Coinfection with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* alters the course of murine Lyme borreliosis

Joppe W.R. Hovius, Xin Li, Nandhini Ramamoorthi, Alje P. van Dam, Stephen W. Barthold, Tom van der Poll, Peter Speelman & Erol Fikrig

1 Section of Rheumatology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, Connecticut, USA
2 Department of Internal Medicine, Division of Infectious Diseases, Tropical Medicine and AIDS, and Center for Experimental and Molecular Medicine, University of Amsterdam, AMC, Amsterdam, The Netherlands
3 Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands
4 Center for Comparative Medicine, University of California, Davis, California, USA
Chapter 8

Abstract

Ixodes ricinus ticks and mice can be infected with both Borrelia burgdorferi sensu stricto and Borrelia garinii. The effect of coinfection with these two Borrelia species on the development of murine Lyme borreliosis is unknown. Therefore, we investigated whether coinfection with the nonarthritogenic B. garinii strain PBi and the arthritogenic B. burgdorferi sensu stricto strain B31 alters murine Lyme borreliosis. Mice simultaneously infected with PBi and B31 showed significantly more paw swelling and arthritis, long-standing spirochetemia, and significantly higher numbers of B31 spirochetes than did mice infected with B31 alone. However, the number of PBi spirochetes was significantly lower in coinfected mice than in mice infected with PBi alone. In conclusion, simultaneous infection with B. garinii and B. burgdorferi sensu stricto results in more severe Lyme borreliosis. Moreover, we suggest that competition of the two Borrelia species within the reservoir host could have led to preferential maintenance, and a rising prevalence, of B. burgdorferi sensu stricto in European I. ricinus populations.
Introduction

Lyme borreliosis is a common tick-borne zoonosis in many parts of Asia, Europe and the United States. A main clinical feature of early human Lyme borreliosis is an expanding red cutaneous lesion called erythema migrans. Later manifestations of Lyme borreliosis include cardiac conduction system delays, arthritis, neurologic symptoms, and cutaneous manifestations, such as acrodermatitis chronica atrophicans (ACA) (Steere, 2001; Steere et al., 2004). Joint disease has been associated with *Borrelia burgdorferi* sensu stricto infection, while neuroborreliosis and ACA are associated with *Borrelia garinii* and *Borrelia afzelii* infection, respectively (van Dam et al., 1993; Busch et al., 1996). *Borrelia burgdorferi* sensu stricto is prevalent in the United States, whereas all three pathogenic *Borrelia* species are encountered in Europe. The European vector for Lyme borreliosis, *Ixodes ricinus*, can be simultaneously infected with more than one *Borrelia* species (Rijpkema et al., 1995; Hovius et al., 1998; Misonne et al., 1998; Rauter et al., 2002). Depending on the area, up to 45% of infected ticks harbour more than one *Borrelia* species and, in a study by Misonne and colleagues, the majority of coinfected ticks were simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* (Misonne et al., 1998).

The development of Lyme borreliosis is dependent on many factors, including pathogen burden (Yang et al., 1994), spirochetal virulence factors (Hughes et al., 1993; Carroll et al., 1996; Sellek et al., 2002; Yang et al., 2004), *Borrelia*-arthropod-interactions (Ramamoorthi et al., 2005), and host innate and adaptive immune responses (Matyniak and Reiner, 1995; Kang et al., 1997; Gross et al., 1998; Hirschfeld et al., 1999; Potter et al., 2000; McKisic and Barthold, 2000a; McKisic et al., 2000b; Anguita et al., 2002; Wooten et al., 2002; Wang et al., 2004; Guerau-de-Arellano et al., 2005). Moreover, co-infection of *B. burgdorferi* sensu stricto with other tick-borne pathogens, such as *Anaplasma phagocytophilum* (Thomas et al., 2001), enhances the severity of murine Lyme arthritis and increases *Borrelia* burden. The effect of co-infection of *B. burgdorferi* sensu stricto with another species from the *B. burgdorferi* sensu lato group on the outcome of murine Lyme borreliosis has not been investigated. However, in Europe the simultaneous presence of multiple *Borrelia* genospecies has been observed in both human and canine Lyme borreliosis cases (Demaerschalck et al., 1995; Hovius et al., 1999; Ruzic-Sabljic et al., 2005). In a study by Demaerschalck and collaborators, *B. burgdorferi* sensu stricto and *B. garinii* DNA was simultaneously detected in six out of 18 individuals with neuroborreliosis (Demaerschalck et al., 1995). The course of disease in mice, with the development of arthritis and carditis, partially mimics human disease (Barthold et al., 1990). In addition, infection with *B. burgdorferi* sensu stricto (strain B31), but not with *B. garinii* (strain PBi), readily causes arthritis and carditis in mice (Barthold, 1999). Here we assessed the role of co-infection with *B. burgdorferi* sensu stricto and *B. garinii* in the development of murine Lyme borreliosis.
Chapter 8

Materials and methods

Mice, spirochetes and infection

C3H/HeJ and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in filterframed cages. Six-week-old mice were infected by intradermal syringe inoculation with *B. burgdorferi* sensu stricto strain B31 clone 5A11 (Purser and Norris, 2000) and/or *B. garinii* strain PBi (Barthold, 1999). Spirochetes were cultured at 33 ºC in complete Barbour-Stoenner-Kelly (BSK)-H medium (Sigma-Aldrich, Saint Louis, MO) and grown to the stationary phase (Barthold et al., 1993). Mice were inoculated by a single injection in the midline of the back according to established protocols with 1 x 10^5 spirochetes of strain B31 or PBi alone, with 1 x 10^5 of each strain simultaneously, with 1 x 10^5 PBi spirochetes followed by 1 x 10^5 B31 spirochetes 4 days later, or with BSK-H medium as a control (Fikrig et al., 1992). Spirochetes were enumerated using a Petroff-Hauser bacterial counting chamber (Barthold et al., 1999). In a small control experiment we did not find a difference in pathogen burden, paw swelling, arthritis or immune responses when mice were simultaneously infected with 5 x 10^4 instead of 1 x 10^5 spirochetes of each strain (data not shown). Mice were euthanized by CO_2_ inhalation followed by cardiac exsanguination. Sera were stored at -20 ºC for future use. Skin (inoculation site), urinary bladder, spleen, spine, heart and tibiotarsi were saved for histopathological examination, species-specific quantitative PCR (q-PCR) or culture. We used C3H/HeJ mice to assess the effect of coinfection on the severity of Lyme borreliosis. Mice were therefore sacrificed 3 weeks postinfection, i.e. at the expected peak of arthritis. In C57BL/6 mice, arthritis usually peaks 4 weeks postinfection (Yang et al., 1994; Ma et al., 1998; Potter et al., 2000), and this strain is relatively more resistant to Lyme disease. C57BL/6 mice were sacrificed either at 2 weeks postinfection, to assess whether simultaneous or sequential infection with PBi and B31 altered the initial development of Lyme borreliosis, or late in the course of infection, i.e. at 6 weeks postinfection, to determine whether coinfection of PBi and B31 prolonged the manifestations of Lyme borreliosis.

Culture

Fifty microliter of blood and c. 20 mg of spleen, bladder and spinal cord were cultured in BSK-H medium containing rifampicin (50 mg/mL), amphotericin (2.5 mg/mL) and phosphomycin (2 mg/mL) (Sigma-Aldrich). Cultures were checked weekly for the presence of viable spirochetes using dark-field microscopy, as previously described (Barbour, 1984). The presence of at least one viable spirochete in 10 microscope fields indicated a positive culture.

Quantitative PCR

DNA was extracted from tissues using a DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR for B31 *ospC*, PBi *dbpB*, and mouse *β-actin* was performed with the primers listed in Table 1. The *ospC* and *dbpB* primers were newly designed based on the sequence of *B. burgdorferi* B31 *ospC* and *B. garinii dbpB* and are species-specific: the *ospC* primers amplify from B31 but not from PBi DNA, whereas the *dbpB* primers amplify from
B. burgdorferi and B. garinii coinfection

PBi but not from B31 DNA. Each 50-mL real-time PCR reaction contained a 1-mL DNA sample, 0.3 mL of each 50-mM primer, 23.4 mL of H2O, and 25 mL of Hot Star SYBR Green I PCR Master Mix (BioRad Laboratories, Richmond, CA). PCR was performed with an initial denaturing step of 5 min at 95 °C, followed by 50 cycles of three-step amplification consisting of 30 s at 95 °C, 30 s at 56 °C (ospC) or 60 °C (dbpB and β-actin), and 45 s at 72 °C. Standards were produced by cloning B31 ospC (397 bp), PBi dbpB (376 bp) or mouse β-actin (252 bp) PCR products into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Amplification of test samples and 10-fold dilutions of gene-specific standards were visualized and quantified using the software ICYCLER provided with the thermocycler (iCycler iQ real-time PCR detection system; Biorad Laboratories). To validate PCR products we assessed the melting temperature of the amplified fragment or performed electrophoresis on agarose gels. Similar PCR methods were used by others (Li et al., 2006). Copy numbers of B31 ospC and PBi dbpB were normalized to the copy number of β-actin. All measurements were performed in duplicate. In one separate experiment, flaB primers and a Taqman probe were used to amplify B31 DNA (Ramamoorthi et al., 2005). We did not use these primers in experiments in which we coinfected mice with both B31 and PBi: because flaB primers amplify from B31 as well as PBi DNA it would have been impossible to distinguish between B31 and PBi spirochete levels in coinfected animals.

Paw swelling, arthritis and carditis

Inflammation of tibiotarsi was evaluated by assessing the degree of oedematous swelling of the tibiotarsi. Visual inflammation scores were assessed and scored from 0 to 3, with 0 being no swelling and 3 maximal oedematous swelling (Barthold et al., 1990). Joints were scored by the same independent observer blinded to the experimental design and several times throughout the course of the infection. In addition, right tibiotarsi were fixed with formalin, embedded in paraffin, haematoxylin- and eosin-stained, and examined microscopically. Arthritis was assessed as described previously (Fikrig et al., 1992). Disease severity was scored as 0 (no disease), 1 (mild disease), 2 (moderate disease), or 3 (severe disease). Mild disease consisted of neutrophil infiltration. Moderate disease was marked by neutrophil infiltration and at least some evidence of fibrin exudation. Severe disease was marked by neutrophil infiltration with fibrin exudation and synovial hypertrophy or hyperplasia. All measurements were made in a blinded fashion. Half of

---

**Table 1. Primers (and probes) used for real-time PCR.**

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
<th>Taqman probe 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31 ospC</td>
<td>ATACCGAAAATAAT</td>
<td>CTGAATTAGCAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAACATTGGA</td>
<td>CATCTCTTTAG</td>
<td></td>
</tr>
<tr>
<td>PBi dbpB</td>
<td>TGCTGGACGCCCTG</td>
<td>TTAGCTTCCCTCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTAATTTTG</td>
<td>AAATGGAGCCTT</td>
<td></td>
</tr>
<tr>
<td>mouse β-actin</td>
<td>GGGACCTGCACAGA</td>
<td>AAGAAGGAAGGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTACCTC</td>
<td>TGGAAAG</td>
<td></td>
</tr>
<tr>
<td>B. burg flaB</td>
<td>TTGCTGATCAAGC</td>
<td>TTGAGACCCCTGA</td>
<td>CAGCTGAAGAGCT</td>
</tr>
<tr>
<td></td>
<td>TCAATATAACCA</td>
<td>AGTGATGC</td>
<td>TGGAATGCAGCCT</td>
</tr>
</tbody>
</table>

---
Chapter 8

the sagittally dissected hearts were processed and stained with haematoxylin and eosin by routine histological techniques. Carditis was considered active when acute inflammatory cell infiltrates were seen in heart base tissues (Armstrong et al., 1992).

IgG enzyme-linked immunosorbent assay and cytokine analysis

*Borrelia burgdorferi* sensu stricto (strain B31) lysates were prepared from large quantities of spirochetes cultured for 14 days. Spirochetes were pelleted at 13 000 x g, followed by three washes with phosphate-buffered saline (PBS). The pellet was resuspended in 500 mL of H2O and disrupted by sonication with five 10-s pulses. Insoluble material was pelleted at 16 000 x g for 1 min. The supernatant was collected and used for enzyme-linked immunosorbent assay (ELISA). *Borrelia burgdorferi* sensu stricto strain B31-specific total IgG and IgG subclasses were measured in sera from infected animals and controls by ELISA (Anguita et al., 1996; Thomas et al., 2001). *Borrelia burgdorferi* B31 lysate (50 ng) in 100 μL of coating buffer (0.1M sodium bicarbonate, pH 9.6) was added to 96-well ELISA plates (ICN Biochemicals Inc., Costa Mesa, CA) and kept overnight at 4 °C. Plates were washed three times with wash buffer (PBS with 0.05% Tween 20) and blocked with 200 μL of blocking buffer (10% fetal calf serum in PBS) for 2 h at room temperature. Blocking buffer was removed and 50 μL of sera (diluted 1:100) was incubated for 1 h at room temperature. The plates were washed five times with wash buffer, followed by the addition of biotinylated IgG-, IgG1-, or IgG2a-specific antibodies (Pharmingen, San Diego, CA) at a 1:2000 dilution in blocking buffer. Plates were then incubated at room temperature for 1 h and washed eight times with wash buffer. Streptavidin-conjugated horseradish peroxidase was then added to the wells and left for 45 min, followed by eight washes with wash buffer. The plates were tapped to remove excess solution, 100 μL of tetramethylbenzidine (TMB) solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added, and the reaction was stopped with 100 μL of TMB stop solution. Optical density (OD) was read at 450 nm. SHAM inoculated mouse serum was used as a negative control. Several pro-inflammatory and Th1/Th2 cytokines, namely IFN-γ, TNF-α, IL-10, IL-6, IL-12, MCP-1, IL-2, IL-4 and IL-5, were measured in serum using cytometric bead array kits (CBA; BD Biosciences) according to the manufacturer's recommendations. Measurement was performed using a fluorescence activated cell sorting (FACS) flow cytometer (BD Biosciences) and data were acquired and analysed using the CBA software (BD Biosciences). All measurements were performed in duplicate.

Statistical analysis

Differences in spirochete levels between the groups were analysed using the two-sided Mann Whitney test. For other parameters, differences between groups were analysed by two-sided one-way ANOVA, implementing a one-way ANOVA with Tukey's multiple comparison test using GRAPHPAD PRISM software (GRAPHPAD PRISM Software version 4.0, San Diego, CA). For comparison of the number of bloodculture-positive mice between the various groups, a two-sided chi-square was applied. A P value of ≤ 0.05 was considered significant.
Results

*Borrelia burgdorferi sensu stricto* and *B. garinii* burden

All C3H/HeJ and C57BL/6 mice infected with B31 alone, or simultaneously or sequentially with PBi and B31 were culture-positive. Three weeks postinfection, 4/6 C3H/HeJ mice infected with PBi alone had at least one positive tissue culture, and 2 weeks postinfection all (6/6) C57BL/6 mice infected with PBi alone had at least one positive tissue culture.

Next, we assessed whether spirochetes were also present in the blood of the mice. Two weeks postinfection, 6/8 simultaneously infected C57BL/6 mice had positive blood cultures, whereas only 1/5 of mice infected with B31 alone and 0/6 of mice infected with PBi had positive blood cultures (Table 2). In 2/4 simultaneously infected C3H/HeJ mice blood cultures were positive, whereas none of the mice infected with either B31 or PBi alone had positive blood cultures (Table 2). In a separate experiment, mice syringe inoculated with 1 x 10⁵ B31 alone had detectable spirochetes in their blood from day 4 until day 8 postinfection, as detected by culture (Fig. 1a). This indicates that, under normal circumstances, B31 spirochetes are only detectable in the blood early in infection. The time-points at which blood cultures were positive correlated with peak spirochete numbers as detected by q-PCR (Fig. 1b).

We also assessed spirochete burden in several organ tissues by q-PCR. Three weeks postinfection, C3H/HeJ mice simultaneously infected with PBi and B31 had higher levels of B31 spirochetes in several tissues than mice infected with B31 alone, as detected by B31-specific q-PCR (Fig. 2a). The difference was significant in bladder and skin (P < 0.05, Mann Whitney test). In C57BL/6 mice 2 weeks postinfection, B31 spirochete levels were higher in mice simultaneously infected with PBi and B31 in all tissues examined as compared with mice infected with B31 spirochetes alone (Fig. 2b). The differences were statistically significant for bladder and heart tissue (P < 0.05, Mann

Table 2. Blood cultures of mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains simultaneously.

<table>
<thead>
<tr>
<th>Bloodcultures</th>
<th>C3H/HeJ Pos/Tot</th>
<th>C57BL/6 Pos/Tot</th>
<th>Total Pos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBi</td>
<td>0/6</td>
<td>0/6</td>
<td>0%</td>
</tr>
<tr>
<td>B31</td>
<td>0/5</td>
<td>1/5</td>
<td>10%</td>
</tr>
<tr>
<td>Sim</td>
<td>2/4</td>
<td>6/8</td>
<td>67%*</td>
</tr>
</tbody>
</table>

C3H/HeJ and C57BL/6 mice were sacrificed three and two weeks after syringe inoculation, respectively. Mice were inoculated with PBi (PBi) or B31 (B31) alone, or simultaneously with PBi and B31 (Sim). 50 μL of blood was cultured in BSK-H medium containing antibiotics. Cultures were checked weekly for one month for viable and motile spirochetes using dark-field microscopy. The cumulative number of positive blood cultures in C3H/HeJ and C57BL/6 mice simultaneously infected with PBi and B31 was compared with the number of positive blood cultures in mice infected with B31 alone. Statistical analysis was performed using a two-sided chi-square test. * A P-value < 0.05 was considered significant.
The same trend was observed in C3H/HeJ and C57BL/6 mice sequentially infected with PBi and B31 (data not shown). We observed a similar trend in other tissues examined, such as spleen and spine (data not shown). It should be noted that, in the spine, spirochetes were present in the surrounding structures such as vertebrae, muscles and ligaments, rather than in the spinal cord (data not shown).

In contrast, the levels of PBi spirochetes in several tissues from C3H/HeJ and C57BL/6 mice infected simultaneously with PBi and B31 were significantly lower compared with levels in mice infected with PBi alone (Fig. 2c and d; \( P < 0.05 \), Mann Whitney test). In contrast to simultaneously infected mice, sequentially infected mice had relatively high PBi levels in skin and bladder tissue (data not shown), indicating that the outcome of the competition between \( B. burgdorferi \) sensu stricto strain B31 and \( B. garinii \) strain PBi in this experimental setting is dependent on the timing of inoculation.

**Paw swelling and arthritis**

During the course of infection we evaluated swelling of the hind paws as a parameter for disease. As expected, C3H/HeJ and C57BL/6 mice infected with B31 spirochetes developed significant joint swelling (Fig. 3a and b). At early timepoints, i.e. 2 or 3 weeks postinfection, swelling was more pronounced in mice simultaneously, and also sequentially, infected with PBi and B31 as compared with mice infected with either PBi or B31 alone (Fig. 3a and b). At later timepoints, i.e. 6 weeks postinfection, swelling was more severe in simultaneously infected C57BL/6 mice than in sequentially infected animals. C3H/HeJ and C57BL/6 mice infected with PBi spirochetes alone did not develop, or only developed minor, swelling (Fig. 3a and b). We did not infect mice with B31 followed by PBi spirochetes. We speculate that this type of sequential infection does not influence...
B. burgdorferi and B. garinii coinfection

the outcome of Lyme borreliosis symptoms, because the host immune response against PBi evoked in the presence of B31, at least in simultaneous infection of these two Borrelia species, readily kills PBi. In the mouse model, B31 is arthritogenic and PBi is not.

In order to assess whether swelling could be correlated with disease we investigated haematoxylin-and eosin-stained sections of tibiotarsal joints microscopically. At three weeks postinfection, arthritis was significantly more severe in C3H/HeJ mice infected simultaneously with PBi and B31 than in mice infected with B31 alone (Fig. 4a; \( P < 0.05 \), one-way ANOVA with Tukey’s multiple comparison test). At 6 weeks postinfection there was a trend towards more severe arthritis in C57BL/6 mice simultaneously infected with B31 and PBi as compared with mice infected with B31 alone, albeit not statistically significant (Fig. 4b; \( P > 0.05 \), one-way ANOVA with Tukey’s multiple comparison test). Two weeks postinfection there was no arthritis in any group of infected C57BL/6
In both C3H/HeJ and C57BL/6 mice, sequential infection with PBi and B31 did not result in more severe arthritis (Fig. 4a and b). Carditis was evident in C3H/HeJ mice infected with B31 (4/4), and in those simultaneously (3/4) or sequentially (3/5) infected with PBi and B31, but not in mice infected with PBi alone (0/6).

**Immune responses**

In search of an explanation for our findings we determined total IgG antibodies directed against *Borrelia burgdorferi* sensu stricto strain B31 in mouse sera. The results were similar for all groups of C3H/HeJ and C57BL/6 mice (Table 3). However, in C57BL/6 mice simultaneously infected with PBi and B31, OD values of IgG1 directed against B31 were increased and OD values of IgG2a were decreased, as compared with mice infected with B31 alone (Table 3). Antibodies from PBi-infected animals also cross-reacted with B31 antigens (Table 3). Besides determining antibody responses we determined systemic cytokine responses, but we found no relevant differences in serum cytokine profiles between the various groups of mice (data not shown). Despite very sensitive detection methods (CBA), TNF-α, IL-4, IL-5, IL-6, IL-10 and IL-12 were below detection limits for all groups.
**B. burgdorferi and B. garinii coinfection**

The European Lyme borreliosis vector, *I. ricinus*, can transmit more than one Borrelia genospecies through a single tick bite. Misonne and colleagues found that the majority of coinfected ticks were simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* (Misonne et al., 1998). Demaerschalck and collaborators showed that 33% of patients with neuroborreliosis were infected with both *B. burgdorferi* sensu stricto and *B. garinii* (Demaerschalck et al., 1995). Despite the frequent simultaneous occurrence of these two Borrelia species in ticks and human Lyme borreliosis cases, the role of coinfection with *B. garinii* and *B. burgdorferi* sensu stricto in the pathogenesis of Lyme borreliosis has never been investigated in an experimental setting.

In mice simultaneously infected with PBi and B31, there is long-lasting spirochtemia (Table 2). Interestingly, PCR analysis of in vitro positive blood cultures showed that, in simultaneously infected mice, the species identified was *B. burgdorferi* sensu stricto strain B31 (data not shown). This is unlikely to be the result of the preferential growth of B31 over PBi in vitro, because, when equal numbers, i.e. 1 x 10^5 spirochetes, of B31 and PBi were simultaneously cultured in BSK-H medium for two weeks, both strains grew to a density of c. 1 x 10^7 spirochetes as detected by q-PCR (data not shown). We have shown that in (C3H/HeJ) mice infected with B31 alone, spirochetes are only detectable in the blood during the initial stages of infection (Fig. 1a). However, in C57BL/6 mice simultaneously infected with PBi and B31, but not in mice infected with B31 alone, B31 spirochetes could be readily detected in the blood at 2 weeks postinfection (Table 2). Despite

**Figure 4.** Arthritis in mice infected with *Borrelia burgdorferi* B31, *Borrelia garinii* PBi, or both *Borrelia* strains. C3H/HeJ (a) and C57BL/6 (b) mice were syringe-inoculated with 1 x 10^5 spirochetes per strain. Mice were infected with PBi (PBi), B31 (B31), PBi and B31 simultaneously (Sim), or PBi followed by B31 four days later (Seq). Five to six mice per group were used. Haematoxylin- and eosin-stained sections from tibiotarsi from C3H/HeJ mice 3 weeks postinfection and C57BL/6 mice 6 weeks postinfection were examined microscopically for disease. Disease severity was scored on a scale of 0-3: 0, no disease; 1, mild disease; 2, moderate disease; and 3, severe disease. Error bars represent SDs. Arthritis from mice infected simultaneously or sequentially with PBi and B31 was compared with arthritis in mice infected with B31 alone. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test (a *P* value < 0.05 was considered significant).

**Discussion**

The European Lyme borreliosis vector, *I. ricinus*, can transmit more than one Borrelia genospecies through a single tick bite. Misonne and colleagues found that the majority of coinfected ticks were simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* (Misonne et al., 1998). Demaerschalck and collaborators showed that 33% of patients with neuroborreliosis were infected with both *B. burgdorferi* sensu stricto and *B. garinii* (Demaerschalck et al., 1995). Despite the frequent simultaneous occurrence of these two Borrelia species in ticks and human Lyme borreliosis cases, the role of coinfection with *B. garinii* and *B. burgdorferi* sensu stricto in the pathogenesis of Lyme borreliosis has never been investigated in an experimental setting.
the fact that C3H/HeJ mice infected simultaneously with PBi and B31 were sacrificed 3 weeks postinfection, we could still detect spirochetes in the blood (Table 2).

Coinfection of PBi and B31 results in higher B31 spirochete levels in several tissues of C3H/HeJ and C57BL/6 mice (Fig. 2). In contrast, PBi levels were significantly lower in mice coinfected with PBi and B31 than in mice infected with PBi alone. Thus, there appears to be competition of the two strains within the murine host, resulting in higher B31 spirochete levels and diminished PBi spirochete levels. It has previously been shown that nymphal *I. ricinus* ticks fed upon mice naturally infected with *B. garinii* and *B. burgdorferi* sensu stricto preferentially take up *B. burgdorferi* sensu stricto (Kurtenbach et al., 1998). Originally, this was explained by differential complement sensitivity of the two *Borrelia* species (Kurtenbach et al., 1998). However, competition between two complement-resistant *Borrelia* strains within the host, as in our study, could be an additional explanation for this phenomenon. This competition and preferential uptake of one *Borrelia* species over the other by ticks feeding on coinfected mice could contribute to the preferential maintenance of *B. burgdorferi* sensu stricto in European *I. ricinus* populations. This would increase the risk of human infection with this *Borrelia* species over time. Genomic analysis by Baranton and collaborators suggests that *B. burgdorferi* sensu stricto might have originated in the United States of America and migrated to Europe in the post-Columbian era (Marti et al., 1997; Farlow et al., 2002). Thus, sporadic introductions of *B. burgdorferi* sensu stricto into the ‘Old World’ are expected, and it is not remarkable that the prevalence of *B. burgdorferi* sensu stricto in Europe varies from 0% (Junttila et al., 1999) to c. 20% (Kirstein et al., 1997) of infected questing ticks. Furthermore, we suggest that local high prevalences of *B. burgdorferi* sensu stricto in Europe might be explained by competition of *B. burgdorferi* sensu stricto with other *Borrelia* species within the reservoir host followed by preferential uptake of *B. burgdorferi* sensu stricto by *I. ricinus* ticks.

the fact that C3H/HeJ mice infected simultaneously with PBi and B31 were sacrificed 3 weeks postinfection, we could still detect spirochetes in the blood (Table 2).

Coinfection of PBi and B31 results in higher B31 spirochete levels in several tissues of C3H/HeJ and C57BL/6 mice (Fig. 2). In contrast, PBi levels were significantly lower in mice coinfected with PBi and B31 than in mice infected with PBi alone. Thus, there appears to be competition of the two strains within the murine host, resulting in higher B31 spirochete levels and diminished PBi spirochete levels. It has previously been shown that nymphal *I. ricinus* ticks fed upon mice naturally infected with *B. garinii* and *B. burgdorferi* sensu stricto preferentially take up *B. burgdorferi* sensu stricto (Kurtenbach et al., 1998). Originally, this was explained by differential complement sensitivity of the two *Borrelia* species (Kurtenbach et al., 1998). However, competition between two complement-resistant *Borrelia* strains within the host, as in our study, could be an additional explanation for this phenomenon. This competition and preferential uptake of one *Borrelia* species over the other by ticks feeding on coinfected mice could contribute to the preferential maintenance of *B. burgdorferi* sensu stricto in European *I. ricinus* populations. This would increase the risk of human infection with this *Borrelia* species over time. Genomic analysis by Baranton and collaborators suggests that *B. burgdorferi* sensu stricto might have originated in the United States of America and migrated to Europe in the post-Columbian era (Marti et al., 1997; Farlow et al., 2002). Thus, sporadic introductions of *B. burgdorferi* sensu stricto into the ‘Old World’ are expected, and it is not remarkable that the prevalence of *B. burgdorferi* sensu stricto in Europe varies from 0% (Junttila et al., 1999) to c. 20% (Kirstein et al., 1997) of infected questing ticks. Furthermore, we suggest that local high prevalences of *B. burgdorferi* sensu stricto in Europe might be explained by competition of *B. burgdorferi* sensu stricto with other *Borrelia* species within the reservoir host followed by preferential uptake of *B. burgdorferi* sensu stricto by *I. ricinus* ticks. The

<table>
<thead>
<tr>
<th>C3H/HeJ</th>
<th>SHAM Mean</th>
<th>SD</th>
<th>B31 Mean</th>
<th>SD</th>
<th>Sim Mean</th>
<th>SD</th>
<th>Seq Mean</th>
<th>SD</th>
<th>PBi Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>total IgG</td>
<td>0.15</td>
<td>0.02</td>
<td>0.38</td>
<td>0.04</td>
<td>0.37</td>
<td>0.04</td>
<td>0.40</td>
<td>0.04</td>
<td>0.33</td>
<td>0.03</td>
</tr>
<tr>
<td>IgG1</td>
<td>0.08</td>
<td>0.00</td>
<td>0.19</td>
<td>0.05</td>
<td>0.15</td>
<td>0.03</td>
<td>0.17</td>
<td>0.02</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>IgG2a</td>
<td>0.06</td>
<td>0.00</td>
<td>0.17</td>
<td>0.03</td>
<td>0.15</td>
<td>0.04</td>
<td>0.15</td>
<td>0.03</td>
<td>0.12</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C57BL/6</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>total IgG</td>
<td>0.07</td>
<td>0.01</td>
<td>0.42</td>
<td>0.04</td>
<td>0.39</td>
<td>0.03</td>
<td>0.38</td>
<td>0.02</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>IgG1</td>
<td>0.07</td>
<td>0.00</td>
<td>0.18</td>
<td>0.01</td>
<td>0.24*</td>
<td>0.06</td>
<td>0.19</td>
<td>0.03</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>IgG2a</td>
<td>0.07</td>
<td>0.00</td>
<td>0.30</td>
<td>0.03</td>
<td>0.23*</td>
<td>0.05</td>
<td>0.20*</td>
<td>0.02</td>
<td>0.14</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Mice were infected with BSK-H-medium (SHAM), strain B31 (B31), strain PBi (PBi), strain PBi and B31 simultaneously (Sim), and strain PBi followed by strain B31 four days later (Seq). OD values of total IgG, IgG1 and IgG2a directed against B31 were determined in sera three (C3H/HeJ) or six (C57BL/6) weeks post-infection by enzyme-linked immunosorbent assay using whole B31 lysate as an antigen. Sera were diluted 1:100 and OD was read at 450 nm. Antibody responses in mice infected simultaneously or sequentially with PBi and B31 were compared with antibody responses in mice infected with B31 alone. Statistical analysis was performed using one-way analysis of variance with Tukey’s multiple comparison test. *A P value < 0.05 was considered significant.

Table 3. IgG antibodies against *Borrelia burgdorferi* sensu stricto strain B31 in C3H/HeJ and C57BL/6 mice.
different strains of *B. burgdorferi* sensu stricto, *Borrelia afzelii* and *B. garinii* that exist in Europe and the timing of the coinfection may influence the final outcome of this competition.

Simultaneous infection with PBi and B31 spirochetes also alters systemic immune responses. C57BL/6 mice show higher OD values of IgG1 and lower OD values of IgG2a levels directed against B31 6 weeks after simultaneous infection with B31 and PBi, as compared with mice infected with B31 spirochetes alone (Table 3). However, the altered IgG responses do not seem to be important for the differences in disease severity between the different groups of infected mice, because no difference in IgG1 and IgG2a responses was observed in C3H/HeJ mice (Table 3). We could not find relevant differences in levels of several cytokines in sera from the different groups of infected mice (data not shown). It is noteworthy that MCP-1 levels in sera from C3H/HeJ mice simultaneously infected with PBi and B31 (74.4 pg/mL ± 16.9) were significantly higher than MCP-1 levels in mice infected with B31 alone (31.4 pg/ml ± 9.2). However, because PBi-infected mice that did not develop arthritis or carditis had similar MCP-1 levels (58.6 pg/mL ± 9.6) as compared with coinfected mice, we could not associate increased MCP-1 levels with arthritis or carditis. In contrast, Guerau-de-Arellano and collaborators have shown that MCP-1 production by dendritic cells following *B. burgdorferi* sensu stricto stimulation is likely to contribute to the development of Lyme carditis (Guerau-de-Arellano et al., 2005).

Thus, the exact mechanism of the competition between the two *Borrelia* species remains unknown. We show that in single infection, PBi and B31 have, at least in part, tropisms for similar tissues, i.e. skin, bladder, joints and heart tissue. However, when injected simultaneously, B31 dominates PBi resulting in higher B31 spirochete levels and in lower PBi spirochete levels. The competition of the two *Borrelia* species is unlikely to be caused by competition for host factors, such as receptors on synovial tissue such as decorin, fibronectin, or integrins, because these are abundantly present in the host (Coburn et al., 2002). However, the relative affinity for these receptors might differ between *Borrelia* species, but, to our knowledge, this is currently unknown (Heikkila et al., 2002). The competition might also be driven by the immune response of the host. We focussed on the adaptive host immune response and were unable to find relevant differences in systemic cytokine production and IgG antibody production. Perhaps a more local approach, i.e. restimulating local lymphocytes with *Borrelia* antigen, might have revealed differences in cytokine responses between the experimental groups. Alternatively, altered innate immune responses in coinfected animals might have been the cause of the competition between the two *Borrelia* species. An adequate innate immune response is essential for clearance of *B. burgdorferi* (Wang et al., 2004). In mice coinfected with PBi and B31, innate immune responses might be mostly triggered by, and directed at, PBi, resulting in clearance of PBi, and to a lesser extent at B31, allowing for higher B31 spirochete levels. Thus, PBi would serve as a decoy for B31. Another explanation could be differences in resistance to complement-mediated killing (Brooks et al., 2005). Although both *Borrelia* strains are known to be partially serum-resistant, coinfection could have changed complement activation in a way that favours the persistence of the more complement-resistant *B. burgdorferi* strain B31 (Breitner-
Chapter 8

Ruddock et al., 1997; van Dam et al., 1997). Finally, Liang and collaborators have shown that *B. burgdorferi* changes its surface antigenic expression in response to host immune responses (Liang et al., 2004). It is possible that, in our studies, the altered immune response in coinfected mice changed the expression of proteins important for early infection of, and persistence in, the host, for example OspC, dbpA and VlsE in PBi and/or B31.

Regardless of the underlying mechanism, we have shown that simultaneous, and to a lesser extent sequential, infection with *B. burgdorferi* sensu stricto and *B. garinii* alters the course of murine Lyme borreliosis. Paw swelling was more pronounced in C3H/HeJ mice simultaneously and sequentially infected with B31 and PBi than in mice infected with B31 spirochetes alone (Fig. 3a). In C57BL/6 mice, paw swelling persisted longer in simultaneously infected mice (Fig. 3b). In C3H/HeJ mice, arthritis was significantly more severe in mice simultaneously infected with PBi and B31 than in mice infected with B31 alone (Fig. 4a). We have also shown that these coinfected mice have higher B31 spirochete levels in several tissues. This implies that higher overall B31 spirochete levels result in more local oedema formation and more neutrophil influx and inflammation in tibiotarsal joints. Apparently, oedema formation and immune cell influx are not caused solely by high levels of just any *Borrelia* strain, because we have clearly shown that mice infected with *B. garinii* strain PBi, despite high PBi spirochete levels in the joint, do not develop arthritis, a result that was to be expected (Barthold, 1999). In our studies, coinfected mice showed higher B31 spirochete levels in various tissues; however, B31 spirochete levels were not significantly higher in tibiotarsal joints. The increased inflammation we have observed in coinfected mice could be the result of an altered immune response by the host caused by coinfection of B31 and PBi. Others have postulated that in Lyme borreliosis, as in other systemic diseases, local precipitation of immune complexes in the joint contributes to inflammation (Hardin et al., 1984). In C3H/HeJ mice, sequential infection of PBi and B31 resulted in more pronounced paw swelling, but not arthritis. Others have previously shown that swelling and arthritis do not always coincide in murine Lyme borreliosis (Anguita et al., 1996).

In summary, this study shows that coinfection of *B. burgdorferi* sensu stricto and *B. garinii* alters the outcome of murine Lyme borreliosis. Coinfection is likely to occur in Europe and could be of medical importance because animals, and possibly also humans, simultaneously infected with *B. burgdorferi* sensu stricto and *B. garinii* could develop more severe Lyme borreliosis symptoms. Coinfection could also be of ecological importance, because, in nature, competition between *B. burgdorferi* sensu stricto and *B. garinii* within the animal host could lead to preferential maintenance of *B. burgdorferi* sensu stricto in European *I. ricinus* populations.
Acknowledgments

The authors are grateful to Dr K.E. Hovius for discussions on the ecological implications of our findings, to K.A. de Groot for his assistance with the graphics, and to V. Thomas for thoroughly reviewing the manuscript and making useful suggestions. The authors are also grateful to Debby Deck for her excellent technical assistance. J.W.R.H. received support from the Infectious Disease Society of the Netherlands. This work was also supported by grants from the National Institutes of Health.
References


Chapter 8