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Infection by *Borrelia burgdorferi* and cutaneous B-cell lymphoma

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In past years, association of primary cutaneous B-cell lymphoma (CBCL) with infection by *Borrelia burgdorferi* has been reported in a few patients. The evidence for a pathogenetic role was based on clinical grounds or raised titre of antibodies in serum. Both methods, however, do not prove the association between the micro-organism and the CBCL, especially in countries where infection by *Borrelia burgdorferi* is endemic. Moreover, the exact percentage of *Borrelia burgdorferi*-positive CBCL is not known. We retrieved from our files 50 cases of CBCL to perform PCR analysis of *Borrelia burgdorferi* DNA on paraffin-embedded tissue sections. Only patients with primary CBCL were selected. In all cases, monoclonality of the infiltrate was confirmed by immunohistological pattern of immunoglobulin light chains or molecular analysis of J_H gene rearrangement, or both. Specific DNA sequences of *Borrelia burgdorferi* were identified in cutaneous lesions from 9 patients (follicle center lymphoma: 3/20; immunocytoma: 3/4; marginal zone B-cell lymphoma: 2/20; diffuse large B-cell lymphoma: 1/6). Specificity was confirmed by Southern blot hybridisation in all positive cases. We could show that *Borrelia burgdorferi* DNA is present in skin lesions from a small proportion of patients (18%) with various types of CBCL. Our results may have therapeutic implications. In analogy to *Helicobacter pylori*-associated MALT-lymphomas, which in some cases can be cured by eradication of *Helicobacter pylori* infection, a proportion of CBCL may be cured with antibiotic therapy against *Borrelia burgdorferi*. Although yet speculative, adequate antibiotic treatment for patients with primary CBCL should be considered before more aggressive therapeutic options are applied, particularly in countries where infection by *Borrelia burgdorferi* is endemic. PCR analysis of *Borrelia burgdorferi* DNA is a fast test that should be performed in all patients with CBCL to identify those who more likely could benefit from an early antibiotic treatment.

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In past years, association of primary cutaneous B-cell lymphoma (CBCL) with infection by *Borrelia burgdorferi* has been reported in a few patients (1, 2). The evidence for a pathogenetic role was based on clinical grounds (B-cell lymphomas arising on skin affected by acrodermatitis chronica atrophicans) or raised titre of antibodies in serum. Both methods, however, do not prove the association between the micro-organism and the CBCL, especially in countries where infection by *Borrelia burgdorferi* is endemic. Moreover, the exact percentage of *Borrelia burgdorferi*-positive CBCL is not known. We retrieved from our files 50 cases of CBCL to perform PCR analysis of *Borrelia burgdorferi* DNA on paraffin-embedded tissue sections.

Material and methods

Selection of cases: Formalin-fixed, paraffin-embedded biopsy specimens from 50 patients with prima-

Table 1. Classification of cases and results of PCR analysis of *Borrelia burgdorferi* DNA

Classification	Total number of cases	*Borrelia burgdorferi* positive
Follicle center lymphoma	20	3
Lymphoplasmacytoid lymphoma/ Immunocytoma	4	3
Marginal zone B-cell lymphoma	20	2
Diffuse large B-cell lymphoma	6	1

Table 2. Oligonucleotide primers for nested PCR analysis of *Borrelia burgdorferi* DNA (7)

Bb1	5'AAAACGAAGATACTCGATCTGTAATTGC 3'
Bb2	5'TTGCAGAATTTGATAAAGTTGG 3'
Bb3	5'TCTGTAATTGCAGAAACACCT 3'
Bb4	5'GAGTATGCTATTGATGAATTATTG 3'
Bb-hyb	5' TTGAATTAAATTTTGGCTT(G/A)TCAGGAGC(C/T)TATGGAA 3'
actin 1	5' CCTTCCTGGGCATGGAGTC CTG 3'
actin 2	5' GGAGCAATGATCTTGATCTTC 3'

ry CBCL were retrieved from the files of the Department of Dermatology of the University of Graz, Austria. The city lies in a region where infection by *Borrelia burgdorferi* is endemic (about 15% of the population reveals positive serological tests). Only patients with B-cell lymphoma arising primary in the skin were selected for the study. Primary skin involvement was defined as the presence of cutaneous lymphoma without nodal and/or visceral involvement over a period of at least 6 months after complete staging procedures have been performed. Extracutaneous lymphomas with secondary skin manifestations were excluded from this study. Classification of our cases was based on the criteria proposed in the recently published "Revised European American Lymphoma Classification" (3). Details on classification of the cases are provided in Table 1. In all cases, monoclonality of the infiltrate was confirmed by immunohistological pattern of immunoglobulin light chains or molecular analysis of J_H gene rearrangement, or both, as detected according to standard immunohistochemical and molecular genetic analyses (4, 5).

Controls: DNA of three cultured borrelial strains kindly provided by Immuno AG Vienna served as positive controls. *Borrelia burgdorferi* strain B 31 was obtained from the American Type Culture Collection (ATCC # 35210); the other two strains were *Borrelia afzelii* VS 461 and *Borrelia garinii* 20047. Ten samples of normal skin served as negative control.

DNA isolation: DNA isolation was performed as described (6, 7). Briefly, five 5-µm sections were cut from each biopsy specimen and coated on slides. In order to avoid cross-contamination, the blade of the microtome was changed after cutting each of the samples. Slides were subsequently deparaffinized by xylene and ethanol. After air drying sections were scraped off the slides and resuspended in 50 to 100 µl of digestion buffer (0.5% Tween 20, 50 mM Tris pH 8.5, 1 mM EDTA pH 8.0, and 1 µg/µl Proteinase K – Boehringer, Mannheim, Germany), depending on the size of the specimen. After incubation at 55°C for 24 h samples were heated to 94°C for 15 min and stored at –20°C.

Oligonucleotide primers for PCR: Oligonucleotide primers for PCR amplification reactions (Table 2) were designed by Wienecke et al. (7) on the basis of the *Borrelia burgdorferi*-specific gene described by Rosa et al. (8). The outer primer pair (Bb1 and Bb2) is flanking a 171-base pair (bp) fragment (nucleotide position (np) 143–np313), while the inner primer pair (Bb3 and Bb4) spans a 92-bp amplification product (np160–np251). For internal hybridisation control, oligo Bb-hyb, annealing between nucleotides 182 and 217, was used.

To prove the quality of DNA extracts, a 202-bp fragment of the human β-actin gene was amplified according to Fleet et al. (9).

PCR amplification and Southern blot analysis: For analysis of *Borrelia burgdorferi* DNA we used a nested PCR technique (7). The two-step nested PCR procedure substantially increases both sensitivity and specificity of the assay. An aliquot of the first PCR product, produced by the outer primer pair, is amplified by an internal set of primers annealing to *Borrelia burgdorferi*-specific sequences. In this way, only *Borrelia burgdorferi*-specific PCR products obtained in the first assay are further amplified, considerably increasing the specificity of the technique.

Briefly, 4 µl of each DNA sample served as template for PCR. The reaction cocktail (25 µl) contained 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of desoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1 u of Taq DNA polymerase (Perkin Elmer Corporation) and 5 pM each of the outer primers Bb1 and Bb2. After initial denaturation at 94°C for 5 min, samples were subjected to 40 cycles of PCR at 94°C for 1 min, 60°C for 30 s and 72°C for 30 s.

Subsequently, 2 µl of external PCR product were submitted to nested PCR for 40 cycles using inner primers Bb3 and Bb4 under the same temperature profile as above.

Inner PCR products were analysed by an ethidium bromide stained 2.5% agarose gel and visualised by UV light.

Specificity of products was tested by hybridisation and restriction endonuclease analysis. For hybridisation PCR products were vacuumblotted

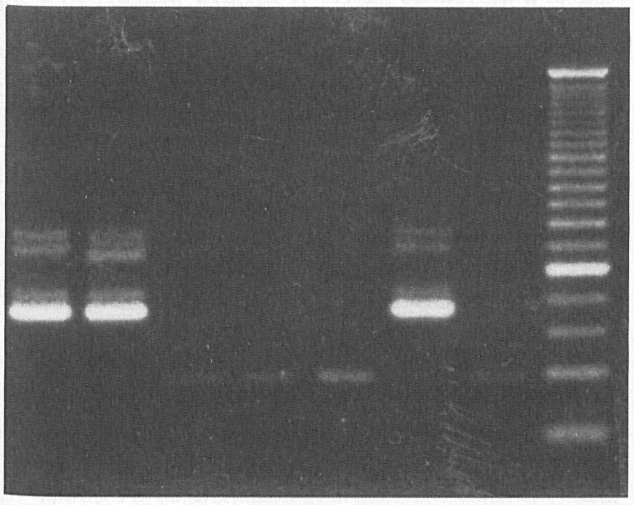

Fig. 1. 3.5% agarose gel of *Borrelia burgdorferi* PCR products. *Lanes 1–5*: DNA from specimens of primary cutaneous B-cell lymphoma; 1–2: specific band at the expected length; the higher, weaker signal represents a dimer of the specific amplification product; 3–5: negative cases; *Lane 6*: positive control (*Borrelia burgdorferi* strain B 31-ATCC # 35210); *Lane 7*: negative control (normal skin); *Lane 8*: 25-bp ladder (the strongest band is a 125-bp fragment).

onto nylon membrane (Boehringer, Mannheim, Germany) and hybridised with an internal digoxigenin 3'-tailed oligoprobe (Bb-hyb, see Table 2). The hybridisation buffer consisted of 5×SSC, 1% blocking reagent (Boehringer), 0.1% N-laurylsarcosine, 0.02% SDS, 0.1 mg/ml poly(A) and 2 pM/ml of labelled probe. After overnight incubation at 37°C, two washing steps at the same temperature were performed following non-isotopic detection according to the Nucleic Detection Kit of Boehringer, Mannheim, Germany.

Additional inner PCR products (92 bp) were subjected to Alu I restriction endonuclease digestion resulting in two fragments of 49 bp and 43 bp (data not shown).

Control amplification of human β-actin gene was performed at 95°C for 1 min, 58°C for 30 s and 72°C for 30 s.

Results

Specific DNA sequences of *Borrelia burgdorferi* were identified in cutaneous lesions from 9 patients (Table 1). Fig. 1 shows 2 representative examples (Fig. 1). Upon gene electrophoretic analysis, a clear amplification product was seen as a band at the expected length. The intensity of the PCR product was identical in all positive samples. Specificity was confirmed by Southern blot hybridisation using oligonucleotide Bb-hyb (Fig. 2). Negative control reactions using biopsy specimens of normal skin were negative. Positive control reactions using primers actin-1 and actin-2 were positive in all biopsy specimens (data not shown). Analysis of DNA from *Borrelia burgdorferi* B31 (ATCC # 35210), *Borrelia afzelii* and *Borrelia garinii* revealed a clear product of the expected size after nested PCR.

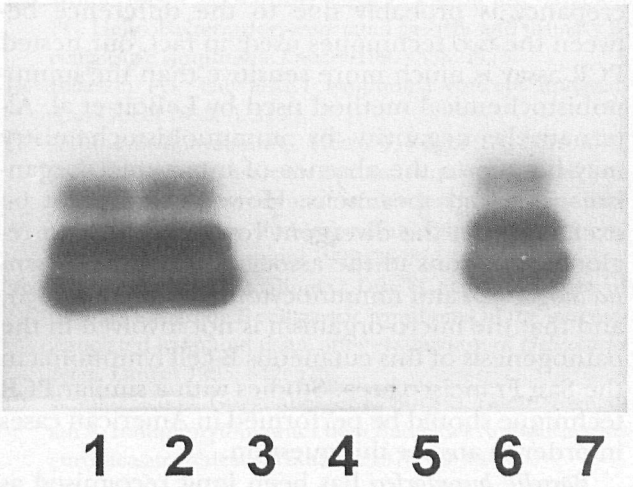

Fig. 2. Southern hybridisation of amplification products shown in Fig. 1 (Lanes 1–7 as in Fig. 1). The additional band represents an amplification dimer.

Discussion

The spirochete *Borrelia burgdorferi* has been identified as the etiologic agent of Lyme disease and of several skin manifestations of it including erythema chronicum migrans, lymphocytoma cutis, and acrodermatitis chronica atrophicans (10, 11). Recently, other skin disorders have been linked with infection by *Borrelia burgdorferi*, such as localised scleroderma and lichen sclerosus et atrophicus among others. Our data clearly show that *Borrelia burgdorferi* DNA is present also in skin lesions from a small proportion of patients (18%) with various types of CBCL. Subclassification of our cases revealed a high percentage (3 out of 4) of positivity among lesions of immunocytoma; follicle center lymphoma, marginal zone lymphoma and diffuse large cell B-cell lymphoma showed a much lower percentage of positive cases (15%, 10% and 17%, respectively). In a recent study on 5 patients with primary cutaneous immunocytoma, LeBoit et al. could not detect *Borrelia burgdorferi* by immunohistochemical staining of tissue sections using an antiserum to the micro-organism (12). This dis-

crepancy is probably due to the difference between the two techniques used: in fact, our nested PCR assay is much more sensitive than the immunohistochemical method used by LeBoit et al. Alternatively, negativity by immunohistochemistry may be due to the absence of intact microorganisms in tested specimens. However, it cannot be excluded that the divergent results are due to regional variations in the association between *Borrelia burgdorferi* and immunocytoma of the skin (13), and that the micro-organism is not involved in the pathogenesis of this cutaneous B-cell lymphoma in the San Francisco area. Studies with a similar PCR technique should be performed in American cases in order to answer this question.

Borrelia burgdorferi has been long recognised as one of the etiologic factors of the so-called cutaneous pseudolymphomas, particularly of cutaneous B-cell pseudolymphomas such as lymphocytoma cutis (10, 14). Diagnosis of lymphoma in our cases was based on clinico-pathological features and was confirmed in each patient by monoclonality of the B-cell infiltrate as detected by immunohistology, molecular biology, or both. It has been suggested recently that several of the cases classified in the past as B-cell pseudolymphoma of the skin represent in fact CBCL of low-grade malignancy (14). In this context, the finding of *Borrelia burgdorferi*-positive CBCL is not surprising, and can be paralleled to the *Helicobacter pylori*-associated low-grade B-cell lymphomas (marginal zone lymphoma, monocytoid B-cell lymphoma) of the gastric mucosa (15). Also some of these lymphoma have been considered in the past to be reactive processes (pseudolymphomas), and only recently have their true nature and the link to *Helicobacter pylori* been demonstrated (16).

Although the figure of 18% might seem relatively low, the true incidence of *Borrelia burgdorferi* infection in CBCL patients may be higher, because PCR analysis may fail to detect specific DNA in some cases (due to absence of the micro-organism in the small part of the block analysed, sensitivity of primers, or quantity of DNA below the level of detection) (7). In fact, in 4/12 specimens (33.3%) obtained from patients with *Borrelia burgdorferi*-associated erythema chronicum migrans, Wienecke et al. could not detect the micro-organism using the nested PCR technique described above, indicating that the true rate of positivity might be higher (7). It is of interest to note that recently *Borrelia burgdorferi* was successfully cultivated in one case of CBCL where PCR analysis yielded negative results (17).

Our results may have therapeutic implications. In analogy to *Helicobacter pylori*-associated MALT-lymphomas, which in some cases can be cured by eradication of *Helicobacter pylori* infection (18), a proportion of CBCL may be cured with antibiotic therapy against *Borrelia burgdorferi*. This seems likely especially for patients with cutaneous immunocytoma, because specific DNA sequences were present in 3 out of 4 cases. Cutaneous immunocytoma may also simulate clinically acrodermatitis chronica atrophicans (19). Although yet speculative, adequate antibiotic treatment for patients with primary CBCL should be considered before more aggressive therapeutic options are applied, particularly in countries where infection by *Borrelia burgdorferi* is endemic. A complete response to antibiotic therapy has been observed recently in one patient with *Borrelia burgdorferi*-associated CBCL, whereas a second one did not show any response (17). It is important to treat patients at onset of disease, because in later stages lesions may no longer be sensitive to systemic antibiotics (2). PCR analysis of *Borrelia burgdorferi* DNA is a fast test that should be performed in all patients with CBCL to identify those who more likely could benefit from an early antibiotic treatment.

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