Arthritogenicity of *Borrelia burgdorferi* and *Borrelia garinii*: Comparison of Infection in Mice

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Abstract. Arthritogenicity, as determined by joint swelling and synovial histology, was compared between or within two *Borrelia* genospecies that cause Lyme arthritis in humans. The spirochete burden in bladder tissue (a site of spirochete persistence) was documented by quantitative polymerase chain reaction, and immune responses were analyzed. In C3H/HeJ mice, three *B. burgdorferi* isolates and two of the three *B. garinii* isolates induced severe arthritis and swelling. Previous designation as invasive or noninvasive *B. garinii*, or RNA spacer type of *B. burgdorferi* did not determine arthritis severity induced by isolates. Compared with the other five isolates, the *B. garinii* PBi isolate induced significantly less arthritis, a lower humoral immune response, and persisted at a much lower level in bladder tissue. However, *B. garinii* PBl isolates induced similar *Borrelia* antigen-specific inflammatory T cell responses from the local draining lymph node.

Thus, diverse *B. burgdorferi* and *B. garinii* isolates were highly arthritogenic in immune competent mice.

INTRODUCTION

*Borrelia burgdorferi* is the only cause of Lyme disease in the United States, whereas *B. garinii* and *B. afzelii* are the cause of most Lyme borreliosis in Europe and Asia. Although all three species have been associated with Lyme arthritis in mice or humans,

The three genospecies seem to differ in the frequency of certain manifestations and persistence at particular sites. *Borrelia garinii* is especially neurotropic, *B. afzelii* may persist for long periods in the skin, and *B. burgdorferi* seems to be particularly arthritogenic. For example, in one U.S. study of 55 untreated patients with erythema migrans, 28 (51%) developed intermittent attacks of arthritis, and 6 (11%) developed chronic synovitis.

In contrast, in a Swedish study, only 1 of 16 untreated patients with erythema migrans developed brief arthritis. Although largely descriptive, this early experience raised the possibility of significant differences between genospecies in arthritogenicity.

A well-established mouse model exists for Lyme arthritis, but severe arthritis in immune competent mice infected with *B. garinii* has not been reported. After *B. burgdorferi* infection, C57BL/6 mice develop mild or no arthritis, BALB/c mice develop moderate arthritis, and C3H/He mice develop severe arthritis. Susceptibility was shown to be heritable and dominant. Although a tick isolate of *B. garinii* was able to induce arthritis in severe combined immunodeficient mice, arthritis was reported to be inconsistent and mild in immune competent C3H/He mice infected with *B. garinii* Pbi. Moreover, the occurrence of arthritis was not investigated in a previous study of *B. garinii* infection using multiple isolates in immune competent mice.

The RNA spacer type (RST) of *B. burgdorferi* has been associated with disseminated infection in human Lyme disease and in murine model systems. When mice were infected by needle inoculation, RST 1 strains reached greater densities in the blood, were present for longer durations, and caused greater organ system involvement than RST 3 strains. Of 22 *B. garinii* groups defined by the outer surface protein C (ospC) gene sequence, 9 contained all isolates from human blood or tissue and only 4 groups contained all isolates from disseminated human sites of infection.

Designation of invasive or noninvasive groups of *B. garinii* was then based on human tissue origin or relatedness of ospC sequences to patient isolates.

Previous investigations of experimental Lyme arthritis have delineated protective and pathogenic immune responses that determine outcomes of infection. Primed T cells may help prevent or resolve arthritis as part of an adaptive immune response that results in antibodies to spirochetes. Conversely, studies have also suggested a pathogenic role for T cells in acute Lyme arthritis. Thus, *Borrelia* spp. induces adaptive immune responses that may control the infection or contribute to arthritogenicity.

This study compared the arthritogenicity of genetically diverse *B. burgdorferi* or *B. garinii* isolates. *Borrelia afzelii* isolates were not tested because a study of infection of monkeys with commonly used isolates showed *B. afzelii* at high levels in the skin, but not at sites of dissemination. These available isolates were therefore considered unlikely to cause experimental Lyme arthritis. We found that three tested isolates of *B. burgdorferi*, and two of three *B. garinii* isolates, induced severe arthritis. All isolates that induced marked joint swelling and inflammation persisted at high levels in bladder and also stimulated antibodies to spirochetes.

MATERIALS AND METHODS

Isolates. RNA spacer type of *B. burgdorferi* isolates was determined in our laboratory using previously published methods. Prior designation of *B. garinii* isolates as invasive or noninvasive was based on the isolate’s site of origin and relatedness of ospC sequences. Reference isolates *B. burgdorferi* N40 (tick, U.S., RST 3) and *B. garinii* PBI (human cerebrospinal fluid [CSF], Germany, invasive) were kindly provided by Dr. Stephen Barthold (University of California, Davis, CA). *Borrelia burgdorferi* ACS02 (human erythema migrans, U.S., RST 1) and *B. burgdorferi* 297 (human CSF, U.S., RST 2) were kindly provided by Dr. Allen Steere (Massachusetts General Hospital, Boston, MA). *Borrelia garinii* JEM3 (human erythema migrans, Japan, noninvasive) and JEM5 (human erythema migrans, Japan, unknown) were kindly provided by Drs. Michael Sean Metts and Richard Marconi (Medical College of Virginia/Virginia Commonwealth University, Richmond, VA). Only isolate ACS02 was known to be low
passage. Soluble antigen was prepared from N40 and PBi isolates, as described previously, for use in an enzyme-linked immunosorbent assay (ELISA) and T cell activation assays.

**Infection of mice.** BALB/c and C3H/HeJ mice were obtained at 3–5 weeks of age from the Jackson Laboratory (Bar Harbor, ME) and were housed in the barrier containment facility in the Division of Laboratory Animal Medicine at Tufts-New England Medical Center. All experimental protocols involving mice were reviewed and approved by the Institutional Animal Care and Use Committee. The maintenance and care of laboratory animals complied with the National Institutes of Health guidelines for the human use of laboratory animals.

High-passage isolates frequently lose their ability to infect mice. In our experience, passage through mice restored virulence to our cloned *B. burgdorferi* N40 isolate after multiple in vitro passages. Therefore, to optimize pathogenicity, *Borrelia* spp. were re-isolated from mouse tissues and these low-passage isolates were frozen into aliquots for single experiments in Barbour-Stoenner-Kelly (BSK) II (Sigma, St. Louis, MO) with 30% glycerol. This method will work only if the isolate contains a subpopulation of virulent organisms.

To infect mice, frozen *Borrelia* spp. were thawed rapidly, transferred into 10 volumes of BSK II, and cultured at 32°C until they achieved 75% motility. At that time, spirochetes were enumerated using dark field microscopy. Mice were inoculated in the right hind footpad with 1 × 10^4 organisms of each isolate in 50 µL of BSK II, except for *B. garinii* PBi. There were a number of difficulties in working with this isolate. *Borrelia garinii* PBi had a slower post-thaw recovery and required a longer incubation time (4 days) to achieve 75% motility than the other five isolates (1 day). Because PBi-infected mice often did not develop arthritis (Figure 1) and sometimes did not have polymerase chain reaction (PCR) evidence of spirochete persistence in bladder, they were inoculated with more organisms (5 × 10^4). This amount was the lowest dose of *B. garinii* PBi to at least reproducibly induce seroconversion in all recipient mice. Infection with a higher dose (10^5 organisms) did not induce significantly more severe disease. Isolate PBi might be genetically attenuated (see Discussion). Control mice were mock infected with BSK II alone.

**Joint swelling.** Tibiotarsal joint swelling was measured on a weekly basis with a spring-loaded micropipeter (Federal, Providence, RI). Swelling in the contralateral (left) joint occurred 1–2 weeks after that in the right joint in mice with severe swelling. Thus, the diameters of the right and left joints were added for presentation. Swelling was measured for five weeks to observe persistence.

**Joint inflammation.** After mice were humanely killed at 5 weeks post-infection, the right tibiotarsal joint was fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA). Fixed tissue was decalcified, sectioned, and stained with hematoxin and eosin (Suzanne White, Beth Israel Medical Center, Boston, MA). We independently scored each section in a blinded manner from 0 (no arthritis) to 4 (severe arthritis) based on relative accumulation of infiltrating cells, proliferation of synovial lining, and destruction of cartilage and bone, if any. The final score was the average of the two scores.

**Isolation of DNA and quantitative PCR.** *Borrelia* spp.-infected and mock-infected mice were killed at 5 weeks post-infection and the bladder was stored at −80°C until DNA extraction was performed. The PCR was performed in a spectrophurometeric thermal cycler (MX4000; Stratagene, La Jolla, CA). The final reaction concentration was 1 × Brilliant SYBR Green master mix (Stratagene), 3 mM MgCl₂, 0.25 µM of each primer, and 200 ng of target DNA. The primers used to detect spirochetal *recA* were nTM17F (5'-GTG GAT CTA TTT TAT TAG ATG AGG CTC TCG-3') and nTM17R (5'-GCC AAA GTT CTT CAA CAT TAA CAC TTA AAG-3'). The primers used to detect the mouse *nidogen* gene were nido.F (5'-CCA GCC ACA GAA TAC CAT CC-3') and nido.R (5'-GGA CAT ACT CTG CTT CCA TC-3'). The thermal profile conditions used were 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Emitted fluorescence for each reaction was measured three times during the annealing/extension phase, and amplification plots were analyzed using the MX4000 software version 3.0 (Stratagene). A series of *Borrelia* DNA standards was prepared using the QI Amp DNA Mini Kit (Qiagen, Valencia, CA). The DNA concentration of the standard sample was used to calculate *Borrelia* genome equivalents (1 µg/mL DNA = 6.1 × 10^10 genomes/µL) that were then diluted into DNA from an uninfected mouse to make a standard curve. Because it has been shown that 200 ng of mouse DNA contains approximately 10^4 *nidogen* copies, the number of *recA* copies amplified was normalized to this number, as previously described. *Borrelia* species-specific controls were used to determine *recA* copies for the *B. garinii* and *B. burgdorferi*-infected mice.

**Enzyme-linked immunosorbent assay.** *Borrelia*-specific IgG or IgM antibody in the sera of the mice was tested by ELISA as previously described, using *Borrelia* species-specific soluble antigen for the *B. garinii* or *B. burgdorferi* infected mice. Mice were considered to have a positive ELISA result if the optical density at 450 nm was greater than the mean plus three times the standard deviation of values in uninfected control mice. All infected C3H/HeJ mice were seropositive at 5 week post-infection.

**T cell activation and flow cytometry.** Draining lymph nodes were harvested from C3H/HeJ mice 10–14 days post-infection, at the onset of measurable arthritis (Figure 1A). In preparation for these experiments, irradiated (2,200 rads) spleen cells from mock-infected syngeneic mice were pulsed with *B. burgdorferi* N40 or *B. garinii* PBi soluble antigen (10 µg/mL final concentration) for 60 minutes and washed twice with complete RPMI 1640 medium (BioWhittaker, Walkersville, MD) with 10% heat-inactivated fetal calf serum, 5 × 10⁻³ M 2-mercaptoethanol (both from Sigma), 2× minimal essential medium vitamin solution, 1 mM sodium pyruvate (both from Invitrogen Life Technologies, Grand Island, NY), 1× non-essential amino acids, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (all from BioWhittaker) before use as antigen-presenting cells. Single-cell suspensions from pooled lymph nodes were washed with complete RPMI 1640 medium and plated at a concentration of 5 × 10⁴ cells/well in 96-well plates in the presence of 5 × 10⁴ antigen-pulsed irradiated antigen-presenting cells. After 4–5 days of *in vitro* restimulation, cells were activated for the final 5 hours of culture with soluble antibodies to CD3 (1 µg/mL) and antibodies to CD28 (1 µg/mL) (both from BD Pharmingen, San Diego, CA), in the presence of 3 µM monensin (Sigma) to retard cytokine export from the Golgi. Intracellular cytokine and cell surface marker analyses were performed by flow cytometry, as previously described. Cells that were CD8+ and interferon-γ (IFN-γ)+ were counted as activated. Few lymph node cells and virtually no CD8+ T cells from mock-infected mice survived the culture period, even in the presence of antigen.
RESULTS

Joint swelling induced by infection with *B. burgdorferi* and *B. garinii* reference isolates. Joint swelling was first compared among mice after infection with *B. burgdorferi* N40, the U.S. tick isolate that is used most commonly to study experimental Lyme arthritis, or with *B. garinii* PBi, the genome-sequenced human CSF isolate from Germany that has been used as a reference isolate. Both BALB/cJ and C3H/HeJ mouse strains were initially included because C3H/HeJ mice might be too susceptible to detect differences in arthritogenicity between the *Borrelia* isolates. As shown previously,25 C3H/HeJ and BALB/cJ mice infected with *B. burgdorferi* N40 had significant swelling, and although infected BALB/cJ mice had less swelling, it was...
still significantly more than swelling in mock-infected mice (Figure 1A and B). The swelling was apparent by two weeks and peaked at four weeks for both mouse strains. When infected with B. garinii PBi at a five-5-fold higher dose than with B. burgdorferi N40, C3H/HeJ and BALB/cJ mice developed ankle swelling that was only significantly greater than swelling in mock-infected mice at isolated time points (Figure 1A and B). Thus, B. burgdorferi N40 induced more severe swelling in both mouse strains than B. garinii PBi. Because C3H/HeJ mice did not develop severe swelling after infection with B. garinii PBi, the BALB/cJ mice were not included in subsequent experiments.

**Swelling induced by infection with other isolates of each Borrelia species.** To determine if observations with the two reference isolates reflected Borrelia species-specific differences, arthritis-susceptible C3H/HeJ mice were infected with two additional genetically diverse isolates of each *Borrelia* species. RNA spacer type 1 of *B. burgdorferi* has been associated with more frequent or severe disseminated infection in murine model systems and human disease. Similarly, *B. garinii* isolates have been described as invasive or noninvasive based on tissue of origin, or origin of genetically similar isolates based on *ospC* sequence. *Borrelia burgdorferi* ACS02, a low-passage U.S. erythema migrans isolate, *B. burgdorferi* 297, a U.S. CSF isolate, or *B. garinii* JEM3 or JEM5, isolated in Japan from patients’ erythema migrans lesions, were inoculated (10⁴/mouse) into recipients and swelling was measured longitudinally. Results were compared with swelling induced by the two reference isolates described above.

All *B. burgdorferi* isolates, representing RST 1 (ACS02), RST 2 (297), or RST 3 (N40) isolates, induced severe swelling in the recipient mice (Figure 1C and D). *Borrelia garinii* JEM5 caused severe swelling, JEM3 induced an intermediate amount of swelling, and PBi induced mild swelling in the recipient mice. **Histopathologic analysis.** Edema and infiltration of immune cells into joint tissue are under separate genetic control in mice. When C3H/HeJ ankle histopathology was compared, all *Borrelia* isolates tested, except *B. garinii* PBi, induced significant joint changes by 5 weeks post-infection (Figure 2). These changes included cellular infiltration into muscle, tendon, synovium, and the joint space, and erosion of bone and cartilage. Each of the *B. burgdorferi* isolates and *B. garinii* JEM3 and JEM5 isolates induced severe arthritis, and PBi induced mild arthritis. Ankle histopathology score was significantly associated with ankle swelling for each group (P = 0.005, by Pearson correlation).

**Spirochete burden.** In mice, spirochetes are known to replicate and persist at high levels in the bladder, serving as a marker for spirochete persistence. DNA was collected from bladder tissue of infected mice and subjected to qPCR analysis for genomic spirochetal DNA. Although spirochete burden varied over several log copies/10⁵ murine genomes, as observed by Morrison and others, all *B. burgdorferi* isolates, as well as *B. garinii* JEM3 and JEM5, replicated to a significantly higher level in the bladder than *B. garinii* PBi (Figure 3). This finding suggested that *B. garinii* PBi induced less arthritis than the other isolates because of a limit in the replication or persistence of the organism in vivo. Nonetheless, when results for *B. garinii* PBi were excluded, there was only a trend towards an association between joint swelling and spirochete burden.

**Humoral immunity.** Observation of spirochete dissemination and persistence in the host results from the intrinsic capacity of the spirochete combined with success in evading the immune system. It was possible that the more arthritogenic isolates elicited less adaptive immunity in the host. As shown in Figure 4, mice infected with each of the *B. burgdorferi* and *B. garinii* isolates mounted a significant anti-spirochetal humoral immune response. However, the response of mice is depicted in Figure 3. Quantitative polymerase chain reaction (qPCR) analysis of bladder tissue for *Borrelia* spp. *recA*. Bladders were removed from mice five weeks after *Borrelia* spp. or mock infection and DNA was analyzed by qPCR for murine glyceraldehyde 3-phosphate dehydrogenase and *Borrelia* spp. *recA*. Results are presented as the ratio of *Borrelia* spp. genomes per 10⁵ mouse cells to normalize for differences in sample size. Results for individual mice (black circles) and median and third quartile values (boxes) are presented. No *recA* copies were detected in bladders from mock-infected mice. *Borrelia burgdorferi* N40, 297, and ACS02-infected, and *B. garinii* JEM3- and JEM5-infected mice had significantly greater arthritis (P ≤ 0.01). *Borrelia garinii* PBi infection induced only slight arthritis in some mice that was not significantly different from background.
infected with *B. garinii* PBi was lower than that mounted by mice infected with the other two *B. garinii* or the three *B. burgdorferi* isolates. There was no significant correlation between IgG titer and joint swelling or arthritis severity, or IgG titer and spirochete numbers in bladder. Thus, severe arthritis was not associated with a failure to mount an immune response against the spirochete.

**Cellular immunity.** CD8+ T cells in C3H/He mice seem to contribute to inflammation after *B. burgdorferi* infection, possibly by producing large amounts of the inflammatory cytokine IFN-γ. When the T cell responses of C3H/HeJ mice infected with *B. burgdorferi* N40 or *B. garinii* PBi were compared, both isolates activated antigen-specific CD8+IFN-γ+ T cells (Table 1). It is not possible to elicit these cells from lymph node mononuclear cells from uninfected mice. Thus, infection with either of these *Borrelia* species induced inflammatory T cells that did not correlate with severity of joint swelling or arthritis.

**DISCUSSION**

A predominance of descriptive studies and lack of direct comparisons have left gaps in our understanding of what makes a *Borrelia* spirochete arthritogenic. Although strict differences in tissue tropism have been described for isolates of *B. turicatae*, the related spirochetal species that causes relapsing fever, *B. burgdorferi* and *B. garinii* sometimes cause neurologic complications and arthritis in human patients. Although previous studies determined that *B. garinii* induced severe arthritis in SCID mice and that *B. garinii* PBi did not induce arthritis in immune competent mice, the only studies to compare murine infection with other *B. garinii* isolates did not study arthritis. The current study demonstrates unequivocally that *B. garinii* can induce severe arthritis in immune-competent mice. Furthermore, *B. burgdorferi* isolates uniformly induced severe swelling and joint inflammation in mice when compared with a range of outcomes of infection with the *B. garinii* isolates.

We selected isolates in an effort to span the known genetic diversity of the two species, including one isolate of each *B. burgdorferi* RST type and *B. garinii* isolates from different groups based on relatedness of *ospC* sequences. The *B. garinii* isolates were previously described as invasive (PBi) or noninvasive (JEM3) and derived from a wide geographic range including Germany and Japan. We included *Borrelia* isolates from tick, human skin, or CSF (a disseminated site). It is possible that comparing more isolates would have identified a *B. burgdorferi* isolate from the United States or Europe that would not induce severe arthritis. However, this study clearly shows that all RST groups of *B. burgdorferi* and a previously designated noninvasive *B. garinii* isolate can be highly arthritogenic. Fingerprinting studies have shown that *B. garinii* is the most genetically heterogeneous of the *Borrelia* species causing Lyme disease. In a direct comparison of *B. burgdorferi*, *B. garinii*, and *B. afzelii* isolates, *B. burgdorferi* N40 and 297 replicated to higher levels in outbred Swiss-Webster mice. Moreover, in nonhuman primates, these two *B. burgdorferi* isolates induced more inflammation than any of the tested *B. garinii* isolates. This finding agrees with our experience that joint swelling with this species was more variable. It is possible that examination of a larger number of isolates, different inoculation routes, or infectious dose might show differences between the genospecies.

One limitation is the possibility of attenuation of the isolates caused by variable passage number. In the present study, *B. garinii* PBi exhibited poor growth *in vitro* and impaired arthritogenicity, despite efforts to adjust experimental conditions to optimize *B. garinii* PBi arthritogenesis. This finding suggests that it grew slowly or persisted less well *in vivo*. When tested in a recall assay, this isolate induced inflammatory IFN-γ production as well as *B. burgdorferi* N40. Thus, the lack of arthritis induced by this isolate was unlikely to stem from a loss of inflammatory capacity. Rather, the data suggest that this isolate had become genetically attenuated. Thus, high-passage PBi may not be characteristic of the original low-passage isolate, and it might be a poor choice for those interested in studying *B. garinii*.

Because immunity plays a role in controlling arthritis susceptibility, it was important to consider the possible role of different immune responses in the arthritogenicity of the two *Borrelia* genospecies tested here. An adaptive humoral response occurred in response to all six *Borrelia* isolates, although it was lower in some of the *B. garinii* PBi-infected mice. In this case, arthritis was not inversely related to the intensity of the immune response. This finding does not exclude differences at a finer specificity, for example differences in innate responses or recognition of specific antigens contributing to disease resolution. An arthritogenic isolate of *B. afzelii* will probably be identified, although available data suggests that this is uncommon. Some studies have identified *B. garinii* and even *B. afzelii* by PCR analysis of synovial fluid samples from German patients with Lyme arthritis. In another German study, *B. burgdorferi* predominated in patients with Lyme arthritis, but was not identified in patients with neuroborreliosis or acrodermatitis.

### Table 1

| CD8+ T cell responses in C3H/HeJ mice after a two-week infection with *Borrelia burgdorferi* N40 or *B. garinii* PBi and five days restimulation with isolate-specific soluble antigen | CD8+ interferon-γ+ T cells, % |
|---|---|---|
| Isolate | No antigen | Borrelia spp. antigen | Ratio |
| *B. burgdorferi* N40 | 7.0 | 13.0 | 1.9 |
| *B. garinii* PBi | 8.2 | 22.8 | 2.8 |

**Figure 4.** Antibody titers against *Borrelia burgdorferi* N40 or *B. garinii* PBi soluble antigen in mouse sera five weeks post-infection measured by enzyme-linked immunosorbent assay. Infection with each of the six isolates tested induced seroconversion in C3H/HeJ mice (P ≤ 0.01). Results from median and third quartile values are presented.
chronic atrophicans. Similarly, in a consecutive series of 10 patients with Lyme arthritis from northeastern France, B. burgdorferi DNA was identified in nine synovial fluid samples and B. garinii in only one sample. In comparison, only B. garinii or B. afzelii DNA was identified in 10 consecutive erythema migrans samples. Thus, B. burgdorferi seems to be consistently arthritogenic, but B. garinii and perhaps B. afzelii may also cause arthritis.

Received June 6, 2008. Accepted for publication October 6, 2008.

Acknowledgments: We thank Dr. Allen C. Steere for advice during the study and in preparing the manuscript for publication.

Financial support: This study was supported by a research grant from the Harold G. and Leila Y. Mathers Foundation (Lisa J. Glickstein) and a training award (T32 RA07570) from the National Institutes of Health (Kathleen A. Craig-Mylius).

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