Molecular Subtyping of *Borrelia burgdorferi* sensu lato Isolates from Five Patients with Solitary Lymphocytoma

Roger N. Picken,* Franc Strle,† Eva Ruzic-Sabljic,‡ Vera Maraspin,† Stanka Lotric-Furlan,† Joze Cimperman,† Yu Cheng,* and Maria M. Picken§

*Section of Infectious Disease, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois, U.S.A.; †Department of Infectious Diseases, University Medical Center, Ljubljana, Slovenia; ‡Institute of Microbiology, University of Ljubljana, Ljubljana, Slovenia; and §Department of Pathology, Loyola University Medical Center, and Hines Veterans' Administration Hospital, Maywood, Illinois, U.S.A.

Solitary lymphocytoma is a rare cutaneous manifestation of Lyme borreliosis that has been reported almost exclusively from Europe. This suggests that its etiologic agent may be absent or extremely rare on the North American continent. All three species of *B. burgdorferi* sensu lato known to be associated with human Lyme borreliosis (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) have been isolated in Europe, whereas only *B. burgdorferi* sensu stricto has been found in North America. This suggests that either *B. garinii* or *B. afzelii* might be the etiologic agent of borrelial lymphocytoma. To investigate this hypothesis we characterized five strains of *B. burgdorferi* sensu lato isolated from lymphocytoma lesions of patients residing in Slovenia. The methods used included: large restriction fragment pattern analysis of restriction enzyme *Mlu*I-digested genomic DNA, plasmid profiling, protein profiling, ribotyping using 5S, 16S, and 23S rDNA probes, and polymerase chain reaction amplification of the *rrf* (5S)-*rrl* (23S) intergenic spacer region. Molecular subtyping showed that four of the five isolates belonged to the species *B. afzelii*; however, this species is the predominant patient isolate in Slovenia and, therefore, may not represent a preferential association with lymphocytoma. The fifth isolate appeared to be most closely related to the DN127 genomic group of organisms. Further characterization of the isolate revealed that it possessed a unique molecular "fingerprint." The results not only show that borrelial lymphocytoma can be caused by *B. afzelii* but also demonstrate an association with another genomic group of *B. burgdorferi* sensu lato that is present in North America as well. *Key words: LRFP-analysis, ribotyping,* Borrelia afzelii, *DN127-group. J Invest Dermatol 108:92–97, 1997*

Lymphocytoma is a cutaneous manifestation of Lyme borreliosis that usually presents as a bluish-red plaque or nodule with a diameter of one to a few centimeters. Histologic examination typically reveals a dense dermal lymphocytic infiltrate, frequently with germinal centers (Abele and Anders, 1990; Hovmark, 1993). Together with erythema migrans (EM) and acrodermatitis chronica atrophicans (ACA), it represents one of the dermatologic hallmarks of the disease; however, whereas lymphocytoma and EM are typical of early disease, ACA is a late, chronic manifestation. Of the three, lymphocytoma is the most rarely seen. Although it may occur concurrently with EM, it typically develops later and lasts longer. In some cases, lymphocytoma is the first and only dermatologic manifestation. Like EM, but unlike ACA, lymphocytoma lesions eventually heal, even in the absence of treatment. Preferred sites include the earlobe in children and the nipple/areola mammae in adults, but other sites, such as the nose or scrotal area, may also be involved. This association with extremities has led to the speculation that the etiologic agent may prefer cooler body sites (Hovmark, 1993).

The role of *Borrelia burgdorferi* sensu lato in lymphocytoma was unequivocally demonstrated in 1986 when spirochetes were isolated in culture from a lymphocytoma lesion (Hovmark *et al*, 1986). Further clinical observations and laboratory tests throughout the 1980s demonstrated that most cases of typical solitary lymphocytoma occurring in Lyme borreliosis–endemic regions of Europe are of borrelial origin. Recent additional evidence has included elevated antibody titers (Wilske *et al*, 1984; Weber *et al*, 1985; Hovmark *et al*, 1986; Weber and Neubert, 1986; Åsbrink *et al*, 1989; Strle *et al*, 1992, 1996), the demonstration of spirochetal organisms in silver-stained sections of tissue biopsies (De Koning and Hoogkamp-Korstanje, 1986), and the amplification of borrelial DNA by the polymerase chain reaction (PCR) (Ranki *et al*, 1994). In addition, several recent studies of patients with a clinical diagnosis of solitary lymphocytoma have been published in which the concomitant presence of EM (Weber *et al*, 1985; Hovmark *et al*,

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Reprint requests to: Dr. Maria M. Picken, Department of Pathology, Room 2242, Building 110, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153.

Abbreviations: EM, erythema migrans; ACA acrodermatitis chronica atrophicans; BL, borrelial lymphocytoma; PFGE, pulsed-field gel electrophoresis; LRFP, large restriction fragment pattern; rRNA, ribosomal RNA; rDNA, ribosomal DNA.

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Table I. *Borrelia* Species and Strains Used and Results of Molecular Subtyping Studies

Species	Strain Designation	LRFP[a]	Biological Origin	Geographic Location
Reference strains				
B. burgdorferi sensu stricto	B31 [ATCC 35210]	MLb1	Tick *(Ixodes scapularis)*	U.S.A.
B. burgdorferi sensu stricto	297 [ATCC 53899]	MLb2	CSF[b]	U.S.A.
B. garinii	20047	MLg1	Tick *(Ixodes ricinus)*	France
B. afzelii	PGau	MLa1	Skin (ACA[c])	Germany
B. burgdorferi sensu lato	25015	MLx	Tick *(Ixodes scapularis)*	U.S.A.
Strains from patients				
B. burgdorferi sensu lato	SL-78	MLx	Skin (lymphocytoma)	Slovenia
B. afzelii	SL-82	MLa1	Skin (lymphocytoma)	Slovenia
B. afzelii	SL-84	MLa1	Skin (lymphocytoma)	Slovenia
B. afzelii	SL-86	MLa1	Skin (lymphocytoma)	Slovenia
B. afzelii	SL-87	MLa1	Skin (lymphocytoma)	Slovenia

[a] LRFP, Large Restriction Fragment Pattern. Nomenclature follows the system devised previously (Belfaiza *et al*, 1993) for bands > 70 kb in size. Thus, all LRFPs derived from restriction endonuclease *Mlu*I digestion are designated ML: *B. burgdorferi* sensu stricto LRFPs are designated MLb, *B. garinii* LRFPs are designated MLg, and *B. afzelii* LRFPs are designated MLa. For *B. burgdorferi* sensu lato isolates of unknown species designation we use the term MLx.
[b] CFS, cerebrospinal fluid.
[c] ACA, acrodermatitis chronica atrophicans.

1986; Weber and Neubert, 1986; Åsbrink *et al*, 1989; Strle *et al*, 1992, 1996) or ACA (Strle *et al*, 1996) was noted. To precisely define cases with substantial evidence of borrelial etiology, the term borrelial lymphocytoma (BL) was introduced (Weber *et al*, 1985; Åsbrink and Hovmark, 1987; Åsbrink and Hovmark, 1988; Hovmark *et al*, 1993); however, the organism has yet to be implicated in some other forms of solitary lymphocytoma and multiple disperse lymphocytoma lesions. The subject has recently been reviewed (Hovmark *et al*, 1993).

Although BL is known throughout Europe, there have been few reports from North America. A recent report from Canada (Albrecht *et al*, 1991) described a case in which the infection was almost certainly contracted in Switzerland. To date, there has been only one single convincing report of BL acquired in the United States (Finkel and Johnson, 1990). Reasons for the disparate geographic distribution of cases and the distinctive body localization of lesions are not completely understood. It has been surmised that lymphocytoma may be associated with a distinct species or distinct strains of *B. burgdorferi* sensu lato that are present on one continent but not on the other. For example, three species of *B. burgdorferi* sensu lato have been found in Europe (*B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto) (Baranton *et al*, 1992; Marin Canica *et al*, 1993), whereas only the latter organism is known to be from the United States. Up to the present time, only a single isolate derived from BL has been described in the literature (Hovmark *et al*, 1986). In this report we describe the culture isolation of *B. burgdorferi* sensu lato from the lymphocytoma lesions of five patients residing in Slovenia and present the molecular characteristics of the strains.

MATERIALS AND METHODS

Patients and Tissue Samples From January 1993 to May 1995, 32 adult patients with BL were seen at the Lyme Borreliosis Outpatient's Clinic, University Medical Center, Ljubljana. This compares with 1833 patients with typical EM lesions seen during the same time interval. The sites of lymphocytoma lesions were the nipples/areola mammae (23 patients), earlobe (six patients), and nonclassic locations (shoulder, upper arm, chest) (three patients). Lesions from 13 previously untreated patients were cultured, and *B. burgdorferi* sensu lato was isolated from five (38.5%). Biopsy of lymphocytoma nodules was performed after disinfection of the skin with 70% alcohol and local anesthesia with 2% xylocaine. The specimen was divided into several pieces: one piece was submitted for histologic examination, and two other pieces were placed in modified Barbour-Stoenner-Kelly II medium (Strle *et al*, 1995) and Modified-Kelly-Pettenkofer (MKP) medium (Preac-Mursic *et al*, 1986), respectively. Cultures were incubated at 33°C and were evaluated weekly for the presence of *Borrelia* by darkfield microscopy.

Antibodies to *B. burgdorferi* Sensu Lato For all patients IgM and IgG antibody titers against *B. burgdorferi* sensu lato were determined on the day of skin biopsy by immunofluorescence assay (Wilske *et al*, 1984). A local isolate of *B. afzelii* was used as antigen. Titers of 1:256 or higher were interpreted as positive.

Patient Isolates and Reference Strains Patient isolates and reference strains of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* included for comparison in molecular characterization studies are listed in **Table I**. The source of reference strains has been described previously (Picken, 1992).

Protein Profiling by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Determination of the total protein profile of isolates by SDS-PAGE was performed as described previously (Picken *et al*, 1995) except that spirochetes were washed in 20 mM HEPES/10 mM NaCl buffer, pH 7.6, instead of phosphate-buffered saline. Prestained molecular-mass markers (containing polypeptides of 21.9, 29.7, 35.1, 50.0, 97.2, and 142.9 kDa) were obtained from Bio-Rad Laboratories (Richmond, CA).

Large Restriction Fragment Pattern (LRFP) Analysis Pulsed-field gel electrophoretic (PFGE) separation of restriction enzyme *Mlu*I-digested genomic DNA and determination of LRFPs were performed as described previously (Picken *et al*, 1995; Strle *et al*, 1995). Concatamers used as size markers in **Fig 1B** were purchased from FMC Bioproducts Corp. (Rockland, ME). The monomer size of these markers is 48.5 kb, resulting in bands of the following approximate sizes: 49, 97, 146, 194, 243, 291, 340, 388, 437, and 485 kb.

Species Typing by PCR For species typing we used 16S ribosomal RNA (rRNA)-specific PCR primers designed to differentiate *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. These primers and the amplification methodology have been described previously (Kuiper *et al*, 1994). Standard precautions applicable to the amplification and analysis of isolates by PCR were employed to preclude carry-over and cross-contamination of PCR reactions.

Plasmid Profiling by PFGE Pulsed-field gel electrophoretic separation of total genomic DNA, including chromosome and plasmids, was performed as described previously (Picken *et al*, 1995; Strle *et al*, 1995). The "high molecular weight" markers used in **Fig 1C** were purchased from GIBCO-BRL (Life Technologies, Inc., Gaithersburg, MD) and produce bands of the following sizes: 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb.

Ribotyping Ribosomal DNA (rDNA) gene restriction patterns for 16S and 23S rRNA genes were determined as described previously (Baranton *et al*, 1992). Total genomic DNA from strains was digested with the restriction enzymes *Eco*RV, *Hin*dIII, *Eco*RI, and *Pst*I. The products of restriction enzyme digestion were separated by agarose gel electrophoresis, transferred to Nylon membrane as previously described (Picken, 1992), and probed using ^{32}P–end-labeled *Escherichia coli* 16+23S rRNA (obtained from Boehringer, Mannheim, Germany). The size of hybridizing fragments was determined by autoradiography and by comparison to molecular weight markers (bacteriophage λ digested with *Hin*dIII). 5S rDNA-linked restriction fragment length polymorphism (RFLP) analysis was performed as previously described (Liveris *et al*, 1995). For this, genomic DNA from

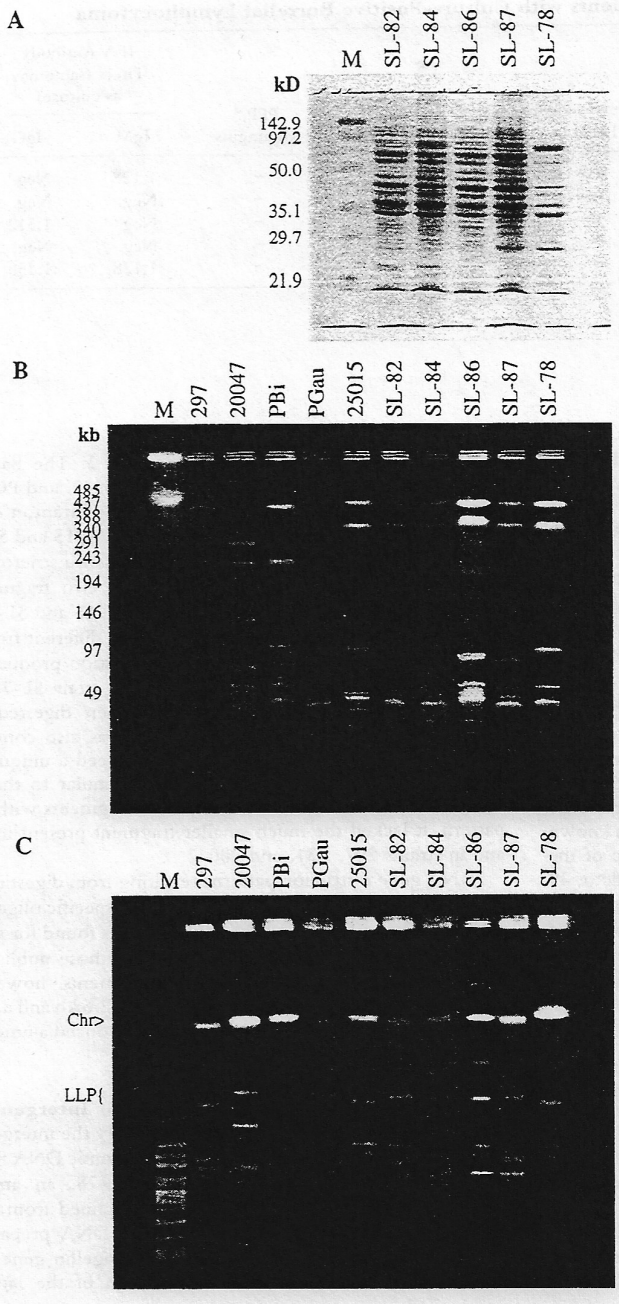

Figure 1. Molecular characterization of patient lymphocytoma isolates and reference strains by PAGE and PFGE. *(A)* SDS-PAGE of total cellular proteins from the five borrelial lymphocytoma isolates SL-82, SL-84, SL-86, SL-87, and SL-78. Strain designations are shown above their respective lanes. *Lane M* contains prestained protein molecular mass markers of 21.9 to 142.9 kDa. *(B)* PFGE of *Mlu*I restriction enzyme-digested genomic DNAs from strains SL-82, SL-84, SL-86, SL-87, and SL-78 in comparison to reference strains of *B. burgdorferi* sensu stricto (strain 297), *B. garinii* (strains 20047 and PBi), *B. afzelii* (strain PGau), and the DN127 genomic group (strain 25015). Strain PGau illustrates the MLa1 LRFP typical of *B. afzelii*. Strain 25015 illustrates the superficially similar ML*x* LRFP. Strain designations are listed above their respective lanes. *Lane M* contains DNA molecular size markers (λ DNA concatamers of 48.5 to 485 kb). *(C)* PFGE of undigested genomic DNAs from the *B. burgdorferi* sensu lato isolates shown in *(B)*. Strain designations are shown above their respective lanes. *Lanes M* contain DNA molecular size markers of 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb and are too closely spaced for them to be marked at the side of the figure. Chr indicates the position of the 950 kb chromosome. LLP indicates the position of the 49- to 56-kb largest linear plasmids.

isolates was digested with *Hpa*I, separated on a 0.8% agarose gel, transferred to Nylon membranes, and hybridized with a ^{32}P-end-labeled oligonucleotide probe specific for 5S rDNA (Schwartz *et al*, 1992).

PCR Amplification of *rrf* (5S)–*rrl* (23S) Intergenic Spacer Region Previously described primers (Postic *et al*, 1994) were used to amplify the variable intergenic spacer region between the 3′-end of the 5S rRNA gene (*rrf*) and the 5′-end of the 23S rRNA gene (*rrl*). Amplification reactions were performed as described (Postic *et al*, 1994) using standard precautions against carry-over and cross-contamination. Amplification products were analyzed by agarose gel electrophoresis and staining with ethidium bromide.

RESULTS

Patient Characteristics and Skin Manifestations Basic data on patients with culture-positive lymphocytoma lesions are presented in **Table II**. In all four patients with lymphocytoma of the breast, the nipple on the involved side was mildly to moderately edematous, and a nodule with a diameter of 1–3 cm was palpated in the area of the areola mammae. In the case of one patient with lymphocytoma of the chest (patient 5), a nodule was present in the cutis and subcutis of the skin at the center of a large annular EM lesion. In all cases, biopsy material for histologic examination and for borrelial culture was taken from nodule tissue. In all five patients histologic examination revealed dense lymphocytic infiltration, and in patients 2, 3, and 5 germinal centers were also observed.

In the case of patient 2, *B. burgdorferi* sensu lato was concomitantly isolated in culture from a biopsy of normal-appearing skin at the site of a previous EM lesion (this lesion had resolved 6 mo prior to biopsy without antibiotic treatment). Such isolations have been reported previously (Kuiper *et al*, 1994; Strle *et al*, 1995). The molecular characteristics of both isolates were the same. In two other patients (patients 4 and 5) *B. burgdorferi* sensu lato was cultured from both a lymphocytoma lesion and the advancing margin of a concomitant EM lesion. Again, the molecular characteristics of the two isolates were identical. In all cases, data for the lymphocytoma isolates are shown in **Fig 1**.

In all five patients, EM and/or BL lesions resolved subsequent to antibiotic treatment; resolution of EM lesions preceded that of BL lesions by periods ranging from 0.5 to 3 wk **(Table III)**.

Molecular Characterization of Lymphocytoma Isolates All isolates were characterized by the following procedures: (i) protein-profiling by SDS-PAGE separation of total proteins; (ii) genospecies typing with PCR primers designed to differentiate *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*; (iii) genospecies typing by LRFP analysis of *Mlu*I-digested total genomic DNA; (iv) plasmid profiling by PFGE separation of total genomic DNA. Strain SL-78 was additionally characterized by RFLP analysis of 5S, 16S, and 23S rDNA genes (ribotyping), and by PCR amplification of the *rrf* (5S)-*rrl* (23S) intergenic spacer region.

Protein Profiling by SDS-PAGE Four of the five lymphocytoma isolates (SL-82, SL-84, SL-86, and SL-87) possessed very similar protein profiles as shown in **Fig 1*A***. The fifth isolate (SL-78) differed in the pattern of its higher molecular mass proteins. All five isolates possessed prominent proteins of 35 and 32 kDa. Proteins of this size are typical of the OspA and OspB proteins of both *B. afzelii* and reference strain 25015. Strains SL-87 and SL-78 also possessed a prominent 25-kDa protein, which is in the appropriate size range for OspC.

Species Typing by PCR and LRFP Analysis Using species-specific PCR primers, four of the five lymphocytoma isolates (SL-82, SL-84, SL-86, and SL-87) typed as *B. afzelii*. These isolates

Table II. Characteristics of Patients with Culture-Positive Borrelial Lymphocytoma

Patient No.	Age	Sex	Location of BL[a]	Tick Bite[b]	Symptoms		EM[c]	PFP,[d] Meningitis	IFA Antibody Titers (same day as culture)		Isolate
					Local	General			IgM	IgG	
1	72	F	breast	+	+	+	+	−	1:128	Neg.[e]	SL-78
2	43	F	breast	+	−	+	+	+	Neg.	Neg.	SL-82
3	40	F	breast	+	−	+	−	−	Neg.	1:512	SL-84
4	38	F	breast	−	+	−	+	−	Neg.	Neg.	SL-86
5	58	F	chest	+	+	+	+	−	1:128	1:256	SL-87

[a] BL, borrelial lymphocytoma.
[b] As recalled by the patient.
[c] EM, erythema migrans.
[d] PFP, peripheral facial palsy.
[e] Later seroconversion.

also possessed the highly conserved MLa1 LRFP typical of this species. This is illustrated in **Fig 1*B***, which also shows the LRFPs of reference strains representing the species *B. burgdorferi* sensu stricto (297), *B. garinii* (20047 and PBi), and *B. afzelii* (PGau). The MLa1 LRFP is characterized by three fragments of 440, 320, and 90 kb. The fifth isolate SL-78 typed as *B. burgdorferi* sensu stricto by species-specific PCR and possessed the same LRFP as the reference strain 25015. This is also shown in **Fig 1*B***. Since strain 25015 belongs to a distinct genomic group of *B. burgdorferi* sensu lato (DN127-group), which has not yet been assigned a species designation, we will refer to this LRFP as ML*x*. The ML*x* LRFP is characterized by three fragments of 440, 300, and 100 kb.

Plasmid Profiling by PFGE **Fig 1*C*** presents the complete genomic complement (chromosome and plasmids) of the five lymphocytoma isolates in comparison to the same five reference strains used previously. The largest plasmid of *Borrelia* spp. is known to be linear (LLP in **Fig 1*C***), and its size is characteristic of the species. The calculated sizes are: for *B. burgdorferi* sensu stricto, 49 kb; for *B. garinii*, 55 kb; and for *B. afzelii*, 56 kb (Samuels *et al*, 1993). Strain 25015 has a large linear plasmid that corresponds in size to that of the largest of *B. burgdorferi* sensu stricto. The largest linear plasmids of strains SL-82, SL-84, SL-86, and SL-87 correspond to that of *B. afzelii* strain PGau whereas the largest linear plasmid of strain SL-78 is equivalent in size to that of reference strain 25015.

Ribotyping Because of the similarity of the molecular profile of strain SL-78 to that of strain 25015 and because the latter strain belongs to a distinct genomic group of *B. burgdorferi* sensu lato, we undertook a more extensive genetic characterization of strain SL-78. For this we utilized rRNA gene restriction patterns resulting from digestion with the enzymes *Pst*I, *Eco*RI, *Hin*dIII, and *Eco*RV and probing with 16+23S rRNA probes. The results for strain SL-78 and five reference strains representing *B. burgdorferi* sensu stricto (B31, 297), *B. garinii* (20047), *B. afzelii* (PGau), and the DN127-group (25015) are shown in **Fig 2**. The band patterns found for the reference strains B31, 297, 20047, and PGau were in agreement with those published previously (Baranton *et al*, 1992).

On the basis of *Eco*RV digestion, strains 25015 and SL-78 could not be distinguished from the *B. burgdorferi* sensu stricto strains B31 and 297. Thus, all four strains produced two fragments. After digestion with *Hin*dIII, however, strains 25015 and SL-78 showed identical patterns of fragments, which were different from those of the other three species. While *Eco*RI digestion produced a single fragment from strains B31, 297, and 25015, strain SL-78 produced a unique pattern of three fragments when digested with this enzyme. One of these *Eco*RI fragments was also common to *B. afzelii* strain PGau. Strain SL-78 also produced a unique fragment pattern after *Pst*I digestion. It was most similar to that of strain 25015; however, although it shared two fragments with the 25015 pattern, it lacked the much smaller fragment present in this strain and in strains 297, B31, and 20047.

rRNA gene restriction patterns resulting from digestion with the enzyme *Hpa*I and probing with a 5S rRNA-specific oligonucleotide probe are shown in **Fig 3**. The band patterns found for strains B31, 20047, PGau, and 25015 closely resemble those published previously (Liveris *et al*, 1995). In our experiments, however, strain 25015 produced major fragments of 3.0 and 2.6 kb and a weak band of 1.9 kb. In contrast, strain SL-78 again produced a unique pattern characterized by a single band of 2.6 kb.

PCR Amplification of *rrf* (5S)-*rrl* (23S) Intergenic Spacer Region When primers designed to amplify the intergenic spacer region were used in PCR reactions with genomic DNA from strains B31, 297, 20047, PGau, 25015, and SL-78, an amplification fragment of approximately 250 bp was obtained from all isolates except strain SL-78. The SL-78 genomic DNA preparation was tested using other primer pairs based on flagellin gene sequences and 16S ribosomal RNA sequences. Both of the latter sets of

Table III. Patient Antibiotic Treatment Regimens and Disease Outcome

Patient No.	Clinical Diagnosis	Duration of BL[a] Before Culturing	Treatment History	Duration Until Resolution of EM[b]	Duration Until Resolution of BL
1	EM/BL	14 wk	Azithromycin (3 g total dosage)[c]	2.5 wk	4.5 wk
2	EM/BL	Unknown[d]	Ceftriaxone (2g/d iv, 14 d)	5 wk[e]	6 wk
3	BL	5 wk	Amoxicillin (500 mg t.i.d., 14 d)	n/a[f]	2 wk
4	EM/BL	4 wk	Amoxicillin (500 mg t.i.d., 14 d)	1 wk	1.5 wk
5	EM/BL	6 wk	Ceftriaxone (2g/d iv, 14 d)	5 d	8 wk

[a] BL, Borrelial lymphocytoma.
[b] EM, Erythema migrans.
[c] Day 1, 500 mg b.i.d.; days 2 to 5, 500 mg o.d.
[d] The patient was not aware of the BL lesion and was examined because of neurologic involvement and a history of EM. Lymphocytoma was found incidentally at examination.
[e] EM resolved spontaneously before institution of antibiotic treatment.
[f] n/a, Not applicable.

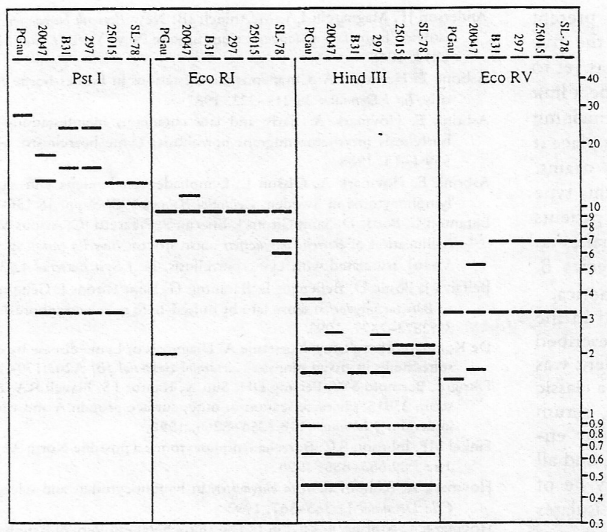

Figure 2. 16S and 23S rRNA ribotyping studies of strain SL-78 and reference strains of *B. burgdorferi* sensu lato. Diagrammatic compilation of the results from four separate autoradiographs, showing the migration patterns of rRNA gene restriction fragments after digestion with the restriction enzymes *Pst*I, *Eco*RI, *Hin*dIII, and *Eco*RV followed by hybridization with ^{32}P-labeled *E. coli* 16+23S RNA. *Thick* and *thin lines* represent strong and weak bands, respectively, as seen on the original autoradiographs. Strain designations are shown above their respective lanes. Results for strain SL-78 are shown in comparison to representatives of four *B. burgdorferi* sensu lato genomic groups. These include: *B. afzelii* (strain PGau), *B. garinii* (strain 20047), *B. burgdorferi* sensu stricto (strains B31 and 297), and the DN127-genomic group (strain 25015).

primers produced amplification products, indicating that inhibitors of amplification were not present in the SL-78 DNA preparation.

DISCUSSION

In Lyme borreliosis–endemic regions, it is generally considered that the majority of solitary lymphocytoma cases are caused by *B. burgdorferi* sensu lato (Hovmark *et al*, 1993). Usually, lymphocytoma is interpreted to be of borrelial origin in patients with typical EM (or a reliable history thereof) and/or with serum antibodies to *B. burgdorferi* sensu lato. Although we also use these criteria routinely as indicators of borrelial infection (Strle *et al*, 1992, 1996), it is apparent that isolation of the causative agent from involved tissue represents the most reliable method of establishing etiology. Up to the present time, however, *B. burgdorferi* sensu lato has been cultured from solitary lymphocytoma in only a single case (Hovmark *et al*, 1986) and detected by PCR amplification of borrelial DNA in one other case (Ranki *et al*, 1994). In this study, we describe findings for five patients with lymphocytoma lesions from which spirochetes were isolated in culture and characterized by a variety of molecular methods.

In this study, five of 13 patients presenting with BL (38.5%) were culture positive. Thus, the isolation rate was lower than that typically found for EM skin lesions, which in the same time period was 50% (unpublished data). In the two previously reported series of patients with BL, seropositivity at first examination was 74% (Åsbrink *et al*, 1989) and 69.4% (Strle *et al*, 1992), respectively; some, but not all, seronegative patients later seroconverted (Strle *et al*, 1992). In the current study, only two of the five patients with culture-confirmed BL lesions had positive IgG antibody titers at the time of skin biopsy (patients 3 and 5); in two patients (1 and 5) low IgM antibody titers were detected. Later IgG seroconversion occurred in one of these patients (patient 1). Two further patients were seronegative at the time of skin biopsy and remained so during follow-up; in both of these patients, a positive *B. burgdorferi* sensu lato culture result was the only laboratory proof of borrelial infection. The presence of a concomitant EM lesion in four of the five patients (patients 1, 2, 4, and 5) supported the notion that lymphocytoma was of borrelial origin, as did the successful resolution of lesions after antibiotic treatment. Patient 1 was exceptional in that a lymphocytoma lesion appeared before the emergence of EM.

The single lymphocytoma isolate of *B. burgdorferi* sensu lato reported to date (Hovmark *et al*, 1986) was described as appearing the same by darkfield microscopy as other borrelial isolates cultured from ticks, EM lesions, and ACA lesions in the same region (Sweden). We are not aware that this isolate was investigated further by molecular or other methods. This single, previous isolation also predates the recognition of separate genomic groups (Postic *et al*, 1990) and the eventual designation of three separate species within what was formerly *B. burgdorferi* (Baranton *et al*, 1992; Marin Canica, 1993).

Of the five lymphocytoma isolates reported here, four appeared to be typical *B. afzelii* isolates. Thus, the PCR-typing results obtained with these strains, as well as their LRFP profiles after *Mlu*I digestion, plasmid profiles, and protein profiles, were all characteristic of this latter species. The fifth isolate, SL-78, was shown to possess a molecular "fingerprint" that is, thus far, unique. We have obtained eight other isolates from the same region that possess the same distinctive LRFP as strain SL-78 (ML*x*), as well as similar plasmid and protein profiles (unpublished data). None of the eight other isolates share the same ribotyping pattern, however, and, in contrast to strain SL-78, the *rrf* (5S)–*rrl* (23S) intergenic spacer region could be amplified from these strains. Both of these latter findings suggest that strain SL-78 has undergone a profound rearrangement of its ribosomal RNA genes. It is, however, a low-passage isolate from a skin biopsy that was taken before the patient received antibiotic treatment.

The results of these molecular characterization studies appear particularly interesting in light of the longstanding observation that lymphocytoma is a predominantly "European" manifestation of Lyme borreliosis. The recent discovery that *B. burgdorferi* comprises several distinct species, with more species present in Europe than in North America, has fueled speculation that there may be a link

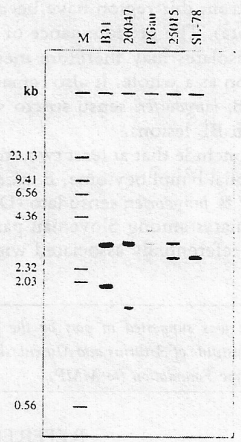

Figure 3. 5S rRNA ribotyping studies of strain SL-78 and reference strains of *B. burgdorferi* sensu lato. Autoradiograph showing the results of digesting genomic DNA from strain SL-78 and four reference strains of *B. burgdorferi* sensu lato with the restriction enzyme *Hpa*I and hybridizing with a ^{32}P-labeled *B. burgdorferi* 5S rRNA-specific probe. Strain designations are shown above their respective lanes. Reference strains include: *B. burgdorferi* sensu stricto (B31), *B. garinii* (20047), *B. afzelii* (PGau), and the DN127 genomic group (25015). *Lane M* contains DNA molecular mass markers derived from *Eco*RI digestion of bacteriophage λ DNA. The molecular size of markers is indicated at the side of the figure.

between "European" manifestations and species that are present only on the European continent. The fact that four of the five strains from this study were identified as *B. afzelii* (which has yet to be isolated from North America) suggests that there may be a link between lymphocytoma and this species; however, the remaining isolate possessed genetic and phenotypic characteristics that place it together with another group of *B. burgdorferi* sensu lato strains, which we have tentatively termed ML*x*. Two strains of this type have been isolated in the United States, although not from patients (Anderson *et al*, 1988; Picken *et al*, 1995). This finding makes an exclusive association between lymphocytoma and the species *B. afzelii* seem less certain and suggests a link with North America.

Recently, a North American case of BL that originated in the expanding midwestern focus of Lyme borreliosis was described (Finkel and Johnson, 1990). In this case, in which the patient was shown to have acquired the disease in western Wisconsin, a classic BL lesion was accompanied by concomitant EM lesions, serum antibodies to *B. burgdorferi* sensu lato were demonstrated by enzyme-linked immunosorbent assay and western blot assays, and all lesions resolved after antibiotic treatment. It may possibly be of significance that one of the two North American ML*x* isolates described previously (Picken *et al*, 1995), and referred to above, was isolated from a white-footed mouse in northern Illinois, at a site proximal to the Illinois/Wisconsin border. This site is on the North/South migration route of numerous avian species. The other North American isolate with the same LRFP as SL-78, strain 25015, was previously tested in a mouse model of Lyme borreliosis and found to be infectious but not pathogenic (Anderson *et al*, 1990). In a later study, however (Fikrig *et al*, 1992), it was shown that strain 25015 was mildly arthritogenic in a murine system. Therefore, the effects of pathogenicity may be relative and not absolute for this strain. As noted, the Slovenian isolate appears to possess an unusual rearrangement of its rRNA genes that makes its molecular "fingerprint" unique, but in its other characteristics it is more similar to the ML*x* group of isolates than to any other species or subtypes of *B. burgdorferi* sensu lato described to date. The extremely low incidence of BL in North America may therefore reflect a correspondingly low incidence of such strains.

Similarly, although *B. afzelii* predominated among the lymphocytoma isolates, the species also predominates in the geographic locale from which the isolates were obtained. Thus, 84% of patient isolates from this region have been identified as *B. afzelii* (unpublished data). The predominance of this species among the lymphocytoma isolates may therefore merely reflect its predominance in the region as a whole. It also remains to be seen whether *B. garinii* and/or *B. burgdorferi* sensu stricto will eventually be found associated with BL lesions.

We conclude that at least two etiologic agents can be responsible for borrelial lymphocytoma, *B. afzelii* and another distinct genomic group of *B. burgdorferi* sensu lato (DN127-group). Because *B. afzelii* predominates among Slovenian patient isolates as a whole, it may not be preferentially associated with borrelial lymphocytoma.

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