Increased severity of disease and persistence of symptoms have been recently reported in some patients with simultaneous infection of *Borrelia burgdorferi* and *Babesia microti* in the northeastern and northern midwest United States. This study used a murine model to examine whether defined disease conditions such as arthritis and carditis differed in severity in mice infected solely with *B. burgdorferi* and in mice coinfected with *B. microti* and *B. burgdorferi*. C3H.HeJ and BALB/c mice cohorts were coinfect ed or singly infected and then monitored experimentally for 15 and 30 days after inoculation. Carditis and arthritis was determined by blinded histopathologic evaluation of myocardium and tibiotarsal joints. Cytokine measurements were made on lymph node and spleen supernatants for interferon-γ, interleukin (IL)-4, IL-10, and IL-13. No differences were observed for C3H.HeJ mice cohorts; however, coinfect ed BALB/c mice had a significant increase in arthritis severity at day 30. This clinical observation was correlated with a significant reduction in expression of the cytokines IL-10 and IL-13.

In the northeastern and northern midwest United States, *Babesia microti* infection is commonly diagnosed in residents of many areas where Lyme disease is endemic [1, 2]. The systemic presentation of Lyme disease and babesiosis can overlap significantly with nonspecific symptoms of fever, fatigue, and malaise [2]. In severe cases of babesiosis, hemolytic anemia, lasting from several days to a few months, may occur, most commonly in asplenic or elderly hosts. However, most cases of human babesiosis in normosplenic immunocompetent persons are probably subclinical and occur as a self-limiting illness [1, 2].

Active coinfection of *Borrelia burgdorferi*, *B. microti*, and a newly described organism related to *Bartonella vinsoni* is a feature of white-footed mouse reservoir (*Peromyscus leucopus*) [3]. However, in humans, direct proof of simultaneous infections has been difficult to demonstrate, because seroreactivity to multiple organisms can arise by sequential, not necessarily simultaneous, infection. Recently published seroprevalence studies suggest that seroreactivity to both *B. microti* and *B. burgdorferi* is not associated with unusually severe presentations of Lyme disease [4]. However, because both organisms are present in the same areas, seroprevalence studies do not identify coincident infection but rather a cumulative risk of exposure for pathogens that each give rise to sustained seroreactivity [5]. In contrast, Krause et al. [6] showed that simultaneous or closely sequential infections resulted in clinically apparent differences in symptom severity. Detection of *B. burgdorferi* DNA in blood by polymerase chain reaction (PCR) appeared to be more common in patients who were actively coinfect ed, but the number of cases analyzed was too small to establish statistical significance [6]. Because of the difficulty in providing direct evidence of simultaneous infection, we used a mouse model to address the proposed synergistic pathogenic effect associated with coinfection. In this article, we present evidence of a mouse strain–dependent increased arthritis severity in a murine model of coinfection.

**Materials and Methods**

**Mice.** Inbred 4–5-week-old BALB/c and C3H.HeJ mice were purchased from Jackson Laboratory and housed at the Mayo Clinic animal facility.

**Babesia.** The *B. microti* MN1 strain originally isolated from a human patient in Minnesota was inoculated in golden Syrian hamsters, and the blood was cryopreserved in liquid nitrogen for later use. When an experiment was performed, cryopreserved blood was inoculated in a hamster, and blood was collected when parasitemia reached ∼80%. This infected blood was utilized for inoculation in mice.

**Spirochetes.** *B. burgdorferi* strain N40 (originally obtained...
from S. Barthold) was used in all experiments. Frozen aliquots of low-passage N40 were thawed and grown to log phase in 10 mL of modified Barbour-Stoenner-Kelly (BSK II) medium in plastic screw-top tubes at 32°C, prior to each experiment. Spirochetes were visualized to assess viability and counted by darkfield microscopy before inoculation into mice.

**Experimental infection of mice.** The mice (5–6 weeks old) were infected intradermally by syringe inoculation behind the left shoulder with 10^7 spirochetes per animal in 100 μL of BSK II. Coinfected animals were also inoculated intraperitoneally with 10^7 B. microti–infected hamster erythrocytes. Fifteen or 30 days after inoculation, animals were killed, and regional draining lymph nodes and spleens were harvested for cytokine analysis. At both time points, the infection status was assessed by culture of ear tissue in 10 mL of BSK II medium at 32°C for 3 weeks. The presence of viable spirochetes was assessed by darkfield microscopy at the end of the culture period. *B. microti* infection confirmation was done by visualization of the parasite on Giemsa-stained blood smears or by PCR from blood collected before the selected time points (10 days after inoculation) [7].

*B. burgdorferi*-specific IgG isotype ELISA. Ninety-six-well polystyrene plates were coated with whole-cell lysates (1 μg/well) and kept at −20°C until further use. Plates were thawed and then blocked by the addition of 1% bovine serum albumin–PBS-Tween solution for 1 h at 37°C. The cells were then washed extensively (PBS–Tween 20). Duplicate samples of murine serum (90 μL/well; 1:150) were applied to the plate and incubated for 1 h at 37°C. Goat anti–mouse IgG1 and IgG2a, each diluted 1:4000 and linked to horseradish peroxidase (Southern Biotechnology Associates), were added to each well, incubated for 1 h at 37°C, and washed. We then added 100 μL of ABTS peroxidase (KPL) substrate and monitored reactions at 405 nm. The reactions were stopped with SDS-containing buffer.

**Cytokine analysis.** Lymph nodes and spleens were individually processed into single-cell suspensions in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Lymph node cells and splenocytes were washed with RPMI 1640 medium, resuspended in RPMI 1640 medium with 10% FCS and supplements at 10^7 cells/mL, and aliquoted into 96-well (total volume, 200 μL) tissue culture plates. Lymph node cells and splenocytes were stimulated with *B. burgdorferi* sonicate at a final concentration of 25 μL/mL or with an equivalent volume of PBS. Supernatants were harvested at 72 h, and interleukin (IL)-4, IL-10, IL-13, and interferon (IFN)-γ were measured by sandwich ELISA, as specified by the manufacturer (PharMingen).

**Histopathology.** Tibiotarsal joints and hearts were fixed in neutral-buffered formalin (pH 7.2), demineralized (joints), and then processed and stained with hematoxylin-eosin by routine histologic techniques. Joints were blindly scored for arthritis severity on a scale of 0 (negative) to 3 (severe), on the basis of the degree of leukocyte infiltration and synovial proliferation, as described elsewhere [8]. Differences in mean values of arthritis severity in the different experiments were analyzed by Student 2-tailed test. Myocardial specimens were evaluated blindly.

**Quantitative *B. burgdorferi* detection by competitive PCR.** *B. burgdorferi* DNA was quantitated as described elsewhere [8]. In brief, DNA was purified from preweighed mouse ear tissue by use of a modified QIAamp tissue kit protocol (Qiagen). A 256-bp region within the *B. burgdorferi* flagellin gene was used as a genomic target for amplification and quantitation. The PCR products generated during the amplification process were detected with a modified PCR ELISA detection kit (Roche Molecular Biochemicals) [8].

**Results**

We infected C3H. HeJ and BALB/c cohorts of 15 mice each with *B. burgdorferi* and *B. microti* alone and in combination. Cardiac and tibiotarsal joint tissue was assessed 15 and 30 days after inoculation. These time points were selected to evaluate peak Lyme arthritis activity [9], as well as peak *B. microti* parasitemia, which occurs ~15 days after inoculation. As observed in previous studies, BALB/c mice infected with *B. burgdorferi* alone developed mild arthritis at 15 and 30 days, reaching maximum (albeit low) arthritis severity scores 15 days after inoculation. In contrast, the severity of arthritis was significantly increased for the coinfected BALB/c group at day 30 (table 1; P < .05). Carditis was present in both groups with equal severity.

C3H.HeJ mice developed moderate-to-severe arthritis with *B. burgdorferi* alone, and no further increase in severity was noted.

To determine whether the increase in arthritis severity in BALB/c mice correlated with an alteration in immune responses to *B. burgdorferi*, we analyzed cellular immune responses at days 15 and 30 against *B. burgdorferi* whole cell antigen sonicates. IL-4, IL-10, IL-13, and IFN-γ were measured as indicators of Th helper (Th) cell responses in splenocytes and lymph node cells. Of interest, we observed a marked and statistically significant (P < .05) decrease of IL-10 and IL-13 in coinfect BALT/c mice 30 days after inoculation, compared with levels in mice infected with *B. burgdorferi* alone (figure 1). Consistent with the histopathologic findings, no significant differences in cytokine levels were observed for C3H.HeJ mice at any time point or in BALB/c mice at day 15. In addition, no significant differences were detected between the groups for IL-4 and IFN-γ cytokines.

To determine whether the differences in cytokine levels were related to antibody isotype switching, we measured *B. burgdorferi* IgG1 and IgG2a isotypes. Lower IgG1 titers were observed in the coinfect cohort of BALB/c mice (optical density, 0.2; SE, of a modified QIAamp tissue kit protocol (Qiagen). A 256-bp region within the *B. burgdorferi* flagellin gene was used as a genomic target for amplification and quantitation. The PCR products generated during the amplification process were detected with a modified PCR ELISA detection kit (Roche Molecular Biochemicals) [8].

<table>
<thead>
<tr>
<th>Mice cohort</th>
<th>Mean severity of arthritis</th>
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<tbody>
<tr>
<td></td>
<td>Day 15</td>
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<tr>
<td>Coinfected</td>
<td>0.9 ± 0.5</td>
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<tr>
<td><em>B. burgdorferi</em> only</td>
<td>1.0 ± 0.3</td>
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**NOTE.** Data are averaged scores (±SD) on a scale of 0–3 of both tibiotarsal joints. Ten mice were analyzed per cohort. The difference was statistically significant (P < .05, 2-tailed test).

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Table 1. Comparison of arthritis severity in BALB/c mice infected with *Borrelia burgdorferi* only or coinfecte with *B. burgdorferi* and *Babesia microti* on days 15 and 30 after infection.
Figure 1. Antigen-specific cytokine production by regional lymphoid organs and spleen in Borrelia burgdorferi–Babesia microti coinfected and B. burgdorferi–only infected BALB/c mice at day 30 of infection. A, Interleukin (IL)–10; B, IL-13. LN, lymph node; SP, spleen.

Discussion

Previous work indicated that the type of Th cell response mounted against B. burgdorferi influences arthritis but not carditis [10]. T cells from arthritis-resistant BALB/c mice usually produce IL-4 and develop a Th2 bias after infection with B. burgdorferi [11]. In contrast, T cells from arthritis-susceptible C3H.HeJ mice generally produce IFN-γ and develop a Th1 dominant phenotype after infection with B. burgdorferi. Treatment of BALB/c mice with antibody to IL-4 increases the severity of arthritis, although treatment of C3H.HeJ mice with either antibody to IFN-γ or recombinant IL-4 reduces arthritis development [11]. Recent reports, however, have indicated that these cytokines may not be absolutely required for disease modulation.

Our data clearly indicate an increase in Lyme arthritis severity in a coinfected BALB/c cohort at 30 days, a time point when arthritis scores usually decrease significantly for this mouse strain. These observations are consistent with an antecedent reduction in IgG1 levels (e.g., a reduction in the Th2-dependent IL-4 driven antibody subclasses) and significant decreases in levels of IL-10 and IL-13, suggesting alteration of the Th cell response.

Th1 cytokine responses are thought to dominate in B. microti infections—at least during acute infection [12]. IFN-γ has been reported to have some role in the resolution of acute infection of mice infected with B. microti and to be involved in protection against other intracellular parasites. It is believed to operate through activation of macrophages and NK cells, which interfere with the development of the parasite within the host erythrocytes, perhaps via activation of innate immune responses [13]. It is quite possible that the immune response to the babesial infection in BALB/c mice could have a nonspecific adjuvant type effect relative to the immune response to other infections, including B. burgdorferi. Recent studies of B. burgdorferi and Anaplasma coinfection suggest an immunologic interaction that ultimately enhances pathogenicity of B. burgdorferi [14]. These effects may have a significant impact on the persistence of B. burgdorferi and the immunologic selective pressure it is subjected to. We did not observe an increase in B. microti parasitemia as a function of coinfection, but B. burgdorferi infection might conversely affect immune responses to B. microti.

Our results did not show a marked increase of IFN-γ for coinfected BALB/c mice, compared with B. burgdorferi–infected mice, probably because of the strong IL-4 cytokine response and corresponding down-regulation of IFN-γ characteristic of B. burgdorferi infections in BALB/c mice. Despite this, we detected significant down-regulation in the anti-inflammatory cytokine IL-10. IL-10 has a major influence on the degree of inflammation and severity of arthritis in B. burgdorferi–infected mice [15]. Thus, it is quite possible that the effect we observed of increased arthritis activity in BALB/c mice is due not as much to increased IFN-γ production as to diminished IL-10 production.

With regard to human infection with B. burgdorferi and B. microti, it is likely the timing of infection with both organisms is the most critical factor in determining whether there is a clinical synergistic effect. Sequential infections spaced months or years apart might be expected to have little, if any, synergistic effect, whereas simultaneous infections might be expected to interact at an immunologic level. Our data support the concept that the adjuvant effects of one tickborne infection can affect the course of another, at least in certain genetically susceptible hosts. Further studies of immunogenetic loci in humans infected with these organisms may shed light onto the possible mechanisms involved.
Acknowledgment

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References