Coinfection with *Borrelia turicatae* Serotype 2 Prevents the Severe Vestibular Dysfunction and Earlier Mortality Caused by Serotype 1

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**Background.** Relapsing fever (RF) is a multisystemic spirochetal infection caused by different *Borrelia* species. Studies in our laboratory have shown that disease severity varies depending on the infecting serotype. However, the relative contribution of each serotype to pathogenesis during mixed infections is not known. To investigate this, we compared the outcome of infection with isogenic serotypes 1 (Bt1) or 2 (Bt2) of the RF agent *B. turicatae* alone or in combination.

**Methods.** B cell–deficient mice were used for these experiments, to avoid serotype clearance by the host’s variable membrane protein–specific antibodies. Observers masked to infection status examined infected and uninfected control mice for clinical disease and functional impairment for up to 65 days.

**Results.** All mice developed persistent infection with the serotypes with which they were originally inoculated. Severe vestibular dysfunction developed in mice infected with Bt1 alone and was associated with increased morbidity and mortality. However, coinfection with Bt2 significantly reduced the severity of vestibular dysfunction and prevented earlier mortality. In contrast, coinfection with Bt1 had little effect on the severe arthritis caused by Bt2 infection.

**Conclusions.** The manifestations of infection with *B. turicatae* are significantly influenced by the combination of serotypes present during mixed infection.

Relapsing fever (RF) is a multisystemic spirochetal infection caused by different *Borrelia* species [1]. In North America, RF is caused by *B. hermsii* in the mountainous West and by *B. turicatae* in the Southwest. The hallmark of RF is several febrile periods and spirochetemia separated by periods of well-being resulting from antigenic variation of the spirochetes in the blood. Antigenic variation is explained by differential expression of variable membrane proteins (VMPs), the immunodominant outer-membrane variable major lipoproteins that are the target of the host’s serotype-specific antibody response [2]. More recently, VMPs have been classified as variable small proteins (Vsps) and variable large proteins (Vlps) on the basis of size and sequence homology [3].

Studies from our laboratory have indicated that VMPs may also be determinants of disease severity and tissue tropism [4]. In mice with severe combined immunodeficiency (*scid* mice), which are B and T cell deficient, we found significant differences in the clinical manifestations of persistent infection with isogenic serotypes 1 (Bt1) and 2 (Bt2) of the RF agent *B. turicatae* [4]. Bt2, defined by expression of the 20-kDa Vsp2, causes higher spirochetemia and neonatal mortality as well as more-severe arthritis, whereas Bt1, defined by expression of the 23-kDa Vsp1, is more neurotropic [4–6]. Because the only detectable difference between Bt1 and Bt2 is their Vsp, this suggests that another consequence of VMP variation is modulation of clinical disease [5–7]. However, most RF *Borrelia* infections...
occur with a mixture of serotypes at the same time [4, 8]. To investigate the virulence of individual serotypes during mixed infection, we compared the clinical and functional consequences of persistent infection with Bt1 or Bt2 alone and in combination. B cell–deficient mice were used, to avoid clearance by the host’s VMP-specific antibody response. The results revealed that the pathogenicity of an individual serotype could be significantly influenced by other serotypes simultaneously present during mixed infection.

METHODS

Strains and culture conditions. Bt1 (formerly serotype A) and Bt2 (formerly serotype B) of B. turicatae have been described elsewhere [4–6, 9]. Clonal populations of both serotypes were cultured in BSK II medium with 12% rabbit serum at 34°C [1] and were counted in a Petroff-Hauser chamber under phase-contrast microscopy [8]. Plasma from infected mice was inoculated into BSK cultures. The identity of the serotypes was assessed before and after infection by Western blotting with serotype-specific monoclonal antibodies [5, 6].

Mouse infections. Groups of eight 4-week-old female mice were inoculated intraperitoneally with B. turicatae spirochetes in 300 μL of PBS. Three types of B cell–deficient mice were used: (1) mice with B and T cell deficiency due to the scid mutation (CB17/lcr–prkdc scid/Crl; Charles Rivers; hereafter, “scid mice”); (2) mice with B and T cell deficiency due to a mutation of the recombination-activating gene 1 (B6.129S7-Rag1tm1Mom; JaxMice; hereafter, “Rag1tm1Mom; JaxMice”); and (3) mice with B cell deficiency only, due to a mutation of the μ chain (B6.129S2-Igh-6tm1Cgn; JaxMice; hereafter, “Igh6−/− mice”). Swiss Webster outbred mice were used as a positive control in a CXCL13 ELISA. Mice sham inoculated with PBS as a negative control. An examiner masked to infection status inspected the scoring scale was 0 (none), 1 (mild twitching when held by the tail), 2 (strong twitching but less than a full circle), and 3 (spinning in circles). The masked examiner also scored tibiotarsal joints as 0 (no swelling), 1 (mild swelling), 2 (moderate/severe swelling), and 3 (moderate/severe swelling plus redness). Mice were weighed 7 days before inoculation and 32 and 65 days after.

Functional studies. scid mice were trained on a stationary horizontal plexiglass beam 48 and 24 h before test trials, as described elsewhere [4]. Test trials consisted of 2 complete crossings of the beam. For each crossing, 2 functional measures were obtained: traversing time in seconds and the number of times the hind legs slipped off the beam. The examiner was masked to infection status. Functional testing was limited to 4 mice/group (1 cage) because of time constraints.

Tissue and fluid collection. Necropsies were performed as described elsewhere [10]. Whole blood was centrifuged for 5 min at 7000 g, and 200 μL of plasma was inoculated into BSK II culture tubes. All plasma cultures were examined by phase-contrast microscopy and SDS-PAGE. CXCL13 ELISA was performed as described elsewhere [11], using a commercial kit (MCX130; R&D Systems) with plasma diluted at 1:50.

Statistical analysis. One-way analysis of variance (ANOVA) for independent samples was used to compare changes in clinical and functional scores over time between groups. χ2 or Fisher’s exact tests were used to compare differences in percentages, and t tests were used to compare individual means. Correlation coefficients were calculated using Pearson’s method. P < .05 was considered to be significant.

RESULTS

Vestibular dysfunction in mice persistently infected with Bt1 or Bt2 alone or in combination. Previous studies have shown that scid mice infected with B. turicatae for up to 1 month develop prominent vestibular dysfunction beginning 3 weeks after inoculation and that this may occur more often with Bt1 than with Bt2 [4]. We began our study of the effects of mixed infection on clinical disease by examining vestibular function. For this, groups of 8 scid mice were inoculated with 1 × 104 Bt1 or Bt2 spirochetes or with the same total number of spirochetes (1 × 105) but with half of them being Bt1 (5 × 104) and the other half being Bt2. Another group of 8 scid mice was sham inoculated with PBS as a negative control. An examiner masked to infection status (E.G.) performed clinical examination and functional testing every 1–3 days for up to 10 weeks. Mice underwent necropsy 10 weeks after inoculation or earlier if they became very ill. Cultures of blood obtained during necropsy showed that all mice inoculated with B. turicatae developed persistent infection with the serotypes with which they were originally inoculated. This is consistent with the previous finding that serotype switching is not apparent during infection of scid mice with RF spirochetes for several weeks [4, 9, 12].

Clinical examination showed that none of the uninfected mice developed any signs of vestibular dysfunction for the entire observation period. In contrast, clear signs developed in all infected groups, notably head tilt and spinning in the air when lifted off the ground by the tail (figure 1). Earlier during the infection, from days 12–25 after inoculation, vestibular dysfunction was more severe in mice infected with Bt2 alone or coinfected with Bt1 than in mice infected with Bt1 alone: the mean vestibular scores were 1.44 (95% confidence interval [CI], 1.23–1.66) for Bt2 alone and 1.37 (95% CI, 1.13–1.60) for Bt1
in combination with Bt2, compared with 0.64 (95% CI, 0.40–0.87) for Bt1 alone (P<.0001, ANOVA) (figure 2). However, beginning ∼25 days after inoculation, the vestibular dysfunction became increasingly more severe in mice infected with Bt1 alone and remained so until their death, which occurred either spontaneously (n = 4) or by euthanasia (n = 4) 21–46 days after inoculation. The mean vestibular scores for days 25–46 after inoculation were 2.23 (95% CI, 1.90–2.55) for Bt1 alone, 1.12 (95% CI, 0.90–1.33) for Bt2 alone, and 1.11 (95% CI, 0.86–1.35) for Bt1 in combination with Bt2 (P<.0001, ANOVA). Unlike the group infected with Bt1 alone, vestibular dysfunction significantly improved beginning 29 days after inoculation in mice infected with Bt2 alone or in combination with Bt1 (figure 2). Remarkably, for a period of ∼12 days between days 36 and 48, the vestibular dysfunction completely resolved in these 2 groups (figure 2). However, 50 days after inoculation, it relapsed and continued with fluctuating severity until death, which occurred spontaneously (n = 1/group) or by euthanasia (n = 7/group) 61–65 days after inoculation. We concluded that persistent infection with Bt1 caused severe vestibular dysfunction. However, coinfection with Bt2 prevented this from happening.

Because scid mice are both B and T cell deficient, it was not clear whether T cells could ameliorate or worsen vestibular dysfunction during persistent infection with Bt1. To investigate this, we inoculated 2 groups of 8 C57BL/6 mice—one with a targeted mutation of the recombination-activating gene 1 (Rag1<sup>−/−</sup> mice, which are both B and T cell deficient) and one with a targeted mutation of the μ chain (Igh6<sup>−/−</sup> mice, which are B cell deficient only)—with either 10<sup>6</sup> Bt1 spirochetes or PBS as negative control. We used 10 times fewer spirochetes to investigate whether the severity of vestibular dysfunction was influenced by the original inoculum. A masked observer (H.G.) examined all mice every 2–3 days for up to 5 weeks for signs of vestibular dysfunction. The results showed that both the Rag1<sup>−/−</sup> and Igh6<sup>−/−</sup> mice infected with Bt1 but that none of the PBS-inoculated control mice developed severe vestibular dysfunction over time. We concluded that B cell deficiency causes increased susceptibility to vestibular dysfunction in RF borreliosis independently of T cells.

**Functional impairment in scid mice persistently infected with Bt1 or Bt2 alone or in combination.** Next, we investigated whether coinfection with Bt2 reduced the functional impairment caused by persistent infection with Bt1. For this, we analyzed the performance on an equilibrium bar of groups of 4 scid mice persistently infected with Bt1 or Bt2 alone or in combination or sham inoculated with PBS as negative control. We measured both the number of foot slips per crossing of the bar and the number of seconds needed to cross the bar. A masked examiner (E.G.) tested all mice every 1–3 days for up to 65 days. The results showed that all infected mice had more foot slips and longer crossing times than did the uninfected control mice for the entire observation period (P<.0001, ANOVA) (figure 3). During the period from day 12 to day 22 after inoculation, when the clinical vestibular scores were higher in mice infected with Bt1 alone or in combination with Bt1, we found that functional impairment was also worse in these groups: the mean number of foot slips was 1.9 (95% CI, 1.2–2.6) for Bt2 alone, 1.3 (95% CI, 0.6–2.1) for Bt1 in combination with Bt2, and 0.7 (95% CI, 0–1.4) for Bt1 alone (P = .05, ANOVA). The corresponding values for crossing times (in seconds) were 21.3 (95% CI, 17.4–25.3) for Bt2 alone, 19.5 (95% CI, 15.5–23.4) for Bt1 in combination with Bt2, and 10.0 (95% CI, 5.9–14.1) for Bt1 alone (P = .0002, ANOVA).

However, during the second period of infection (days 25–46 after inoculation), when vestibular dysfunction was more severe in mice infected with Bt1 alone, we found that these...
mice were significantly slower crossing the bar: the mean crossing time (in seconds) was 21.0 (95% CI, 18.0–24.2) for Bt1 alone, 14.0 (95% CI, 11.3–16.6) for Bt2 alone, and 10.6 (95% CI, 8.0–13.3) for Bt1 in combination with Bt2 (P < 0.0001, ANOVA). Unlike crossing times, we found no differences in the number of foot slips during this period: 4.2 (95% CI, 3.1–5.3) for Bt1 alone, 4.2 (95% CI, 3.3–5.1) for Bt2 alone, and 3.5 (95% CI, 2.6–4.4) for Bt1 in combination with Bt2 (not significant by ANOVA). We concluded that persistent infection with Bt1 resulted in severe functional impairment after 3 weeks that was significantly reduced by coinfection with Bt2.

The finding that crossing times but not the number of foot slips worsened during the second period of infection with Bt1 suggested that crossing time was a better functional measure of vestibular dysfunction than foot slips. To investigate this, we calculated the correlation of vestibular dysfunction with foot slips and crossing times. The results showed a higher value for crossing times than for foot slips: 0.38 versus 0.31 for days 12–22 after inoculation and 0.50 versus 0.42 for days 25–46 after inoculation (P < 0.01, for both comparisons; ANOVA). These results confirmed that crossing time was a better measure of vestibular dysfunction than foot slips.

Arthritis in scid mice persistently infected with Bt1 or Bt2 alone or in combination. The correlation coefficients indicated that other aspects besides vestibular dysfunction were contributing to functional impairment in infected scid mice. It was likely that one such contributing factor was tibiotarsal arthritis [4, 13]. To investigate this, we analyzed the severity of clinical arthritis that had been recorded by the same masked examiner (E.G.) in the same mice studied for vestibular dysfunction. The results showed that all infected mice but none of the uninfected control mice developed signs of tibiotarsal arthritis. Furthermore, there were significant differences between mice infected with Bt2 alone or in combination with Bt1 and mice infected with Bt1 alone: the mean clinical arthritis scores for the groups infected with Bt2 alone or in combination with Bt1 during the first month of infection were 0.96 (95% CI, 0.87–1.05) and 1.06 (95% CI, 0.96–1.16), respectively, compared with 0.33 (95% CI, 0.26–0.40) for the group infected with Bt1 alone (P < 0.0001, ANOVA). The corresponding scores for uninfected control mice were 0.03 (95% CI, 0.01–0.05) (P < 0.0001, for the differences with any of the infected groups; ANOVA). During the second month of infection, arthritis persisted in mice infected with Bt2 alone or in combination with Bt1 but completely resolved in mice infected with Bt1 alone (figure 4); the mean clinical arthritis score was 0 for both uninfected control mice and mice infected with Bt1 alone, compared with 0.90 (95% CI, 0.75–1.05) and 1.04 (95% CI, 0.90–1.19) for mice infected with Bt2 alone or in combination with Bt1, respectively (P < 0.0001, ANOVA). Measurement of the tibiotarsal joints with a caliper confirmed that there was more swelling in mice infected with Bt2 alone or in combination with Bt1 than in mice infected with Bt1 alone (data not shown). These results revealed significantly more tibiotarsal arthritis during the first month of infection with Bt2 that was not influenced by coinfection with Bt1. We concluded that coinfection with Bt2 suppressed the severe vestibular dysfunction caused by Bt1 but that coinfection with Bt1 did not influence the severe arthritis caused by Bt2.

To investigate the extent to which tibiotarsal arthritis contributed to functional impairment on the equilibrium bar, we calculated the correlation coefficients for foot slips and crossing times. The results showed that the correlation coefficient for days 12–25 after inoculation was 0.23 for foot slips and 0.11 for crossing times. The corresponding values for days 25–46 were 0.25 and 0.30. These correlation coefficients were higher for foot slips than for crossing times for days 12–25, when mice had more arthritis (figure 4) than vestibular dysfunction (figure 2). However, the overall values were lower than for vestibular dysfunction.

Weight change in scid mice persistently infected with Bt1 or Bt2 alone or in combination. Previous studies have shown that another consequence of persistent infection with B. turicatae in scid mice is changes in weight. We investigated the
Figure 3. Performance in an elevated narrow-beam walking test of scid mice persistently infected with *Borrelia turicatae* serotypes 1 (Bt1) or 2 (Bt2) alone or in combination and in control mice that were sham inoculated with PBS. Mice were tested by an examiner masked to infection status every 1–3 days for up to 65 days. Results are shown as the mean no. of foot slips for each crossing of the bar (*A* and *B*) or as the mean no. of seconds needed to cross the bar (*C* and *D*) per group during the first (*A* and *C*) and second (*B* and *D*) months of infection. Each group had 4 mice.

degree to which coinfection influenced weight change. We measured weight change by calculating the mean percent weight change between before infection (day 7 before inoculation) and after 1 (day 32) or 2 (day 65) months of persistent infection. Because half of the mice infected with Bt1 alone experienced earlier mortality, we could only study weight change in this group during the first month. The results showed that all infected mice failed to gain weight, compared with the uninfected control mice, during the first month (for all comparisons; *t* test) (figure 5). The weight change in coinfected mice resembled closely the weight change in mice infected with Bt2 alone. Mice infected with Bt1 alone failed to gain weight during the first month significantly more than did mice infected with Bt2 alone or in combination with Bt1 (*t* test). This severe failure to gain weight may have contributed to their earlier mortality: 4 (50%) of 8 mice infected with Bt1 alone had died spontaneously by day 46 of infection, compared with 0 of 16 mice infected with Bt2 alone or in combination with Bt1 (*P* < .001, Fisher’s exact test). Mice infected with Bt2 alone or in combination with Bt1 gained significantly more weight during the second month of infection than did uninfected mice (*P* < .001). Clinical exams and necropsy revealed that a contributor to weight gain was hepatosplenomegaly. These results revealed that coinfection with Bt2 prevented the severe loss of weight observed early during infection with Bt1 alone.

**Effect of coinfection on pathogen load.** Although we did not directly measure pathogen load in the blood obtained at necropsy [4, 14], we were able to indirectly investigate the effect of coinfection on pathogen load by measuring the levels of the B cell chemokine CXCL13 in plasma. This was a valid alternative because we recently found that levels of this chemokine strongly correlate with pathogen load for *B. turicatae* [11] and *B. burgdorferi* [15]. Although we did not have plasma available from mice infected with Bt1 alone because of their earlier mor-
Figure 4. Severity of arthritis in scid mice persistently infected with *Borrelia turicatae* serotypes 1 (Bt1) or 2 (Bt2) alone or in combination and in control mice that were sham inoculated with PBS. Mice were examined by an observer masked to infection status (E.G.) every 1–3 days for up to 65 days. Results are given as mean clinical scores for 8 infected or sham-inoculated mice per group. The total no. of mice in the group infected with Bt1 alone decreased over time because of earlier mortality. Note the significant difference between the groups infected with Bt1 alone or in combination with Bt2.

Figure 5. Percent weight change in scid mice persistently infected with *Borrelia turicatae* serotype 1 (Bt1) or serotype 2 (Bt2) alone or in combination and in control mice that were sham inoculated with PBS, as measured by an examiner masked to infection status between 7 days before and 32 or 65 days after inoculation. Results are given as mean ± SD values. The group infected with Bt1 alone had significantly less weight gain during the first month than did any of the other 3 groups. The groups infected with Bt2 alone or in combination with Bt1 had significantly more weight gain than did the uninfected control mice during the second month of infection. There are no data for weight change after 65 days for the group infected with Bt1 alone because of earlier mortality.

**DISCUSSION**

Several human pathogens are characterized by great diversity of serotypes. For many of them, only certain serotypes are associated with human disease. A well-known example is *Streptococcus pneumoniae*, for which 7 serotypes account for nearly 80% of cases of bacteremia, meningitis, and otitis media [16]. In pneumococcal disease, infection with serotype 3 is associated with an increased risk of death, whereas infection with serotype 1 is associated with a decreased risk [17]. Furthermore, otitis media caused by serotype 3 is more severe than that caused by serotype 23 and results in more complications [18]. Significant differences in the pathogenicity of serotypes have also been observed for *Escherichia coli*, for which only certain O serotypes can produce bacteremia [19]. However, much less is known about how the outcome of bacterial infections is influenced by the presence of >1 serotype at the same time.

The phenomenon of antigenic variation in RF results in the simultaneous presence of different serotypes in the blood [4, 10]. The generation of multiple serotypes is the result of a change in the expression of VMPs in the outer membrane of RF spirochetes [2, 20]. Work in our laboratory has shown that the outcome of RF can vary significantly depending on the infecting serotype [4, 9, 10, 21]. For the present study, we investigated the question of whether the simultaneous presence of >1 serotype influences the outcome of infection with individual serotypes. The results showed significant differences in the clinical course and severity of disease caused by Bt1 depending on whether Bt2 was also present. Significant differences were apparent not only on clinical examination but also on functional testing. The vestibular disorder persisted longer and became more severe in mice infected with Bt1 alone. Unlike vestibular dysfunction with Bt1 infection, the clinical manifes-
tations of Bt2 infection were not significantly altered by coinfection with Bt1. This was true for tibiotarsal arthritis and weight change. These findings indicate that the phenotype of infection with Bt2 predominates during coinfection with Bt1 to the extent that the consequences of Bt1 infection can be suppressed. In a previous study, we found that brain infection was intermediate in coinfected mice, compared with that in mice infected with either Bt1 or Bt2 alone [14]. This suggests that Bt2 coinfection can also lessen the brain infection observed with Bt1 infection alone. The mechanism by which coinfection with Bt2 prevented severe vestibular dysfunction, failure to gain weight, and mortality from Bt1 infection is not known. However, it is likely that the severe vestibular dysfunction made it more difficult for Bt1-infected mice to eat and drink, which may have contributed to their earlier mortality. Recently, we found a strong correlation between pathogen load and production of interleukin (IL)–10 in mice persistently infected with *B. turicatae* [22]. It is possible that, at high pathogen loads—as was seen during infection with Bt2—increased production of IL-10 reduced the clinical manifestations of Bt1 infection. It is unlikely that the worse phenotype observed in mice infected with Bt1 alone resulted from use of half of the Bt1 inoculum used in coinfected mice, given that inoculation with 10 times fewer spirochetes was sufficient to cause severe disease in Rag1−/− and Igh6−/− mice.

During previous studies of scid mice infected with *B. turicatae*, we observed that the only clinically apparent neurological complication was vestibular dysfunction [4]. This was confirmed in the present study: in no mice did we observe signs of facial paralysis, the most common cranial neuropathy of human neuroborreliosis in both RF and Lyme disease [23]. The onset of vestibular dysfunction occurred 1–2 weeks after the clinical manifestations of involvement of the eyes, skin, and joints had begun [4]. This delay is reminiscent of facial paralysis in human neuroborreliosis, which occurs only 3 or more weeks after the onset of erythema migrans in Lyme disease [23] or after the second or third febrile episodes in tickborne RF [24]. The recent observation that some immunocompetent mice inoculated with Bt1 developed vestibular dysfunction with a similar time of onset and severity as observed in scid mice—albeit with lesser frequency [12]—indicates that vestibular dysfunction with *B. turicatae* is not simply an artifact of B cell immunodeficiency but rather an important complication of RF borreliosis in mice.

We conclude that coinfection with Bt2 significantly reduces the vestibular dysfunction and mortality that characterizes persistent infection with Bt1 alone, whereas coinfection with Bt1 does not influence the severe arthritis that characterizes persistent infection with Bt2 alone. This is the first demonstration of the modulation of clinical disease during coinfection with *Borrelia* serotypes.

**References**


