21] was used to detect IgM and IgG antibodies in all samples obtained at the first visit. The method was modified by the use of a local *B. afzelii* isolate as antigen.

At Rush Medical College, an ELISA produced by Cambridge Biotech (Worcester, MA) was used to detect polyclonal antibodies to *B. burgdorferi* sensu stricto in all available serum samples. This kit has been found to be sensitive (89% and 92%, respectively) in other studies [22, 23]. Western blots to detect IgM and IgG antibodies to *B. burgdorferi* sensu stricto were performed according to the instructions of the manufacturer (Cambridge Biotech) on serial serum samples from 80 patients. Blots were regarded as positive on the basis of criteria of the Centers for Disease Control and Prevention [24].

Results

Of the 467 skin biopsy specimens, 231 (49%) yielded *B. burgdorferi* sensu lato. One hundred and ninety-seven isolates were isolated in Slovenia and 99 at Rush Medical College. Sixty-five of the samples yielded spirochetes in both laboratories. The mean age in the culture-positive cases was 47 years. The group consisted of 132 women and 99 men. Only four of the patients had received antimicrobials before biopsy. The characteristics of patients are summarized in table 1.

Table 1. Characteristics of 231 patients in Slovenia from whose erythema migrans (EM) lesions *Borrelia burgdorferi* sensu lato was isolated in 1994.

Characteristic	No. (%) of patients*
Patients	
Median age, y (range, 15–83 y)	46.7
Male	99 (43)
Female	132 (57)
Erythema migrans	
Median diameter (cm) at presentation	16
Ringlike	163 (71)
Homogeneous	68 (29)
Multiple lesions	13 (6)
Location	
Leg	144 (62)
Thorax	41 (18)
Arm	39 (17)
Abdomen	7 (3)
Symptoms	
Local	127 (55)
Itching	104 (45)
Burning	43 (19)
Pain	25 (11)
Systemic	88 (38)
Headache	47 (20)
Fatigue	45 (19)
Malaise	27 (12)
Arthralgia	25 (11)
Myalgia	22 (9)
Dizziness	13 (6)
Fever	10 (4)
Nausea	6 (3)
Rigors	4 (2)
Weight loss	1 (0.4)
Abnormal physical examination findings	33 (14)
Localized lymphadenopathy	16 (7)
Conjunctivitis	10 (4)
Hepatomegaly	7 (3)
Generalized lymphadenopathy	4 (2)
Facial palsy	1 (0.4)
Meningeal signs	1 (0.4)
Pharyngitis	1 (0.4)
Prior Lyme disease (with EM)	15 (6)
Recalled tick bite	136 (59)
Recalled tick attachment time	34 (15)
Of <6 h	9 (4)
Of <24 h	16 (7)
Of <48 h	9 (4)
Median no. of days (post-bite) before EM was noted (n = 136)	17

* Except as otherwise indicated.

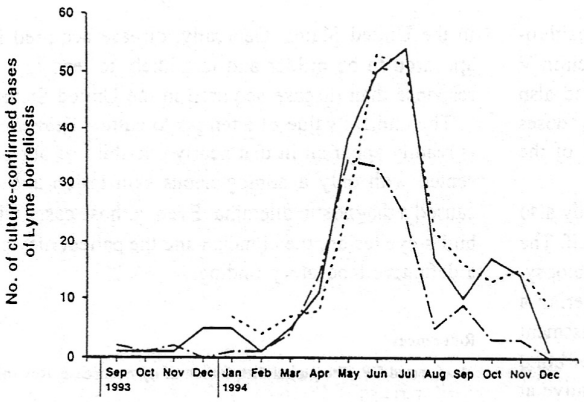

Figure 1. Graph of data regarding culture-confirmed cases of Lyme borreliosis involving 231 patients who presented to the University Medical Center in Ljubljana, Slovenia, with erythema migrans (from September 1993 through December 1994). The number of cases in which the following events occurred, per month, is illustrated: tick bite (n = 136; – – –), EM rash (—), and presentation to the clinic (n = 231; ----).

The majority of the rashes had central clearing (71%), but a fairly large group demonstrated a homogeneous erythema (29%). Rashes appeared most often on the legs (62%). The most common local symptom was pruritus (45%), and the most frequent systemic complaint was headache (20%). Multiple EM lesions (6%) and additional physical examination findings were unusual. Of the 136 patients who recalled a tick bite, the majority noted their rash several weeks following the bite (mean, 24 days; median, 17 days). Figure 1 shows the seasonal nature of this disease and summarizes the months in which patients recalled their bites, noticed the EM, and presented for care.

The 93 samples that were subcultured blindly after remaining negative for growth at 6 weeks of incubation yielded seven additional isolates. Four of the seven new isolates were noted only in subculture, while the original culture remained negative. An additional three new isolates were recovered from the original medium after the additional 4 weeks of incubation.

Serological results obtained by indirect immunofluorescence assay revealed that only 21 (9%) of the 231 patients had IgM antibodies, and there was evidence of IgG antibodies to *B. afzelii* in 48 (21%) on presentation. ELISA of serial samples for polyclonal antibodies to *B. burgdorferi* sensu stricto demonstrated positive serological responses in 47 (31%) of 152 at presentation, in 53 (44%) of 121 at 2 weeks, and in 44 (39%) of 113 at 2 months.

Western blotting with use of *B. burgdorferi* sensu stricto as antigen was performed on serial samples available from 80 patients and was positive for IgM in 32 (40%) of 80, 37 (46%) of 80, and 36 (45%) of 80 patients at presentation, at 2 weeks, and at 2 months, respectively. Western blots for IgG antibodies were positive for only four (5%) of 80 patients.

Discussion

In comparison of the clinical characteristics of our large group of patients with culture-confirmed erythema migrans and the characteristics of patients in the United States, our patients appeared to have a milder early disease course. Steere et al. reported a clinically defined group of patients in the United States with early disease and noted that nearly 80% had systemic complaints [9]. Fever (59%), multiple EM lesions (48%), and localized lymphadenopathy (20%) were reported frequently. Nadelman and Wormser recently described 79 culture-confirmed cases in the United States and reported systemic complaints in >50% [25].

Our patients appear to have fewer systemic complaints, similar to the patients described by Åsbrink and Olsson [26]. In a previous series of culture-confirmed cases in Europe, results were also suggestive of a milder early disease course [27]. It may be that *B. afzelii,* the predominant organism reported from Europe, may initiate a less severe early illness than does *B. burgdorferi* sensu stricto, the pathogen predominant in the United States. Our data from Slovenia for 1993 have shown that >80% of isolates from patients are *B. afzelii* (R.N.P., unpublished data, 1996).

Three other clinical findings deserve mention. First, 15 patients in our series reported having EM previously. At least six of these patients had evidence of IgG antibodies to *B. burgdorferi* sensu lato on presentation, yet they were not protected from a second occurrence of EM. Western blotting was performed on samples from four of these patients. Two samples contained antibodies to outer surface protein A (OspA), and three showed evidence of antibodies to OspC. It is unclear whether or not these patients may have been afforded some protection against disseminated disease, but local disease developed despite the previous occurrence of disease and the presence of some immune memory.

In an animal model, vaccination that induces IgG antibodies to outer membrane proteins of *B. burgdorferi* may offer protection [28]. Antibodies directed against these surface proteins have been induced in human vaccine trials, but evidence that they offer protection will require further study [29, 30]. Our findings regarding the few patients reported herein suggest that the previous occurrence of disease and the demonstrable presence of IgG antibodies may not translate into protection against all subsequent challenges.

A second interesting clinical finding in our group, which contrasts with previous findings in animal studies [31], was that disease transmission occurred despite a tick attachment time of <24 hours. Twenty-five of 136 patients who recalled a tick bite reported a tick attachment time of ≤24 hours. This observation does not negate the general rule that transmission of *Borrelia* spirochetes is uncommon when the time of tick attachment is short, but it does underscore the possibility of early spirochetal transmission in some patients.

Of further interest, we noted that four patients had received brief courses of antimicrobials and yet their specimens still

yielded *Borrelia* organisms. Two patients had received azithromycin (500 mg and 1.5 g) and two had received penicillin V (9 g and 15 g) before the biopsy. The latter patient had also received 200 mg of doxycycline. This suggests that a few doses of antimicrobials will not necessarily preclude culture of the organisms from skin specimens.

In addition to providing clinical information, this study also provided information concerning the culture process itself. The combined laboratory effort led to a 49% yield from skin biopsy, exceeding any serological evidence of disease and offering a definitive diagnosis. These culturing efforts allowed assessment of a large number of samples, as well as of the value of blind subculturing and prolonged incubation of cultures negative at 6 weeks. Both efforts appear to be valuable for increasing the yield from cultures of skin biopsy specimens.

Our work also highlighted the continuing problems associated with use of serological methods for patients with early disease. Fewer than 50% of cases demonstrated seropositivity at any time within the first 2 months. Previous work by Steere et al. showed that 90% of their patients with EM were found to have an IgM titer of $\geq$128, at least once [4]. In previous work with culture-confirmed cases, Mitchell and colleagues noted that the majority of patients, even if negative at baseline, would seroconvert within 1–2 weeks [32, 33].

The work of Dressler et al. suggested the IgG response was present in the majority of patients within a few weeks [34]. Aguero-Rosenfeld et al. have reported that a serological test can be positive for nearly all patients within the first month of illness [35]. The lack of maturation of the serological response in our study may be related in part to the initiation of appropriate antimicrobial treatment early in the course of disease [36].

In addition, we considered the possibility that the use of different species of *Borrelia* as antigens may have influenced the serological results. However, other investigators have suggested this is not a problem [37]. In our study, the immunofluorescent method with use of a local *B. afzelii* isolate as antigen was the least sensitive for detection of serum antibody. The Western blot method with use of *B. burgdorferi* sensu stricto was most sensitive. Our work would suggest that the use of a local strain for antigen may not alter the serological results appreciably but that methods may play an important role.

Of greater import, <50% of our patients developed a serological response to infection. The serological results appear to coincide with our clinical findings. The patients in our series had less systemic illness and developed a serological response less frequently. Recent investigators in the United States have demonstrated greater seropositivity in patients with disseminated disease [35, 37]. It may be that *B. afzelii* has less potential to disseminate early in the disease course.

This series of culture-proven cases has allowed us to assess typical clinical findings in a group of European patients with early Lyme borreliosis and to demonstrate some apparent differences between disease acquired in Europe and that acquired in the United States. Generally, disease acquired in Slovenia appeared to be milder and less likely to lead to a serological response than disease acquired in the United States.

The clinical value of attempts to culture *Borrelia* organisms is readily apparent in that nearly one-third of our patients presented with only a homogeneous skin lesion and could have caused a diagnostic dilemma. Even in those cases with a classic bull's-eye lesion, the clinician and the patient may benefit from a definitive laboratory finding.

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