No Evidence for *Borrelia burgdorferi*–Specific DNA in Lesions of Localized Scleroderma

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A possible association of *Borrelia burgdorferi* with localized scleroderma is currently the focus of intense research and discussion. Skin biopsies from 30 patients with localized scleroderma (28 of the plaque type/morphea; two linear scleroderma) were analyzed for the presence of *Borrelia burgdorferi* using three different polymerase chain reaction systems for amplification of segments of borrelial genes.

Formalin-fixed, paraffin-embedded biopsies of 14 patients and fresh-frozen, cryo-conserved biopsies of 16 patients with localized scleroderma were obtained. Lesions of all patients showed clear signs of scleroderma and disease progression at the time of biopsy. Fresh-frozen as well as formalin-fixed biopsies from patients with erythema migrans or acrodermatitis chronica atrophicans were used as positive controls.

With all three polymerase chain reaction systems, borrelial DNA was detected in none of the 30 specimens of localized scleroderma. In contrast, with one polymerase chain reaction system, *Borrelia burgdorferi*–specific DNA was found in 24 of 27 frozen biopsies from patients with erythema migrans and in all 5 analyzed frozen biopsies of patients with acrodermatitis chronica atrophicans. In approximately half of the paraffin-embedded biopsies from patients with erythema migrans (nine of 23) and acrodermatitis chronica atrophicans (13 of 27), *Borrelia burgdorferi*–specific DNA was identified.

These results question the association of localized scleroderma with known subtypes of *Borrelia burgdorferi*. ***Key words: Borrelia infection/morpheal/polymerase chain reaction. J Invest Dermatol 104:23–26, 1995***

In recent years, several studies analyzing a possible association of *Borrelia burgdorferi (Bb)* with localized scleroderma (LS) based on serologic methods [1–8], bacterial culture [6,9–11], immunohistochemical [6,12], or molecular analysis [13–15] have been published. However, these studies, in total, did not lead to unequivocal results. Several studies found elevated titers of serum antibodies to *Bb* in 30–46% of patients with LS [1–3], others in only a few or no patients with LS [4–6]. *Bb*-like spirochetes have been isolated from at least four patients with LS: one patient with lesions resembling acrodermatitis chronica atrophicans (ACA) [9], two patients with LS, one of them with atrophodermia Pasini and Pierini [10], and a further patient with atrophodermia Pasini and Pierini [11].

To further elucidate a possible association of *Bb* with LS, lesions of 30 patients with LS were analyzed using the highly sensitive polymerase chain reaction (PCR) technique. To increase the overall sensitivity, three different nested PCR assays for amplification of different chromosomal genes of *Bb* were used upon each sample. These systems had previously been shown to detect at least three major subtypes of Bb.

The first PCR system, originally designed for the detection and subtyping of *Bb* in paraffin-embedded tissue [16,17], amplified a short segment of a chromosomal gene of *Bb* [18,19]. Nucleotide sequence analysis and hybridization with an internal oligonucleotide probe had been performed in a representative number of cases, confirming the specificity of the PCR products [16]. In a second PCR system, a segment of the borrelial flagellin gene [20] was amplified. Primers were designed to anneal to regions conserved between different borrelial species to increase the chances for additional detection of spirochetes other than *Bb*. The third PCR system amplifies a relatively large segment of the flagellin gene [14,21].

In all three systems, specificity was assessed by the analysis of DNA from different subtypes of *Bb* or other spirochetes, phylogenetically near or far related to *Bb*. In addition, positive control reactions with DNA from skin biopsies of patients with erythema migrans (EM) or ACA were performed. As internal positive controls, indicating that DNA of sufficient quality and quantity was obtained, a 207-bp fragment of the human beta-actin gene [16,22] was amplified.

MATERIALS AND METHODS

Patients and Specimens Specimens of 28 patients with morphea of the plaque type (22 female, six male) and two female patients with linear scleroderma were analyzed. Lesions were biopsied within the marginal erythematous area (lilac ring) and were either fresh-frozen and cryo-conserved (16 specimens) or formalin-fixed and paraffin-embedded (14 specimens). At the time of biopsy all patients had clinically progressive disease with new inflammatory lesions. Duration of symptoms was less than 1 year in seven patients, 1–5 years in nine patients, 6–10 years in seven patients, and not clearly remembered by the patient in the remaining seven

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Abbreviations: ACA, acrodermatitis chronica atrophicans; *Bb, Borrelia burgdorferi*; EM, erythema migrans; LS, localized scleroderma.

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cases. In 13 patients only the trunk, in five patients only the extremities, and in 12 patients the trunk and extremities were affected. In 11 patients, from whom fresh-frozen biopsies were taken, antibiotic treatment had been performed in the past (in six patients 4–6 months ago, in 5 patients 1–3 years ago). Biopsies from all other patients had been obtained before administration of any antibiotic treatment.

As positive controls, DNA of biopsies from patients with EM (27 fresh-frozen, 23 formalin-fixed) or ACA (five fresh-frozen, 27 formalin-fixed) were used. As negative controls, DNA from normal skin or from patients with lesions not related to *Bb* (T-cell lymphoma, lupus erythematosus) were used.

IgM and IgG serum antibodies to *Bb* had been evaluated in 18 patients with LS by an indirect immunofluorescence assay. The sera were preabsorbed for 'cross-reactive' antibodies with a *Treponema phagedenis* ultrasonicate. IgM or IgG antibody titers at least 1:10 were regarded as elevated [23]. Results were confirmed by a *Bb* flagellum enzyme-linked immunosorbent assay [24]. In one patient with LS, IgM antibodies (1:10), in an other patient IgG antibodies (1:64), and in two patients IgM and IgG antibodies (1:10; 1:80 and 1:10; 1:20) were found to be elevated.

Cultured Strains of *Borrelia burgdorferi* DNA isolated from strains belonging to three different subtypes of *Bb* were used: *Bb* sensu stricto (strains B31, Ip1, Z25), *Borrelia garinii* (strains N34, Ho14, 20047) and the VS 461 group of *Bb* (strains ECM1, Bo23). As specificity controls, strains of *Borrelia parkeri, Borrelia turicatae*, and *Borrelia hermsii* were used [25]. These borrelial strains are known to cause relapsing fever not related to Lyme disease in humans. All borrelial strains were kindly obtained from Dr. Göbel, Freiburg, Germany. As further controls, strains of *Treponema pallidum* (American Type Culture Collection 27087), the causative agent of syphilis, as well as *Treponema denticola* (American Type Culture Collection 33520), which belongs to the normal bacterial flora of the oral cavity, were used.

DNA Isolation Fresh-frozen biopsies were cut into small pieces and transferred into 1.65-ml microcentrifuge tubes and digested with 1 mg/ml Proteinase K (Boehringer, Mannheim) in buffer (0.2 M TrisCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecylsulfate). After incubation at 55°C for 2 *d*, DNA was extracted by phenol/chloroform, ethanol precipitated, and redissolved in 100 μl of destilled and autoclaved water. DNA isolation from formalin-fixed, paraffin-embedded tissue was performed as described [26,27]. Briefly, ten 15-μm–thick tissue sections were cut from each block. To avoid cross contamination, the blade of the microtome was changed between samples. The sections were deparaffinized by xylene, pelleted by centrifugation and resuspended in buffer with Proteinase K (1 mg/ml) and processed as described above. DNA isolation from bacterial cultures was performed by pelleting the heat-inactivated strains and resuspension in digestion buffer with Proteinase K, followed by DNA extraction as described above.

PCR Amplification Reactions Three different nested PCR systems for amplification of segments of borrelial genes were used. Ten microliters of the DNA solution of each sample were used as template for PCR amplification reactions in a final volume of 50 μl containing a PCR reaction mixture as described [16,27]. In a first round, 30 or 40 cycles of PCR using an outer primer pair were performed. Subsequently, an aliquot of the PCR reaction (0.5 μl) was used as template for an additional 30 rounds of PCR using an inner primer pair. Ten microliters of the final product were analyzed by non-denaturing agarose gel electrophoresis (2.5% agarose in Tris-borate-EDTA) and visualized by ultraviolet radiation after ethidium bromide staining.

The first PCR system was designed to detect *Bb* in paraffin-embedded tissue. For this purpose, a relatively short gene segment is targeted and amplified by PCR [16]. In a first round, a 171-bp segment of a chromosomal gene of *Bb* [18,19] was amplified by 30 cycles of PCR. Subsequently, an aliquot of the PCR product was used as a template for an additional 30 cycles of PCR amplifying a 92-bp inner fragment of the first PCR product. Primer sequences and amplification conditions were as described [16].

The second PCR system was designed to detect *Bb* as well as other unknown possible borrelial species. For this purpose, oligonucleotides for PCR were designed to be complementary to a region of the flagellin gene highly conserved between various borrelial specimens [20]. A 194-bp fragment of the flagellin gene was amplified by 30 cycles with an outer primer pair followed by nested PCR amplification of a 164-bp inner fragment by additional 30 cycles. Annealing temperatures were 51°C. Outer primer sequences were 5′ GAA GCG ATT GCT GTA AAT ATT TAT 3′ and 5′ TTA GCA TCA ACT GTG GTT GTA ACA TTA A 3′. Inner primer sequences were 5′ AAT ATT TAT GCA GCT AAT GTT GCA AA 3′ and 5′ GGT TGT AAC ATT AAC AGG AGA ATT AAC 3′.

In a third PCR system, a 730-bp fragment of the borrelial flagellin gene [28] was amplified, followed by nested PCR amplification of a 290-bp inner fragment. Primer sequences and amplification conditions were as described [14,21].

In addition, a 207-bp fragment of the human beta-actin gene was amplified to ascertain isolation of DNA of sufficient quality and quantity from all analyzed clinical specimens. Primer sequences and amplification conditions were used as described [16].

RESULTS

The specificity of PCR primers of all three systems was tested using DNA from cultured strains of the three known subtypes of *Bb* (*Bb* sensu stricto, *Borrelia garinii*, and the VS 461 group of *Bb*) and of spirochetes not related to Lyme borreliosis (*Borrelia hermsii, parkeri, turicatae*, and *Treponema pallidum* and *denticola*). All three PCR systems did not yield any specific products from DNA from *Treponema pallidum* and *denticola* but amplified DNA from all three subtypes of *Bb*. In addition, the second PCR system amplified DNA from *Borrelia hermsii, parkeri*, and *turicatae* and the third PCR system amplified DNA from *Borrelia hermsii*. The first PCR system, being most specific for detection of DNA from *Bb*, did not amplify DNA from any other strains.

With the first PCR system, *Bb* was detected in none of 30 biopsies of patients with LS, but in 24 of 27 analyzed fresh-frozen biopsies of patients with EM and all five analyzed fresh-frozen biopsies of patients with ACA **(Fig 1)**. In addition, *Bb* was detected in nine of 23 formalin-fixed biopsies of patients with EM and in 13 of 27 formalin-fixed biopsies of patients with ACA.

Using the second PCR system, *Bb* was detected in none of 30 biopsies of patients with LS, but in 16 of 21 fresh-frozen biopsies of patients with EM and all five analyzed fresh-frozen biopsies of patients with ACA. In formalin-fixed tissue, *Bb* was detected in two of 12 patients with EM.

Using the third PCR system, *Bb*-specific DNA was detected in none of 30 biopsies of patients with LS, but in 11 of 21 fresh-frozen biopsies of patients with EM and two of five analyzed fresh-frozen biopsies of patients with ACA. In formalin-fixed tissue, *Bb* was detected in one of 12 patients with EM. Positive control reactions, amplifying a segment of the human beta-actin gene, were positive in all clinical samples.

DISCUSSION

In this study, in none of the 30 biopsy specimens of patients with LS borrelial DNA was detected using three different PCR systems. The isolation of DNA suitable for PCR amplification was demonstrated in all specimens by amplification of a 207-bp fragment of the human beta-actin gene.

To validate the PCR systems used, special emphasis was put on extensive negative and positive control experiments including studies for determination of the specificity of the three systems used. None of the PCR systems amplified DNA from spirochetes not closely related to *Bb*, such as *Treponema pallidum* and *Treponema denticola*. Only the first PCR system proved to be highly specific for detection of *Bb* and did not react with any other tested borrelial strains. In addition to studies on LS, as reported here, we are currently using this system for routine assessment of specimens of patients with atypical manifestations of EM or ACA [16]. The second primer set was designed to be considerably more non-specific, to increase the chances for detection of any borrelial DNA in lesions of LS. However, even using this system, borrelial DNA was not detected in any of the 30 analyzed cases of LS. The third PCR system, using conditions as described [14], amplified DNA from *Bb* and *Borrelia hermsii*, but, in our study, did not detect DNA from any of the 30 cases of LS.

As positive control reactions, DNA from lesions of EM and ACA were amplified with all three PCR systems. Using the first system, which amplifies the shortest DNA fragment (171 bp/92 bp) as compared to the other two systems, *Bb*-specific DNA was detected in the vast majority of fresh-frozen biopsies of EM and ACA and in less than half of formalin-fixed, paraffin-embedded cases of EM and ACA. These data indicate that this system is able to detect *Bb* in clinical specimens with a relatively high sensitivity, at least in

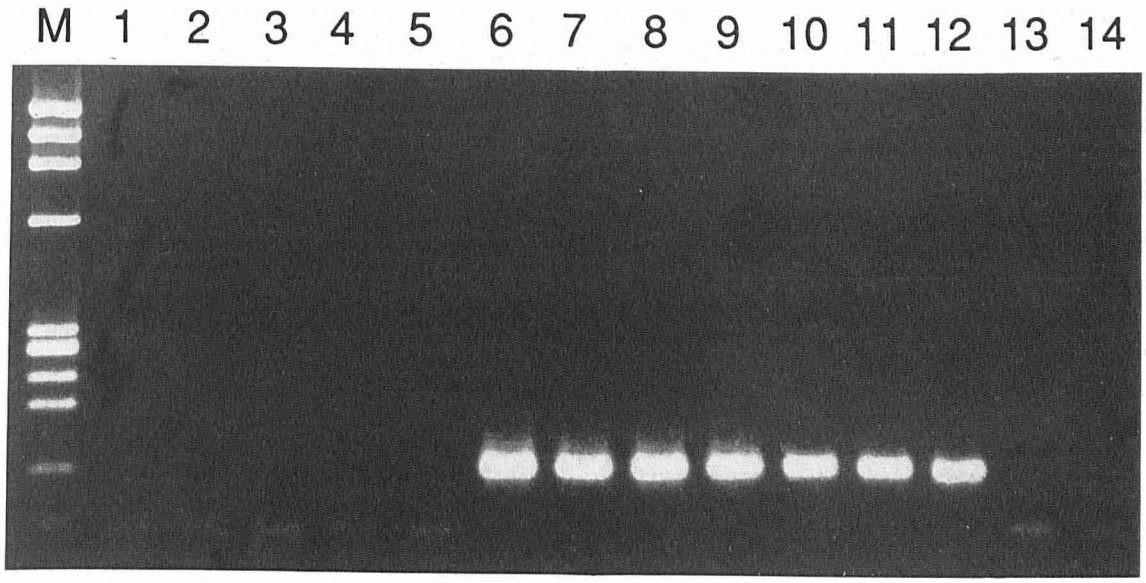

Figure 1. Representative analysis of skin biopsies for the presence of *Borrelia burgdorferi*–specific DNA. A segment of a chromosomal gene of *Borrelia burgdorferi* specific gene was amplified by nested PCR (first system, see text). *Lane M*, PhiX 174 DNA digested with Hae III as molecular size marker. *Lanes 1–5*, localized scleroderma; *lanes 6–10*, erythema migrans; *lanes 11–12*, acrodermatitis chronica atrophicans; *lane 13*, granuloma annulare; *lane 14*, negative control (no DNA). A clear amplification product is seen in *lanes 6–12* (erythema migrans and acrodermatitis chronica atrophicans). No amplification product is seen in *lanes 1–5* (localized scleroderma).

fresh-frozen biopsies. Using the third system, we were not able to detect borrelial DNA in more than 11 of 21 fresh-frozen biopsies of EM and two of five fresh-frozen specimens of ACA or in more than one of 12 formalin-fixed specimens of EM. With this system, a relatively large fragment of borrelial DNA is amplified (730 bp/290 bp). In our experience, as well as that of other groups [26,29], amplification of such large fragments is severely hampered in DNA from formalin-fixed, paraffin-embedded tissue because of extensive degradation of DNA during the process of fixation in formalin. In contrast to a recently published study, which detected borrelial DNA in all analyzed (nine of nine) formalin-fixed, paraffin-embedded lesions of patients with LS using this system [14], we were not successful in amplification of *Bb*-specific DNA in formalin-fixed, as well as fresh-frozen, biopsies of patients with LS.

Our data do not support the concept of the presence of *Bb* in active lesions of LS. They do not exclude that *Bb* may be present in a subset of patients with early sclerotic lesions, initiating a disease process with connective tissue reactions similar or identical to scleroderma. Our data also do not entirely exclude the possibility of an association of LS with unknown spirochetes related to *Bb* or with unknown subtypes of *Bb*, which are not detected by the PCR systems used. Analogously, in studies reporting the molecular detection in *Bb* in lesions of LS and lacking extensive specificity controls for the PCR systems used, the possibility of the detection of spirochetes not identical to *Bb* cannot be excluded.

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ANNOUNCEMENT

The Second Seoul International Dermatology Symposium will be held May 11–14, 1995 in Seoul, Korea. A Post-Congress Meeting will be held May 15–19, 1995 in Beijing, China.

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