No Evidence for *Borrelia burgdorferi* Infection in Lesions of Morphea and Lichen Sclerosus et Atrophicus in Spain*

A Prospective Study and Literature Review

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The possible association of *Borrelia burgdorferi* with morphea and lichen sclerosus et atrophicus has been the focus of research and discussion in dermatology during the last 10 years.

To investigate the etiopathogenic role of *B. burgdorferi* in morphea and lichen sclerosus et atrophicus lesions in Spain, we studied 14 cases: 8 patients with lichen sclerosus et atrophicus and 6 with morphea. For the whole group, a prospective study was performed, including serologic studies by indirect immunofluorescence, histologic evaluation of skin biopsy specimens, culture studies, and polymerase chain reaction with different primers sensitive for detecting virtually all *B. burgdorferi* strains tested to date.

Although one patient with morphea had positive serologic findings at low titer, we were not able to culture or detect borrelial DNA in any of the specimens.

These findings do not confirm an association between *B. burgdorferi* and morphea and lichen sclerosus et atrophicus. *Key words: polymerase chain reaction; scleroderma; serology.*

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Morphea and lichen sclerosus et atrophicus (LSA) are closely related to connective tissue disorders that rarely occur in the same patient (1). The etiology of these disorders is unknown. *Borrelia burgdorferi*, the etiologic agent of Lyme disease (LD) (2), was discovered in 1982 (3).

B. burgdorferi may cause sclerotic skin lesions that clinically and histopathologically are indistinguishable from morphea and LSA in up to 10% of patients with acrodermatitis chronica atrophicans (4). Previously, these skin lesions were included under the term "pseudoscleroderma" (5). However, the relationship of *B. burgdorferi* with the idiopathic forms of morphea or LSA has been a matter of controversy (6, 7). The suggestion of a possible etiopathogenic role of *B. burgdorferi* in patients with morphea was first put forward by Aberer et al. (8) in 1985. Because of the clinical and histopathologic similarities between acrodermatitis chronica atrophicans and morphea, these authors studied the sera of 10 patients with long-standing morphea and found a high frequency of positive titers (50%) against *B. burgdorferi*, using the enzyme-linked immunosorbent assay (ELISA). However, since then, other authors have used not only serologic tests but more sensitive and specific methods and have reported conflicting results.

Diagnostic approaches for detecting B. burgdorferi infection

Serologic tests. Currently, serologic testing is the most important laboratory method for diagnosing LD. However, the results must be interpreted carefully because false-negative results are common, and false-positive cases may be found in endemic areas. The lack of standardization also means that the variations in sensitivies and specificities among laboratories may be great. Currently, the most widely used tests for measuring antibodies to *B. burgdorferi* are the indirect immunofluorescence assay and ELISA. The tests are of low diagnostic value in the earliest phase of LD (9–12). However, almost 100% of patients with acrodermatitis chronica atrophicans are seropositive, and they usually have high serum IgG titers to *B. burgdorferi* (9, 11–13). Western blotting has also been used as a diagnostic tool and may be a sensitive method, but it has the disadvantages of being difficult to standardize and being time-consuming and non-quantitative.

Culture of spirochetes. Use of the Barbour-Stoenner-Kelly (BSK-II) medium (14) to culture *B. burgdorferi* is the most specific and reliable way of diagnosing LD. *B. burgdorferi* has been cultivated successfully from more than 40% of skin biopsy samples from patients with erythema chronicum migrans (15, 16).

Demonstration of spirochetes in tissues. Spirochetes may be demonstrated in biopsy specimens by using different silver stains. Polyclonal or monoclonal antibodies to borrelial antigens have also been used to demonstrate spirochetes in tissue specimens. The results of these techniques are difficult to evaluate, and there is a high risk of both overdiagnosis and underdiagnosis.

Polymerase chain reaction (PCR). Currently, PCR is used in only a few special laboratories and has a high sensitivity and specificity in the samples studied. However, the risk of false-positive results by carry-over contamination has been observed.

MATERIAL AND METHODS

Patients and specimens

Randomly, 14 patients (mean age, 54 years; range, 13 to 73 years) from the central geographic area of Spain were included in the study: 8 of them had LSA and 6 had morphea. A skin biopsy sample from the active border of the lesion or from an area of induration was obtained from each. Ten of these specimens showed marked inflammatory activity. The onset of the clinical lesions was between 1 month and 3 years before the biopsy was performed. No patient was from a geographic area with a known major seroprevalence of LD, and none had a history of previous tick bite or any cutaneous or systemic manifestations suggestive of LD (Table I).

Table I. *Summary of clinical findings and laboratory results in the 14 study patients**†

C, contaminated; LSA, lichen sclerosus et atrophicus; ND, not done.

Patient	Age, yr sex	Duration of disease	Diagnosis	Activity	Localization	Culture
1	62/F	2 yr	LSA	−	Vulvar	C
2	55/M	5 mo	LSA	+	Preputial	C
3	73/F	3 yr	Morphea	+	Leg	−
4	55/F	3 mo	Morphea	+	Thorax	−
5	56/F	2 yr	LSA	−	Vulvar	C
6	46/F	4 mo	Morphea	−	Hip	−
7	53/F	6 mo	LSA‡	+	Vulvar and cutaneous	C
8	13/F	8 mo	Morphea§	+	Trunk and 4 extremities	−
9	65/F	6 mo	Morphea‖	+	Abdomen	−
10	30/M	7 mo	LSA	+	Preputial	ND
11	58/M	9 mo	Morphea	−	Trunk	−
12	61/F	3 yr	LSA	+	Vulvar and cutaneous	−
13	64/M	1 mo	LSA	+	Preputial	ND
14	69/M	3 mo	LSA	+	Preputial	C

* Results of serologic testing were negative in all patients except patient 9.
† The results of the polymerase chain reaction for *B. burgdorferi* were negative for all 14 patients.
‡ LSA extensive, with cutaneous and mucosal involvement.
§ Linear morphea.
‖ Morphea profunda.

Serologic tests

The serum of each patient was studied at the time of diagnosis and, for patient 9, it was studied again 3 months later. The presence of antibodies, class IgM and IgG against *B. burgdorferi*, was determined with indirect immunofluorescence assay. The sera were preabsorbed for cross-reactive antibodies with a *Treponema phagedenis* ultrasonicate. Initially, the screening was started at a titer of 1/64 for IgG, and the reference values were considered positive at titers equal to or higher than 1/256 (*B. burgdorferi* B31, Hillcrest Biologicals, Cypress, CA).

Culture medium BSK-II

An infectious and pathogenic clone of *B. burgdorferi* N40 was obtained with in vitro limiting dilution technique (17). Spirochetes were grown in modified BSK-II medium (14) at 33°C without antibiotics. Inocula were grown to log phase, quantified in a Petroff-Hauser bacterial counting chamber (Baxter Scientific Products, McGaw Park, IL), and diluted to the desired concentration with BSK-II medium. Of the 14 skin samples (see below), 12 were placed in an 8-ml screw-cap glass tube containing 7.0 ml of medium. Cultures were incubated for at least 2 months and examined for spirochetes on a weekly basis with darkfield microscopy.

Tissue collection

Skin biopsy samples, 2 to 3 cm², were obtained using strict aseptic technique to minimize bacterial contamination. The biopsy area was soaked with povidone-iodine solution (Betadine) for 2 to 3 min, rinsed with sterile water, and soaked in alcohol. Each skin sample was placed on a clean paper towel. Instruments were cleaned, dipped in absolute alcohol, passed through a flame, dipped in undiluted chlorine bleach, rinsed in clean water, then dipped twice in alcohol and again passed through a flame. Next, the skin samples were excised, placed in a disposable sterile 50-mm-diameter Petri dish (Falcon 1006; Becton Dickinson, Lincoln Park, NJ), and cut into four equal pieces with a new, sterile scalpel blade. One piece was cultured and another piece was fixed in 5% formalin and processed for routine histologic examination. The other two pieces were placed in separate, sealed plastic centrifuge tubes: one was snap-frozen in liquid nitrogen and stored at −70°C for PCR and the other one was stored in 70% ethanol for PCR testing in another laboratory.

Fresh tissue processing

The dried tissue samples were resuspended in proteinase K digestion buffer (5 mM/l TRIS, pH 8.5, 1 mM/l EDTA, 0.45% Nonidet P40, 0.45% polysorbate 20 [Tween 20], and 100 μg/ml proteinase K) and incubated at 55°C. After digestion, the proteinase was heat-inactivated at 100°C for 10 min and the low-speed supernatant recovered for use as template in the PCR process.

PCR testing

DNA was extracted from digested skin biopsy samples and 5-mm ear punch samples from infected mice, as described elsewhere (17), and all samples were tested in a blinded manner in two laboratories. Extraction was performed with a commercially available kit with conditions modified as described (18). One genetic locus of *B. burgdorferi* was targeted for PCR amplification: primer set 149/319 was used to detect a 195-bp portion (bp 149–343) of the gene encoding the spirochetal outer surface protein (Osp A) (18), whose sensitivity comprises most of the strains tested to date, including those known Spanish strains of *B. burgdorferi* (19). With the nested PCR technique, two different sets of primers were used in the other laboratory to amplify a highly conserved gene sequence in Osp A (20), that also recognized the Spanish strain of *B. burgdorferi* (ESP-1) and isolates from erythema chronicum migrans lesions from autochthonous patients (personal observation). Outer primer sequences were 5′ GAG CTT AAA GGA ACT TCT GAT AA 3′ (bp 334–356) and 5′ GTA TTG TTG TAC TGT AAT TGT 3′ (bp 874–894). Inner primer sequences were 5′ ATG GAT CTG GAG TAC TTG AA 3′ (bp 362–381) and 5′ CTT AAA GTA ACA GTT CCT TCT 3′ (bp 693–713) (21).

Five or 10 μl of processed specimen DNA was added to a reaction mixture containing 50 pmol of each primer, 200 μmol of each dNTP, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 0.01 μg/μl bovine serum albumin, 1.75 mM MgCl₂, 10% glycerol, 0.5% polysorbate 20, and 1.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Isopsoralen compound 10 (100 μg/ml final concentration; HRI, Concord, CA) was added to PCR master mixes for postamplification inactivation of PCR products (22–24). Control samples for each amplification included multiple negative water controls and positive controls containing total *B. burgdorferi* DNA from strain N40. Amplification conditions were as follows: 45 cycles of

denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min. Thermal cycling was preceded by a 4-min incubation at 94°C and followed by a 7-min extension at 72°C. All amplification products were inactivated with isopsoralen; after amplification, the unopened reaction tubes were exposed to 20 mW/cm^2, 300 to 400 nm UV light for 15 to 20 min at 20°C or 4°C and then stored at −20°C until further analysis.

Amplification products were analyzed in an area separate from where PCR reagents were prepared, and the precautions recommended for prevention of false-positive PCR results were observed consistently (22–24). A combination of physical containment of amplification products and photochemical inactivation of the products themselves prevented false-positives in all negative control reactions.

Detection of PCR products

Amplified products were electrophoretically separated in 4% agarose (1% Seaplaque, 3% Nusieve; FMC BioProducts, Portland, ME), stained with ethidium bromide, transilluminated with UV light, and Southern blotted as described elsewhere (18, 22–24). Membranes were hybridized and washed as described elsewhere (18). Amplification products were detected by hybridization to a chemiluminescent internal hybridization probe constructed by amplification of internal sequences (ECL, Amersham Laboratories, Arlington Heights, IL) (18). For full-length probes, the presence of PCR primer sequences in nonspecific amplification products did not result in lower hybridization specificity (data not shown).

Controls

Positive culture controls were obtained from mice ear punch samples infected with *B. burgdorferi* N40. The success rate for culture of the *B. burgdorferi* Spanish strains tested to date with this technique has shown a positive yield of 100% (positive cultures in 13 of 13) (personal observation). Positive PCR controls were used in every amplification in the former laboratory and included weak (10^{-8}) and strong (10^{-5}) isolates prepared from N40 *Borrelia* culture, quantitated, and diluted serially (10^{-1} to 10^{-12}). Three negative PCR controls consisted of PCR master mix solution and water. The second laboratory used as positive PCR controls one ear punch sample from infected mice with *B. burgdorferi* N40 that was run with the clinical samples from the beginning of the procedure. Two negative PCR controls in each run consisted of water.

RESULTS

Serologic tests revealed an IgG anti-borrelial antibody only in patient 9 at a titer of 1:256, which was repeated for confirmation. All the other 13 patients (5 with morphea and 8 with LSA) and 20 patients with other dermatoses (10 with psoriasis and 10 with eczema) were seronegative.

No spirochetes were isolated from any of the 7 skin biopsy samples successfully processed for culture in BSK-II medium (patients 3, 4, 6, 8, 9, 11, and 12). The samples from patients 1, 2, 5, 7, and 14 were contaminated and eliminated from the study, and the samples from patients 10 and 13 were fixed directly in 70% ethanol and not processed for culture. Positive culture results were obtained from mice ear punch samples infected with *B. burgdorferi* N40.

In the former laboratory, a specific amplification product of 195 bp was detected in the two diluted positive controls containing total *B. burgdorferi* DNA from strain N40. In contrast, no specific amplification was seen in the multiple negative controls containing water or in the 14 skin samples from patients with morphea or LSA (Fig. 1). The second laboratory, using a nested PCR technique, obtained specific amplifications from the positive control containing
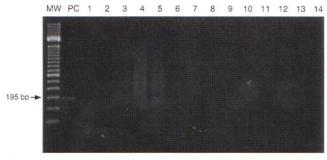

Fig. 1. Polymerase chain reaction (PCR) amplification results obtained from patient specimen DNA extracts. Lanes are as follows: 100-bp molecular weight marker (*MW*) ladder; *PC*, positive control; *lanes 1–6*, morphea; *lanes 7–14*, lichen sclerosus et atrophicus. *Arrow*, position of positive PCR amplification product (195 bp). No amplification product is seen in lanes 1–14. Amplification product appears to be greater than 195 bp because of covalent addition of isopsoralen to DNA product postamplification. (MW, Gibco BRL, Gaithersburg, MD.)

B. burgdorferi N40, and the 14 skin samples as well as the negative controls were negative.

DISCUSSION

An increasing number of cases of LD in Spain have been reported during the last 8 years. Several reviews and seroprevalence studies have demonstrated a clinical awareness of the disease, especially in the north and central part of Spain (25). Recently, a strain of *B. burgdorferi* (ESP-1) was isolated from ticks of *Ixodes ricinus* in northern Spain (26), and a strain of *B. burgdorferi* has been isolated from a lesion of erythema chronicum migrans (observation not published).

For several reasons, a connection has been considered between LD and morphea or LSA (or both). Clinically, there may be some similarities between an annular erythema chronicum migrans and a morphea lesion with a lilac ring. Histopathologically, an admixture of plasma cells may be found in an early morphea lesion, similar to that observed in lesions of erythema chronicum migrans. Also, a good response to antibiotic therapy has been reported in some cases of morphea (27, 28).

It is tempting to consider an infectious agent having a pathogenic role in disorders of unknown cause, such as morphea and LSA. Since the relationship between *B. burgdorferi* and morphea was first suggested (8), many investigations have been conducted in Europe and, more recently, the United States to look for a possible association between this microorganism and the idiopathic forms of morphea or LSA. Different diagnostic approaches have been used and conflicting results have been reported. On the basis of serologic testing, investigators in Austria (28), Switzerland (29), and Germany (30) have found a high frequency of increased serum titers to *B. burgdorferi* in patients with morphea. In contrast, investigators in the United States (31–33), Canada (34), Great Britain (35, 36), Sweden (37), Denmark (38), France (39, 40) and Spain (41) have observed normal titers or nonspecific cross-reacting antibodies (27, 32).

Attempts at using routine microscopy (silver stains and monoclonal or polyclonal antibodies) to detect the microorganism in skin biopsy samples from patients with morphea have

yielded positive results (42–46). Furthermore, spirochetes were cultivated from sclerotic lesions on a lower limb in a patient with *B. burgdorferi* antibodies (43) and from 2 seronegative patients with morphea (47) (although in one of these 2 cases, the pathogen was not definitely identified). However, other attempts to isolate or detect any spirochetal form in tissue samples from patients with morphea have failed (36, 48, 49).

To detect *B. burgdorferi* in skin samples from patients with morphea or LSA, PCR has been performed in different laboratories throughout the world, using very specific primers to react with *B. burgdorferi* or other possible spirochetal organisms hypothetically related to morphea or LSA lesions (or both). A large number of samples, mostly from formalin-fixed and paraffin-embedded tissue, have been examined with this technique, and the results obtained have been consistently negative, both in Europe (50–52) and the United States (53–56). To our knowledge, nine groups of investigators have reported their results (including the present study) (50–57), and only one group (from Germany) has been able to detect borrelial DNA in all the samples studied (57). However, the lack of a humoral immune response in this seronegative group of 9 patients was not explained by the authors (57).

In the present series, patient 9, who had the histologic diagnosis of morphea profunda, had a positive serologic finding, with IgG at low titer (1:256); however, the culture and PCR results were negative. The presence of elevated titers of antibody against *B. burgdorferi* in some patients with morphea has been reported (8, 28–30), specifically in patients with morphea profunda (58). Some have suggested that this represents a cross-reactive phenomenon between *Borrelia* and an unidentified antigenic structure (32, 35, 59).

If *B. burgdorferi* is related to the cause of morphea and LSA, a higher frequency of positive titers would be expected, because these disorders would be late manifestations in the spectrum of dermatologic conditions of LD. Only one of our 14 patients was seropositive at low titer, representing less than 10% of our series. In contrast, a high frequency of positive titers (nearly 100%) has been found in patients with acrodermatitis chronica atrophicans (a well-known late manifestation of LD) (9, 11–13).

Although there are documented cases of seronegative chronic LD in patients who had previously received treatment with antibiotics (60), no patient in our series had a previous history of LD or had received antibiotic therapy from the time of onset of the clinical lesions of morphea or LSA to the time of diagnosis.

Since our study was prospective, we were able to examine fresh tissue samples prepared for culture in BSK-II medium (15, 16), which is fragile and difficult to handle (61). Five of the 12 samples were contaminated and, thus, eliminated from the study; the 7 other samples produced negative results after 2 months of incubation. These findings are concordant with previously reported ones (36, 48–50).

In conclusion, there is no evidence that *B. burgdorferi* has an etiopathogenic role in idiopathic morphea or LSA in Spain. Also, the data are not sufficient to support such a relationship in other geographic areas of Europe or the United States with a higher incidence in LD. Because of the possible presence of cross-reactive antibody against *B. burgdorferi* in the sera of patients with morphea or LSA, as detected by currently used serologic tests (indirect immunofluorescence or ELISA), we believe that only high titers should be considered of diagnostic value. Even in that setting, a detailed medical history, physical examination, and other appropriate tests should be performed to rule out the diagnosis of acrodermatitis chronica atrophicans.

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