Association of Specific Subtypes of *Borrelia burgdorferi* with Hematogenous Dissemination in Early Lyme Disease

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To investigate whether genetic diversity of *Borrelia burgdorferi* sensu stricto may affect the occurrence of hematogenous dissemination, 104 untreated adults with erythema migrans from a Lyme disease diagnostic center in Westchester County, New York, were studied. Cultured skin isolates were classified into 3 groups by a polymerase chain reaction amplification and restriction fragment length polymorphism (RFLP) method. A highly significant association between infecing RFLP type in skin and the presence of spirochetaliaemia was found (P < .001). The same association existed for the presence of multiple erythema migrans lesions (P = .045), proving clinical corroboration that hematogenous dissemination is related to the genetic subtype of *B. burgdorferi* sensu stricto. There were no significant associations between RFLP type and seropositivity or clinical symptoms and signs except for a history of fever and chills (P = .033). These results suggest that specific genetic subtypes of *B. burgdorferi* sensu stricto influence disease pathogenesis. Infection with different subtypes of *B. burgdorferi* sensu stricto may help to explain differences in the clinical presentation of patients with Lyme disease.

Variability in the clinical expression of *Borrelia burgdorferi* infection has been appreciated for many years [1]. In one natural history study of 55 untreated US patients with early Lyme disease associated with erythema migrans, 34 (62%) developed objective signs of arthritis, 10 (18%) developed arthralgias without arthritis, but 11 (20%) remained completely well over a mean follow-up of 6 years [2].

Explanations for differences in Lyme disease manifestations are becoming better understood. Coinfection with other tick-borne pathogens is clearly one factor. Recently, Krause et al. [3] reported that almost all of their early Lyme disease patients who had persistent fatigue 3 months after antibiotic therapy were coinfected with *Babesia microti*. In addition, US patients with Lyme disease who also have cytopenias appear to be coinfected with either the agent of human granulocytic ehrlichiosis or with *B. microti* [4, 5].

Another contributing factor is the genetic diversity of *Borrelia* strains that cause Lyme disease. Although *B. burgdorferi* was originally characterized as a single species, the broad grouping of *Borrelia* spirochetes referred to as *B. burgdorferi* sensu lato consists of ≥8 distinct genospecies. Of these, only *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and the newly named *B. bissettii* sp. nov. have been cultured from persons with Lyme disease [6–13]. Specific clinical manifestations have been associated with certain genospecies [14, 15]. For example, acrodermatitis chronica atrophicans and lymphocytoma, 2 distinctive cutaneous manifestations of Lyme disease caused by *B. afzelii*, are found almost exclusively in Europe [15]. In North America, virtually all characterized isolates are *B. burgdorferi* sensu stricto [6, 16, 17]. However, even among North American isolates of *B. burgdorferi* sensu stricto, there is substantial genetic heterogeneity, as evidenced by restriction fragment length polymorphism (RFLP) analysis, pulsed-field gel electrophoresis, and arbitrarily primed polymerase chain reaction (PCR) [16–18]. We previously characterized 183 skin isolates of *B. burgdorferi* from patients of a Lyme disease diagnostic center in Westchester County, New York, by a PCR-RFLP typing method [19]. The current study addresses whether this typing system identifies genetic subtypes of *B. burgdorferi* sensu stricto that are associated with either different clinical manifestations or an increased likelihood of hematogenous dissemination.

Materials and Methods

Subjects, skin biopsy, and culture. All subjects were adults with erythema migrans enrolled in a prospective study at the Lyme Disease Diagnostic Center of the Westchester Medical Center between 1991 and 1997. Signs and symptoms were recorded as present or
absent. If present, symptoms were characterized by the patient as to severity by use of an 8-cm-long visual analogue scale, previously used elsewhere in a published treatment trial of patients with Lyme disease [20]. For symptomatic patients, a symptom severity index was also calculated which equaled the cumulative symptom score divided by the number of symptoms present.

Skin biopsies (2 mm) were obtained from the advancing border of primary erythema migrans lesions, as described elsewhere [21]. Biopsies were placed in transport medium for later laboratory processing. Tissues were transferred to 0.5 mL of BSK II medium lacking rabbit serum and gelatin (incomplete BSK II) and were ground in a microtissue grinder (Spectrum Medical Industries, Los Angeles). A 0.1-mL aliquot of this suspension was introduced into 6 mL of BSK II medium supplemented with 6% rabbit serum, 1.2% gelatin, and 35% liquid bovine serum albumin (Sigma, St. Louis) (complete BSK II) and was incubated at 33°C for \( \leq \)8 weeks. Cultures were examined at 2-week intervals.

Whole blood, serum, or plasma was collected in sterile tubes, and the aliquots were inoculated within 3 h of collection into complete BSK at a ratio of 1 part specimen to 20 parts BSK [22–24]. Beginning in 1994, antimicrobials (5-fluorouracil and nalidixic acid) were omitted from the BSK medium; in 1997, gelatin was omitted from the BSK medium. Cultures were incubated at 33°C for \( \leq \)12 weeks. Cultures were examined by fluorescence microscopy at 2 weeks and thereafter at 2- to 4-week intervals. Sampling was done as follows: 10-\( \mu \)L aliquots of culture material were mixed with 10 \( \mu \)L of an acridine orange-staining solution (100 \( \mu \)g/mL PBS [pH 7.4]). These mixtures were examined microscopically (magnification \( \times 400 \)) on a slide overlaid with a coverslip. A minimum of 20 high-power fields were viewed for the presence of motile spirochetes. Confirmation that the visualized spirochete was \( B. burgdorferi \) was done by PCR on a sample of the culture medium, as reported elsewhere [25].

To remove nonborrelial microorganisms selectively, contaminated cultures were filtered twice, first through a 0.45-\( \mu \)m and then through a 0.2-\( \mu \)m-pore-size filter (Nalgene; Nalge, Rochester, NY) [26]. In successive years, different experiments were performed in order to find the optimal volume of blood and the preferred blood component to maximize the yield of culture. In most years, comparison studies were done in which the same patient had multiple cultures established (e.g., whole blood vs. serum [23]).

**DNA isolation.** DNA from 0.2 mL of \( B. burgdorferi \) cultures (primary or at passage 1 or 2) was prepared by use of a commercial nucleic acid extraction kit (IsoQuick; Orca Research, Bothell, WA) [27]. Purified DNA was resuspended in a total volume of 50 \( \mu \)L of water, and 10 \( \mu \)L was used for PCR.

**PCR amplification and RFLP analysis.** A 941-bp region of the \( B. burgdorferi \) 16S–23S rDNA spacer region was amplified by nested PCR, as described elsewhere [19, 28]. First-round amplification used PA (5'-GGTATGTTTAGTGAGGG-3') and P95 (5'-GGTTAGGCCGAGGCTGT-3') as the forward and reverse primers, respectively. Ten microliters of either l/1000 dilution of the first-round PCR product was employed as a template in a second PCR using PB (5'-CGTACTGGGAAAGTGCGGCTG-3') as the forward primer and P97 (5'-GATGTTCAACTCATCCTGTTGCC-3') as the reverse primer [19]. Ten-microliter aliquots of the nested PCR products were subjected to RFLP analysis by digestion with 2 U of either Hybrid or MseI, and digested fragments were resolved by agarose gel electrophoresis in TBE buffer, as described elsewhere [19, 28].

**DNA sequencing and phylogenetic analysis.** Individual rDNA spacer PCR products were cloned, and the DNA sequence of 5 separate clones from each \( B. burgdorferi \) isolate analyzed was determined by automated DNA sequencer (Visible Genetics, Toronto, Canada) after cycle sequencing using the Thermosequenase kit from Amersham Life Sciences (Cleveland). Cloning of the amplified products ensures that specimens do not contain multiple genotypes. Sequence alignments were done by use of the multiple sequence alignment software Clustal X, and phylogenetic trees were constructed by the neighbor-joining method [29]. The nucleotide sequences of the 16S-23S rDNA spacers for the \( B. burgdorferi \) isolates analyzed in figure 1 were deposited in GenBank (accession numbers, AF139507–AF139517).

**Serology.** The serologic results reported are for an EIA [30], except for 8 subjects who were also participants in recombinant outer surface protein A (OspA) vaccine trials (6 received a placebo...
Results and 2 the OspA vaccine preparation). The vaccine trial participants had immunoblots instead of EIA: separate IgM and IgG immunoblots were done by test kits (MarDX Diagnostics, Carlsbad, CA) according to the manufacturer’s instructions. Immunoblots were interpreted by criteria of the Centers for Disease Control and the Association of State and Territorial Public Health Laboratory Directors [31].

Statistical analysis. For categorical variables, P values were based on the likelihood ratio test. For continuous variables that were normally distributed, we used Student’s t test or the one-way analysis of variance. If data were nonnormally distributed, we used the Mann-Whitney U test or the Kruskal-Wallis test with adjustment for ties. To control for potential bias because of the use of different blood culture methods during the course of the study, several multivariate analyses were performed. A 2-step logistic regression analysis was used to control for the method of culture in which the independent significance of RFLP type was assessed with regard to blood culture positivity. Other multivariate models were carried out in which the individual study year was regarded as a continuous variable. All tests were 2-tailed. Analysis was done with statistical packages (SPSS [version 4.0], SPSS, Chicago [32]; True Epistat [version 5.1], Epistat Services, Richardson, TX) [33]. P < .05 was considered significant.

Results

This analysis was of 104 persons with a skin biopsy specimen culture positive for B. burgdorferi of a single RFLP type. For all subjects, borrelial blood cultures were performed. All were adults who were evaluated at the Lyme Disease Diagnostic Center, Westchester Medical Center, between 1991 and 1997. Patients were excluded from this analysis if more than a single RFLP type was recovered on the culture of the skin biopsy specimen or if no blood culture was done. To establish that the individual rDNA spacer RFLP types were representative of genetically distinct genotypes, DNA sequences of the amplified rDNA spacer were determined for a group of representative isolates (3 type 1, 4 type 2, and 5 type 3). None of the spacer sequences was identical. A maximum of 19 nucleotide differences was observed between isolates within the same RFLP type, but a minimum of 36 differences was found between the most closely related isolates with different RFLP types. The phylogenetic tree depicted in figure 1 demonstrates that each RFLP type clustered as a distinct phylogenetic entity.

RFLP typing of the 104 B. burgdorferi skin isolates showed type 1 for 28 (27%), type 2 for 44 (42%), and type 3 for 32 (31%). The mean ages (± SD) of patients with infections due to RFLP types 1–3 were 44.1 ± 13.2, 44.4 ± 14.8, and 41.8 ± 12.1 years, respectively. These differences were not significant (P = .696). There was also no significant difference in the proportion of males in any of the 3 groups (P = .936).

The likelihood of having a positive blood culture was significantly associated with the RFLP type of the infecting B. burgdorferi strain (P < .001). Positive blood cultures were most frequently demonstrated with type 1 infection (43%), compared with 20% and 3% for types 2 and 3, respectively (table 1). Patients with type 1 infection were 13.8× more likely to have a positive blood culture compared with persons with type 3 infection (95% confidence interval [CI], 1.9–98.9). To control for the potential bias associated with the higher yields obtained with more recent blood culture methods, several multivariate analyses were done. After adjusting for the blood culture method used, RFLP type 1 was independently associated with blood culture positivity (P = .003). In addition, when the year of the study was controlled for as a continuous variable, RFLP type 1 was still significantly associated with blood culture positivity (P = .006).

We found a similar association between the infecting RFLP type and the presence of multiple erythema migrans lesions (P = .045; table 1). Patients with type 1 infection were 4.6× more likely to have multiple erythema migrans lesions compared with those with type 3 (95% CI, 1.1–19.8).

Serologic results. Acute- and convalescent-phase serum samples obtained 10–30 days after the acute sample were tested for B. burgdorferi antibodies for 103 patients (table 1). Persons with type 1 infection had the highest rate of seropositivity (86%), compared with subjects with type 2 (77%) and type 3 (72%) infection (P = .418).

Clinical symptoms. Clinical symptoms were assessed on the basis of a visual analogue scale (8 cm long) with severity rated from 0 (absent) to 8 (maximum severity) for each symptom. Most patients were symptomatic (76 [73%] of 104) (table 2). In general, persons with type 1 infection had more symptoms and symptoms of greater severity than those with either type 2 or 3 infection, although significance at the P = .05 level was only reached for fever/chills (P = .033). Examination at the time of diagnosis of Lyme disease revealed no significant differences by RFLP type in regard to size or duration of the primary erythema migrans lesion, oral temperature, or presence of lym-

<table>
<thead>
<tr>
<th>RFLP type (%)</th>
<th>Type 1 (n = 28)</th>
<th>Type 2 (n = 44)</th>
<th>Type 3 (n = 32)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture positive</td>
<td>43</td>
<td>20</td>
<td>3</td>
<td>.001</td>
</tr>
<tr>
<td>Multiple EM lesions</td>
<td>29</td>
<td>23</td>
<td>6</td>
<td>.045</td>
</tr>
<tr>
<td>Blood culture positive or multiple EM lesions</td>
<td>57</td>
<td>34</td>
<td>9</td>
<td>.001</td>
</tr>
<tr>
<td>Seropositive on presentation&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46</td>
<td>39</td>
<td>31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.482</td>
</tr>
<tr>
<td>Seroconversion within 30 days&lt;sup&gt;d&lt;/sup&gt;</td>
<td>75</td>
<td>63</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>.414</td>
</tr>
<tr>
<td>Total seropositive</td>
<td>86</td>
<td>77</td>
<td>72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.418</td>
</tr>
</tbody>
</table>

<sup>a</sup> Likelihood ratio test.
<sup>b</sup> Samples tested by EIA or immunoblot.
<sup>c</sup> n = 31.
<sup>d</sup> Convalescent-phase serum samples were obtained 10–30 days after acute samples. Only patients with both acute and convalescent phase samples were considered evaluable.
phadenopathy (table 2). None of the patients had objective evidence of neurologic, rheumatologic, or cardiac disease.

### Discussion

This study suggests that a major determinant of the risk for hematogenous dissemination of *B. burgdorferi* in Lyme disease patients with erythema migrans is the genetic subtype of the infecting strain in the skin. The RFLP typing system devised enabled classification of *B. burgdorferi sensu stricto* clinical isolates into 3 distinct groups. A highly significant association was found between infecting RFLP type in skin and blood culture positivity ($P < .001$). The same association existed for the occurrence of secondary erythema migrans lesions ($P = .045$; table 1). Secondary erythema migrans lesions have long been believed to occur as a result of hematogenous dissemination of *B. burgdorferi* from the site of the original tick inoculation in the skin. Thus, the observed association between RFLP type of the infecting strain of *B. burgdorferi* and the presence of secondary erythema migrans lesions provided corroboration for the blood culture results and further suggests that the RFLP type has objective clinical consequences for the patient.

A limitation of this study is that the blood culture results were obtained during the course of a series of experiments designed to determine how to optimize the yield. Since the infecting RFLP type in skin was not known at the time the blood cultures were done, it was not possible to have each RFLP type equally represented in each experiment. However, based on a retrospective analysis of these data, there was no statistically significant over- or underrepresentation of persons infected with a particular RFLP type for any given experimental blood culture method (data not shown). Although the blood culture methods have resulted in higher rates of recovery of *B. burgdorferi* in recent years, the association of RFLP type and blood culture positivity was independent of this effect. A prospective study using a single method of blood culture, however, should be carried out to confirm the present findings.

To reduce confounding, the study analyzed persons from whom only a single RFLP type was recovered from a culture of a skin biopsy specimen. However, it is unlikely that confounding was completely eliminated, since direct PCR typing of skin samples from erythema migrans lesions indicates that mixed infection with multiple RFLP types may be underestimated by culture [19]. Undiagnosed coinfection with a second RFLP type [19] or with another tickborne infection, such as human granulocytic ehrlichiosis [4], may have contributed to the overlap in clinical symptoms observed among persons infected with the different subtypes (table 2). It is also possible that most of the systemic symptoms that are reported by persons with *B. burgdorferi* infection per se and that these symptoms may occur irrespective of *B. burgdorferi* subtype.

Heterogeneity among persons infected with type 1 or 2 infection may also have contributed to the similarity in symptoms among these groups and the type 3–infected patients. For example, type 1–infected patients had the highest rate of spiro-

### Table 2. Patients’ symptoms and signs by restriction fragment length polymorphism type.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Type 1 (n = 28)</th>
<th>Type 2 (n = 44)</th>
<th>Type 3 (n = 32)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever/chills</td>
<td>50.0</td>
<td>29.5</td>
<td>18.8</td>
<td>.033a</td>
</tr>
<tr>
<td>Tingling/abnormal sensation</td>
<td>21.4</td>
<td>20.5</td>
<td>6.3</td>
<td>.136a</td>
</tr>
<tr>
<td>Joint pain</td>
<td>39.3</td>
<td>25.0</td>
<td>43.8</td>
<td>.192a</td>
</tr>
<tr>
<td>Tired/lack of energy</td>
<td>53.6</td>
<td>52.3</td>
<td>34.4</td>
<td>.216a</td>
</tr>
<tr>
<td>Headache</td>
<td>42.9</td>
<td>38.6</td>
<td>25.0</td>
<td>.292a</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>32.1</td>
<td>15.9</td>
<td>18.8</td>
<td>.257a</td>
</tr>
<tr>
<td>Dizzy</td>
<td>35.7</td>
<td>22.7</td>
<td>18.8</td>
<td>.299a</td>
</tr>
<tr>
<td>Muscle pain</td>
<td>39.3</td>
<td>31.8</td>
<td>43.8</td>
<td>.554a</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>17.9</td>
<td>9.1</td>
<td>9.4</td>
<td>.499a</td>
</tr>
<tr>
<td>Cough</td>
<td>10.7</td>
<td>11.4</td>
<td>6.3</td>
<td>.721a</td>
</tr>
<tr>
<td>Concentration or memory problems</td>
<td>20.0</td>
<td>12.2</td>
<td>11.1</td>
<td>.610a</td>
</tr>
<tr>
<td>Stiff neck</td>
<td>42.9</td>
<td>36.4</td>
<td>37.5</td>
<td>.552a</td>
</tr>
<tr>
<td>Any symptoms present</td>
<td>71.4</td>
<td>72.7</td>
<td>75.0</td>
<td>.950a</td>
</tr>
<tr>
<td>No. of symptoms, median (range)</td>
<td>4.5 (0–11)</td>
<td>3.0 (0–9)</td>
<td>2.0 (0–11)</td>
<td>.415a</td>
</tr>
<tr>
<td>Cumulative score, median (range)</td>
<td>16.0 (0–47.4)</td>
<td>7.6 (0–37.2)</td>
<td>5.4 (0–67.7)</td>
<td>.345a</td>
</tr>
<tr>
<td>Symptom severity index (n = 76), median (range)</td>
<td>3.7 (0.9–5.0)</td>
<td>3.0 (0.5–6.6)</td>
<td>2.5 (1.0–6.2)</td>
<td>.187a</td>
</tr>
<tr>
<td>Baseline temperature, °C ± SD</td>
<td>37.1 ± 0.5</td>
<td>37.2 ± 0.7</td>
<td>37.1 ± 0.7</td>
<td>.699a</td>
</tr>
<tr>
<td>EM duration, days ± SD</td>
<td>7.4 ± 7.7</td>
<td>7.0 ± 6.2</td>
<td>5.4 ± 5.3</td>
<td>.436a</td>
</tr>
<tr>
<td>EM size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, median</td>
<td>9.8</td>
<td>10.0</td>
<td>10.5</td>
<td>.965a</td>
</tr>
<tr>
<td>Width, median</td>
<td>14.8</td>
<td>16.0</td>
<td>16.0</td>
<td>.886a</td>
</tr>
<tr>
<td>Regional lymphadenopathy</td>
<td>34.5</td>
<td>25.0</td>
<td>39.4</td>
<td>.327a</td>
</tr>
</tbody>
</table>

**NOTE.** Values are percentages unless otherwise indicated. EM, erythema migrans.

- a Likelihood ratio test.
- b Kruskal-Wallis test with adjustment for ties.
- c Each symptom scored 0–8 using a visual analogue scale.
- d One-way analysis of variance.
chertemia (43%) and multiple erythema migrans lesions (29%). However, even for type 1–infected patients, more than 40% were neither spirochetal in the culture methods used in this study nor had multiple erythema migrans skin lesions. It is plausible that the spirochete typing system could be refined or improved to identify more specifically the subset of type 1 and 2 \textit{B. burgdorferi} isolates that will disseminate during the early stages of infection.

Two of the 104 subjects received an experimental recombinant OsPA vaccine preparation before they developed Lyme disease. These subjects were not excluded from this analysis, based on previously reported findings that the illness in persons in whom the vaccine fails is similar to the illness in unvaccinated persons [34]. Further reanalysis of the data excluding these 2 patients did not materially affect the results or change the conclusions (data not shown).

The properties associated with the distinct RFLP types of \textit{B. burgdorferi} that influence dissemination are not known. It should be noted, however, that the typing method used here is based on the analysis of a noncoding spacer region within the rRNA gene cluster of \textit{B. burgdorferi} [16, 19, 28, 35]. Such a region is unlikely to have a direct role in invasion or pathogenesis. However, the present analysis indicates that members of distinct RFLP spacer groups behave as distinct phylogenetic entities. Furthermore, in a separate study, a significant correspondence between rDNA spacer RFLP and two other typing methods was found (including whole genome RFLP and a plasmid typing system; R. Iyer, D. Liveris, A. Adams, I. Schwartz, unpublished data). These observations suggest that typing by rDNA spacer RFLP analysis is an accurate reflection of genomic heterogeneity among different \textit{B. burgdorferi} sensu stricto isolates.

In summary, our findings suggest that the specific genetic subtypes of \textit{B. burgdorferi} sensu stricto influence disease pathogenesis. Infection with different subtypes of \textit{B. burgdorferi} may help to explain the diversity of clinical presentations of patients with Lyme disease.

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References


