Arthritis Severity and Spirochete Burden Are Determined by Serotype in the *Borrelia turicatae*-Mouse Model of Lyme Disease

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Immunodeficient mice infected with *Borrelia turicatae*, a relapsing fever agent, have a disorder that resembles disseminated Lyme disease. Two serotypes, A and B, differed in their arthritogenicity in both CB-17 SCID and C3H SCID mice. In CB-17 SCID mice infected with serotype A or B, arthritis was assessed by measurement of tibiotarsal diameter, functional ability on a beam walk test, and microscopic assessment of joint inflammation. Serotype B-infected mice had greater joint swelling, functional disability, and leukocytic infiltration in the joints than serotype A-infected mice. Joint swelling and disability peaked at 2 weeks of infection and then decreased, while leukocyte infiltration in the joints persisted. To investigate the basis for the differences in arthritogenicity of serotypes A and B, spirochete burdens in infected mice were measured by quantitative PCR of spirochete DNA in joints, direct immunofluorescence of spirochetes in joints, and counts of spirochetes in the blood. At 2 weeks of infection there were seven times more spirochetes in the joints of serotype B-infected mice than in those of serotype A-infected mice, measured by both quantitative PCR and direct enumeration. Although serotypes A and B had the same infectivity and growth rate in vivo, serotype B spirochetes were eightfold more abundant in the blood than serotype A spirochetes and produced greater fatality in newborn mice. These findings indicate that differences in disease severity in mice infected with serotype A or B are attributable to differences in the spirochete burden in the joints and blood.

Lyme disease, a zoonosis caused by the spirochete *Borrelia burgdorferi*, involves the skin, joints, heart, and central and peripheral nervous systems of infected humans (31). We found that SCID mice infected with a relapsing fever spirochete, *Borrelia turicatae*, developed at least three of these manifestations, namely, arthritis, myocarditis, and cranial neuritis (13). In addition, we identified two *B. turicatae* serotypes, A and B, which differed in their arthritogenic potential. Two weeks after infection, serotype B-infected mice, but not serotype A-infected mice, had reddened and significantly enlarged tibiotarsal joints and severely impaired locomotor performance (13). Serotype identities of the relapsing fever agents *B. turicatae* and *B. hermsii* are conferred primarily by the variable major proteins (Vmps) expressed on their surfaces (5). Serotypes A and B express two antigenically distinct Vmps, VmpA (23 kDa) and VmpB (20 kDa), respectively. These two Vmps belong to a family of small Vmps that are homologous to OspC of *B. burgdorferi* (12, 14).

Immunocompetent mice infected with *B. burgdorferi* have self-limited arthritis, the severity of which depends on their genetic background (7, 29). The arthritis in mice is attributable to viable spirochetes in the joint tissues rather than autoimmune phenomena. *B. burgdorferi*-infected SCID mice have severe arthritis with persistence of spirochetes in the joints, and the transfer of splenocytes from infected immunocompetent mice prevents rather than exacerbates the arthritis (27, 28, 30). Immunocompetent mice of strains susceptible to arthritis do not develop autoantibodies when infected, and mouse strains that are predisposed to adjuvant-induced arthritis are not more susceptible to *B. burgdorferi*-induced arthritis (29, 33). Mice

from strains, such as C3H/He, that develop more severe arthritis harbor more spirochetes in their joints, for a longer time, than resistant strains, such as BALB/c (34).

Control of infection is determined by both innate and acquired immunity. Early in the infection C3H SCID mice had more severe arthritis than BALB/c SCID mice (10), and C57BL/6-*bg* mice with a mutation affecting granulocyte and NK cell activities had more severe arthritis than congenic wild-type mice (8). This was evidence that strain-specific susceptibilities to arthritis are not solely due to differences in the specific immune response. Later in the infection, spirochete clearance and arthritis resolution appear to be determined more by the type of specific immune response by mice (17, 20, 33).

While host factors are determinants of disease severity, spirochete components may also affect disease outcome. Different isolates of *B. burgdorferi sensu stricto* differ in their potential to cause arthritis (1, 9). However, identification of specific spirochetal determinants of arthritis is difficult, since these strains differ in their genetic backgrounds. An advantage of the *B. turicatae* model of arthritis is that the two serotypes are of the same strain and differ only in their expressed Vmps. This provides an opportunity to focus on individual proteins and their effects on disease severity.

In the present study we investigated the difference in joint disease severity between serotype A-infected and serotype B-infected mice. The more severe arthritis in serotype B-infected mice could be due to more serotype B spirochetes infiltrating the joints, leading to a greater inflammatory response. Alternatively, there could be equal numbers of serotypes A and B in the joints, but serotype B could elicit a greater inflammatory response than A. To discriminate between these possibilities, we quantitated the numbers of spirochetes in the joints and blood of serotype A-infected and serotype B-infected SCID mice by quantitative PCR and microscopy. Using these tech-

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niques, we found that the severity of the arthritis was a function of the spirochete burden in the host. The spirochete burden in turn is determined by the infecting serotype.

MATERIALS AND METHODS

Strains and culture conditions. The origins of *B. turicatae* Oz1 and serotypes A and B have been described previously (13). Both serotypes were cloned by limiting dilution and cultured at 34°C in BSK II medium with 12% rabbit serum (3, 32). Spirochetes were stored frozen in 10% dimethyl sulfoxide at -80°C. For mouse infections, frozen stocks were thawed and incubated at 34°C in an equal volume of BSK II for 1 h. The bacteria were counted in a Petroff-Hausser chamber by phase-contrast microscopy (32) and diluted to 10⁴ spirochetes/ml of phosphate-buffered saline (PBS), pH 7.4, prior to infection. *Escherichia coli* strain INVαF' (Invitrogen, San Diego, Calif.) was grown in LB medium. The serotype identity of isolates in culture and from mice was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and with monoclonal antibodies specific for serotypes A and B (12, 13).

Mouse infections. Four- to six-week-old male CB-17 SCID and C3H SCID mice (Charles River Laboratories, Wilmington, Mass.) were inoculated intraperitoneally with 0.1 ml of either 10³ spirochetes in PBS or PBS alone. Tail vein blood was periodically obtained in a 40-μl heparin-coated capillary tube (Drummond Scientific, Broomall, Pa.). The blood was transferred to a 0.6-ml microcentrifuge tube and centrifuged at 10,000 × g for 2 s. Numbers of spirochetes in the plasma supernatant were counted in a Petroff-Hausser chamber by phase-contrast microscopy. After euthanasia of the mice with methoxyflurane, blood was obtained by cardiac puncture with sodium citrate as an anticoagulant. A 0.2-ml volume of the blood was added to 6-ml culture tubes, and the remainder was transferred to 2-ml capped microcentrifuge tubes and centrifuged at 10,000 × g for 2 s to obtain plasma. Total body perfusion through the exposed heart was then performed with 30 ml of PBS (13). After perfusion, the ankles were obtained by removing the skin and cutting above and below the tibiotarsal joint as described by Yang et al. (34). The left joint was further prepared for histology, and the right joint was processed for DNA extraction (see below). The SCID mice in this study were tested for the presence of antibodies specific for *B. turicatae* serotypes A and B. The plasma was diluted 1:10 and 1:100 in 0.3% (wt/vol) dried nonfat milk in 10 mM Tris-HCl (pH 7.4)-150 mM NaCl and subjected to Western blotting with serotype A and B whole-cell lysates as previously described (25). Positive controls were monoclonal antibodies specific for serotype A or B (12).

Pregnant C3H/HeN mice (Harlan, Indianapolis, Ind.) were observed daily until they gave birth to pups. Three-day-old pups were inoculated subcutaneously with 10³ spirochetes in 0.05 ml of PBS (pH 7.4) or with PBS alone. The mice were observed daily for up to 25 days. Moribund mice were euthanized on the day this was first noted. After euthanasia with methoxyflurane, blood was obtained by cardiac puncture, 0.2 ml of blood was added to 6-ml culture tubes, and the remainder was treated as described above to obtain plasma.

Assessment of joint size and functional impairment. Right and left tibiotarsal joints, from interior to exterior talus, of infected and uninfected mice were measured in triplicate with a vernier caliper. The beam walking test was performed as previously described (13, 26). Briefly, the mice were trained to cross a horizontal beam connecting two platforms 120 cm apart and 50 cm high prior to infection. Each daily trial consisted of three complete crossings, and for each crossing, the traversing time and the number of times the left hind foot slipped were recorded.

Histological studies. Joints were fixed in 10% neutral buffered formalin for 48 h and decalcified in 14% (wt/vol) anhydrous EDTA (Sigma, St. Louis, Mo.), pH 7.2, for 2 weeks with constant stirring and one change of the solution. After decalcification, the joints were dehydrated in ethanol and embedded in paraffin. Slides stained with hematoxylin and eosin were prepared by standard methods, and microscopic examinations were carried out by a pathologist (C.A.) blind to the infecting serotype and degree of disability.

The amount of inflammation in and around the joints was assessed histologically by grading monocyte and polymorphonuclear cell infiltration in the joint space, the joint space capsule, and the periarticular soft tissue and fascia. The degree of infiltration in each of these three locations was graded as 0 (none), 1 (light), 2 (moderate), or 3 (heavy). The average of the three independent scores was assigned as the overall inflammation score for each joint. Also noted was the presence or absence of cartilage erosion and pannus formation. The degree of synovial hyperplasia, if present, was assessed as mild, moderate, or severe.

For direct immunofluorescence, deparaffinized 5-μm-thick sections were incubated with a 1:500 dilution of fluorescein isothiocyanate-labeled polyclonal goat antibody against *Borrelia* spp. (Kirkegaard & Perry, Gaithersburg, Md.) in 1% chicken ovalbumin. The slides were examined in an Olympus BX60 microscope with a BX-FLA reflected light fluorescence attachment (Olympus America Inc., Lake Success, N.Y.). The number of spirochetes in four nonoverlapping high-power fields (magnification, ×400) were counted by an observer blind to the infecting serotype.

DNA extractions. Total DNA from *B. turicatae* was prepared essentially as previously described (21). Plasmid from *E. coli* was extracted with a commercial kit (Qiagen, Chatsworth, Calif.). DNA from mouse ankles was prepared as

described by Yang et al. (34). Briefly, the tissue was digested at 37°C for 4 h in 0.1% collagenase A (Boehringer Mannheim, Indianapolis, Ind.) in PBS. This was followed by an overnight digestion at 50°C with an equal volume of proteinase K (Boehringer Mannheim) solution at 0.2 mg/ml in 200 mM NaCl-20 mM Tris-HCl (pH 8.0)-50 mM EDTA-1% sodium dodecyl sulfate. DNA was extracted with an equal volume of phenol-chloroform and precipitated with ethanol. The pellet was resuspended and digested with 0.1 mg of DNase-free RNase (Sigma) per ml at 37°C for 1 h. The DNA was reextracted and resuspended in water. The DNA concentration of each sample was determined by A₂₆₀ and then adjusted to 50 ng/μl.

PCR. Amplification was performed in 100-μl reaction volumes with 1.25 U of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.), 25 pmol of each primer, 200 μM each deoxynucleoside triphosphate, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin (24). Sample DNA was diluted to 50 μg/ml, and 450 ng was used in the PCR. Mixtures were overlaid with mineral oil and amplified in a thermal cycler (Perkin-Elmer Cetus). All amplifications were done with 30 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The reactions and the DNA extractions were performed in different rooms to prevent contamination. Controls without any DNA and with uninfected mouse DNA were included in every PCR run.

Oligonucleotides and probes. Oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer (Perkin-Elmer Cetus). The flagellin primers used in this study amplify a region that is highly conserved in *Borrelia* spp.; their sequences are 5'-ACA TAT TCA GAT GCA GAC AGA GGT-3' (Bfg1) and 5'-CAA TCA TAG CCA TTG CAG ATT GTG-3' (Bfg2) (6). PCR amplification of *B. turicatae* total DNA with these primers gave a 610-bp fragment, the size expected on the basis of flagellin sequences of other *Borrelia* spp. (22, 23). This product was cloned into the plasmid pCRII and transformed into *E. coli* INVαF'. The identity of the insert was confirmed by sequencing with the Sequenase 2.0 commercial kit according to the procedure described by the vendor (U.S. Biochemicals, Cleveland, Ohio). The sequence obtained was identical to the published sequence of the *B. turicatae* flagellin gene (23). The cloned flagellin fragment was labeled with [α-³²P]dATP by nick translation with a commercial kit (GIBCO BRL, Gaithersburg, Md.).

Quantitative PCR. The flagellin gene sequence was amplified from infected mouse ankle DNA, and the PCR products were dot blotted, hybridized to the *B. turicatae* flagellin gene probe, and quantitated as follows. Ten microliters of the PCR product was boiled in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min. After chilling, an equal volume of 20× SSC was added and the solution was blotted onto a nitrocellulose membrane under vacuum with a manifold (Bethesda Research Laboratories). The membrane was then placed for 10 min on filter paper soaked in denaturing solution (1.5 M NaCl-0.5 M NaOH), after which it was transferred for 5 min to a filter soaked in neutralizing solution (1 M NaCl-0.5 M Tris-HCl [pH 7]) (2). Hybridization with the flagellin probe was performed at 37°C, and washes were performed at 64°C, as previously described (4). A final wash in 0.1× SSC was done before exposure to a PhosphorScreen (Bio-Rad, Hercules, Calif.) for 20 min. Radioactive counts were calculated by using local background in a Molecular Dynamics PhosphorImager equipped with Image Quant software (Molecular Dynamics, Sunnyvale, Calif.).

A standard curve for quantification of the amount of *B. turicatae* DNA in infected joints was obtained by first mixing twofold dilutions of *B. turicatae* serotype A total DNA, ranging from 10 to 1,250 fg, with 450 ng of uninfected ankle DNA. PCR was then performed on this mixture, and the products were blotted on the same blot as the unknown samples. The curve was plotted as log₁₀ femtograms of borrelia DNA per 450 ng of mouse DNA versus log₁₀ radioactive counts (19). The amount of mouse DNA used for quantitation was based on the quantitative PCR developed by Yang et al. (34). Linear regression analysis produced a least-squares line that was used to estimate the amount of borrelia DNA in the joints of infected mice (Fig. 1). The PCR products from infected mouse DNA samples were diluted 10- or 100-fold so that they would fall within the linear range of the standard curve. Dilutions and blots were done in triplicate, and means of the three values were calculated.

RESULTS

Duration of joint swelling and functional impairment in infected mice. A previous study showed that a joint disorder, which peaked after 2 weeks of infection, was more severe in mice infected with serotype B than in those infected with serotype A (13). We undertook the present study to further understand this difference in disease expression. Our aim was to confirm the previous findings and to provide a longer and more detailed record of this phenomenon. CB-17 SCID mice were inoculated with *B. turicatae* serotype A (*n* = 4), *B. turicatae* serotype B (*n* = 4), or PBS alone (*n* = 2). The differences in joint disease were observed for up to 7 weeks of infection by measuring the right and left tibiotarsal joints and by testing

FIG. 1. Standard curve for the quantitation of borrelia DNA in the joints of infected mice. Known amounts of borrelia total DNA were added to uninfected mouse ankle DNA. PCR with flagellin-specific primers was performed, and the product was detected by Southern blotting with a flagellin probe. The bound radioactivity was quantitated as described in Materials and Methods. Each point represents the mean $\log_{10}$ radioactive counts $\pm$ SEM corresponding to the $\log_{10}$ of a known amount of *B. turicatae* total DNA added to mouse ankle DNA. r , Pearson correlation coefficient.

locomotor function on a beam walk, measuring the time it took the mice to cross the beam and the number of times the hind feet slipped as they crossed the bar (13).

The results for joint sizes, crossing times, and number of foot slips are shown in Fig. 2A, B, and C, respectively. Both serotype A- and serotype B-infected mice had swollen ankles, but, as before, the joints of serotype B-infected mice were significantly larger from days 11 to 27. The joints of serotype A- and serotype B-infected mice were the largest on day 13, when the sum of the tibiotarsal joints $\pm$ standard error of the mean (SEM) was 7.47 ± 0.04 mm for serotype B-infected mice, 6.77 ± 0.03 mm for serotype A-infected mice, and 6.10 ± 0.03 mm for uninfected mice. The joints of both serotype A- and serotype B-infected mice gradually returned to normal size from days 32 to 48, when the sum of the joints was 6.2 to 6.3 mm for all mice.

Joint swelling in serotype B-infected mice and, to a lesser extent, in serotype A-infected mice was temporarily accompanied by marked impairment in locomotor abilities. Serotype B-infected mice took the longest times to cross the beam and had the most foot slips during crossing. The greatest difference in crossing times occurred on day 11, when the mean crossing time for serotype B-infected mice was 2.5 times longer than for serotype A-infected mice and 3.5 times longer than for uninfected mice. Day 11 was also the day on which serotype B-infected mice had the greatest number of foot slips, approximately 10 for every 1 by a serotype A-infected mouse. Uninfected mice rarely slipped while crossing the beam. Longer crossing times recurred for both serotype A- and serotype B-infected mice from day 17 to day 25. These later impairments coincided with the appearance of a peripheral vestibular disorder noted previously (13).

Correlation between joint disease severity and spirochete burden on the host. The more severe joint disease in serotype B-infected mice, now consistently observed in two separate studies, could be due to a greater spirochete burden in the joints, leading to a greater inflammatory response. Based on this premise, we next examined if there was a correlation between the spirochete burden and the severity of joint disease.

For this second experiment, joint disease severity was evaluated not only by joint size but also by tissue inflammation. The spirochete burden in the joints was assessed at various stages of disease progression and remission by quantitative PCR and by counting the spirochetes in the joints by immunohistochemistry. The spirochete burden in the blood was also assessed during infection because these spirochetes presumably spread hematogenously to the joints and the number of circulating spirochetes may determine to a greater or lesser extent the number of cells infiltrating the joints.

CB-17 SCID mice were inoculated with *B. turicatae* serotype A ($n = 12$), *B. turicatae* serotype B ($n = 12$), or PBS alone ($n = 6$). The tibiotarsal joints were measured for 21 days of infection. Groups of 3 or 4 infected mice and 2 control mice were sacrificed on days 9, 15, and 21, representing, respectively, a day before arthritis was evident, the day of greatest joint swelling, and a day when swelling was subsiding. Using results of the first experiment, we predicted that the day of peak joint swelling would be the day when the sum of the joint sizes was approximately 7.5 mm. Tibiotarsal joints were obtained from each group of mice. One of the joints was processed for histopathology and assigned an inflammation score. Immunohistochemistry was also performed on these joints to identify the distribution of the spirochetes in the joints as disease progressed and to visually estimate the number of spirochetes in the joints. DNA was extracted from the other joint to estimate the amount of spirochete DNA in the joints by quantitative PCR. Finally, the spirochete burden in the blood was evaluated by collecting tail vein blood every 2 to 3 days from a random sample of 4 to 5 mice and counting the number of spirochetes per milliliter of plasma by phase-contrast microscopy.

(i) Joint disease assessment. Both serotype A- and serotype B-infected mice had joint swelling, but the joints of serotype B-infected mice remained significantly larger than those of serotype A-infected mice after day 14 of infection (Fig. 3A). Joint swelling was first detected on day 11. It increased and reached its peak on day 15, after which it decreased gradually. The severity of the arthritis was also evident by histology (Fig. 3B and 4). Joint inflammation was scored at the indicated time points as described in Materials and Methods. The inflammation scores of serotype A- and serotype B-infected joints increased from day 9 to day 15 of infection, when serotype B-infected joints had significantly higher scores than serotype A-infected joints. There was a correlation between the inflammation scores and the joint sizes on days 9 and 15 ($r = 0.91$, $P < 0.001$), indicating that the joint size can be a useful measure of arthritis severity in the initial stages of the disease. However, on day 21 the joints of serotype B-infected mice had decreased in size, although there was still severe inflammation by histologic criteria.

Figure 4 illustrates some aspects of the joint inflammation observed in serotype A- and serotype B-infected joints on day 15 of infection, in comparison with an uninfected joint. Most evident is the greater leukocytic infiltration in the synovium and cellular exudate in the synovial space in serotype B-infected joints, compared to serotype A-infected joints. Marked bone marrow cellularity was noted in both serotype A- and serotype B-infected joints but not in uninfected joints.

In addition to leukocytic infiltration, pannus formation, cartilage erosion, and synovial proliferation were observed in serotype A- and serotype B-infected joints both on day 15 and on day 21 of infection. Pannus formation occurred in seven of eight joints of serotype B-infected mice but only in two of seven joints of serotype A-infected mice ($P = 0.041$, two-tailed Fisher's exact test). Minimal cartilage erosion was observed in joints of both serotype A- and serotype B-infected mice but not

FIG. 2. Duration of joint swelling and functional impairment in CB-17 SCID mice inoculated with *B. turicatae* serotype A (circles), *B. turicatae* serotype B (squares), or PBS (diamonds). (A) Joint sizes of mice infected with serotype A or B. Each point represents the mean sum of the right and left tibiotarsal joints $\pm$ SEM. (B) Crossing time per run on a high beam during infection with serotype A or B. Each point represents the mean crossing time per run $\pm$ SEM. (C) Number of foot slips per run on a beam during infection with serotype A or B. Each point represents the mean number of foot slips per run $\pm$ SEM. All determinations were done in triplicate for each mouse.

joints on day 15. The mean number of spirochetes in four $\times 400$ fields $\pm$ SEM was 87 ± 37 in serotype B-infected joints and 12 ± 3 in serotype A-infected joints, a ratio of 7:1 ($P = 0.09$, Student's *t* test).

(iii) **Correlation of joint disease and spirochete burden in the blood.** The numbers of spirochetes in the blood were counted over 21 days of infection (Fig. 3D). Spirochetes were microscopically detectable by day 5, when the numbers of serotype A and B cells were equal. Thereafter, there were approximately eightfold more serotype B cells than serotype A cells. The number of spirochetes per milliliter of plasma on day 9 correlated with joint size on day 15 (Fig. 5), an indication that the number of spirochetes in the blood early in the infection determined the extent of arthritis later.

We determined next whether differences in spirochete counts in the blood were due to a faster growth rate for serotype B. Cadavid et al. had previously shown that the doubling time for each serotype in vitro was approximately 7 h (13). For the present study CB-17 SCID mice were infected with serotype A ($n = 4$) or serotype B ($n = 5$), tail vein blood was obtained approximately every 8 h from day 2 to day 6 of infection, and the spirochetes were counted by microscopy. Growth curves were obtained for each mouse. The mean doubling time in the blood (95% CI) was 6.4 (5.6 to 7.2) h for serotype A and 7.7 (6.5 to 8.9) h for serotype B. Thus, the greater number of serotype B cells in the blood and joints could not be attributed to a faster growth rate of serotype B.

Joint disease and spirochete burden in C3H SCID mice. Since severity of arthritis of mice infected with *B. burgdorferi* varies according to the mouse strain used (7), we assessed whether the relative differences in arthritogenicity of *B. turicatae* serotypes A and B observed in CB-17 SCID mice would also occur in C3H SCID mice. C3H SCID mice were inoculated with *B. turicatae* serotype A ($n = 4$), *B. turicatae* serotype B ($n = 3$), or PBS ($n = 2$), their joints were measured, and

in control mice. Moderate synovial proliferation was observed in six of eight serotype B-infected mice and in none of seven serotype A-infected mice ($P = 0.007$).

(ii) **Association of joint disease and spirochete burden in the joints.** The spirochete burden in the joints was assessed by quantitative PCR (Fig. 3C). There was more serotype B than serotype A DNA in the joints on days 9 and 15 of infection. The largest difference between the groups of mice was observed on day 15, when there was 34 ± 13 fg (mean $\pm$ SEM) of serotype A DNA and 233 ± 37 fg of serotype B DNA (Fig. 3C). Assuming that 1 fg of borrelia DNA is equivalent to 3 genomes, that each spirochete contains approximately 10 genomes (18), and that the yield of DNA from the ankle is representative of one joint, we estimate that there were 4.5×10^4 serotype A cells and 3.2×10^5 serotype B cells in each joint on day 15. The ratio (95% confidence intervals [CI]) of serotype B to serotype A DNA in the joints was 4.4 (2.7 to 6.0) on day 9 and 6.9 (3.9 to 9.9) on day 15. On day 21 of infection the ratio of serotype B to serotype A DNA was 2.0 (0.9 to 3.1).

For another measure of spirochete burden we used direct immunofluorescence to estimate spirochete numbers in the

FIG. 3. Disease severity and spirochete burden in CB-17 SCID mice inoculated with *B. turicatae* serotype A (circles), *B. turicatae* serotype B (squares), or PBS alone (diamonds). (A) Joint sizes of mice infected with serotype A or B. Each point is the mean sum of the right and left tibiotarsal joints $\pm$ SEM. The arrows indicate the days when subsets of mice were sacrificed. (B) Tibiotarsal joint inflammation scores on days 9, 15, and 21 of infection. Each point represents the mean score $\pm$ SEM. (C) Amounts of spirochete DNA in the joints on days 9, 15, and 21, estimated by quantitative PCR. Each point represents the mean femtograms of borrelia DNA per 45 ng of mouse DNA $\pm$ SEM. (D) Mean log₁₀ spirochetes per milliliter of plasma $\pm$ SEM in mice infected with serotype A or B.

spirochetemia was monitored as described above. The joint swelling in serotype A- and serotype B-infected C3H SCID mice followed the same pattern as observed in Fig. 2A and 3A for CB-17 SCID mice. The joints of serotype B-infected mice were larger than those of serotype A-infected and uninfected mice from days 7 to 26 of infection. Both serotype A- and serotype B-infected joints were largest on day 17, when the sum of the tibiotarsal joints $\pm$ SEM was 7.1 ± 0.1 mm in serotype B-infected mice, 6.6 ± 0.1 mm in serotype A-infected mice, and 6.3 ± 0.0 mm in uninfected mice. After day 17, both serotype A- and serotype B-infected joints gradually decreased in size until day 26, when they were 6.8 ± 0.2 mm in serotype B-infected mice, 6.5 ± 0.1 mm in serotype A-infected mice, and 6.3 ± 0.1 mm in uninfected mice. From day 7 to day 26 of infection, serotype B cells were 2- to 12-fold more abundant in

the blood than serotype A cells. The greatest difference was observed on day 7, when the mean number of spirochetes per milliliter of plasma $\pm$ SEM was $(1.6 \pm 0.2) \times 10^6$ in serotype A-infected mice and $(1.9 \pm 0.6) \times 10^7$ in serotype B-infected mice.

Virulence in the newborn mouse. Since infant mice infected with *B. burgdorferi* have more severe arthritis than their adult immunocompetent counterparts (7), we assessed whether newborn mice infected with *B. turicatae* would also develop more severe disease. Three-day-old C3H/HeN mice were inoculated with serotype A ($n = 5$), serotype B ($n = 4$), or PBS alone ($n = 2$). They were observed daily, and the blood was terminally cultured to verify infection. By day 11, four of four serotype B-infected mice and none of five serotype A-infected mice were either dead or moribund ($P = 0.008$, two-tailed Fisher's

FIG. 4. Histopathology of tibiotarsal joints of CB-17 SCID mice inoculated with *B. turicatae* serotype A, *B. turicatae* serotype B, or PBS. Joints were obtained on day 15 after inoculation, and paraffin sections were stained with hematoxylin-eosin. There is more pronounced leukocytic infiltration and proliferation in the synovial tissue (Syn) of serotype B-infected mice than in that of serotype A-infected mice and control mice. Increased bone marrow (BM) cellularity was observed in both serotype A- and serotype B-infected mice but not in uninfected mice. Magnification, $\times 44$.

exact test). Serotype A-infected mice remained as disease-free as uninfected mice for at least 25 days.

DISCUSSION

We previously showed that infection of SCID mice with *B. turicatae* produced disease manifestations that are commonly observed during disseminated Lyme disease (13). Notably, one

FIG. 5. The log₁₀ number of spirochetes per milliliter of plasma on day 9 and the sums of the sizes of the right and left joints of mice on day 15 after infection with *B. turicatae* serotype A (circles) or serotype B (squares). *r*, Pearson correlation coefficient.

of the two serotypes, B, caused greater joint enlargement and disability than the other serotype, A (13). In the first part of the present study, we confirmed and extended these findings. Measurements of joint size and tests of locomotor abilities were continued for 7 weeks instead of 4. As before, the joints of serotype B-infected mice were significantly larger than those of serotype A-infected mice for 4 weeks, peaking at 2 weeks after infection and decreasing thereafter to the size of joints of uninfected animals. The locomotor abilities of serotype B-infected mice were most impaired at the time of greatest joint swelling. As the swelling subsided, the performance on the walking beam improved. Since the mice did not have recurrence of joint swelling, the functional disability noted later in the infection was more likely attributable to a vestibular disease than to arthritis (Fig. 2) (13).

Having confirmed the reproducibility of these findings, we next investigated the basis for the differences in joint disease severity between serotype A- and serotype B-infected mice. We asked, is the more severe arthritis in serotype B-infected mice associated with a greater spirochete burden in the host? To answer this, we counted by microscopy and/or PCR the spirochetes in the blood and in the joints at various times during infection. Serotype B organisms consistently reached higher numbers in the blood and in the joints than serotype A organisms. This phenomenon was observed in SCID mice of two different genetic backgrounds, CB-17 and C3H, suggesting that the spirochete levels attained in the blood are more a function of the infecting serotype than of host factors. The level of spirochetemia on day 9 was predictive of the degree of swelling and spirochete infiltration of joints on day 15. Based on these findings, we conclude that the spirochete burden in the blood and joints is a major determinant, if not the sole determinant, of disease severity in this model. It is still possible that serotype B in equal numbers to serotype A is inherently more inflammatory, but this was not detectable under these experimental conditions.

Yang et al. studied spirochete burden and arthritis severity in mice of different strains infected with the same isolate of *B. burgdorferi* (34). In contrast, we looked at spirochete burden and disease severity in mice of one strain infected with either of two serotypes of *B. turicatae*. In both studies mice with more severe disease had more spirochetes in the joints than mice

with milder disease. Yang et al. found that there are host genetic determinants that control the spirochete burden (34), while we found that the spirochete burden is a function of the infecting serotype. Taken together, these data indicate that the disease severity is determined both by the ability of the spirochetes to reach high numbers in the joints and by the ability of the host to control the spirochetes.

Although the joint swelling and the functional disability associated with it were self limited, inflammatory changes in joint tissues persisted. This dissociation of inflammation and joint swelling in the *B. turicatae* model is consistent with observations of Bremell et al., who studied joints of mice infected with *Staphylococcus aureus*, and indicates the importance of using both histologic changes and joint size to evaluate experimental arthritis (11).

The decrease in swelling in spite of persistent inflammation may reflect the kinetics of the local inflammatory response to the spirochetes in the joints. In SCID mice that were persistently infected with *B. burgdorferi*, the arthritis was characterized by a polymorphonuclear and mononuclear cell infiltrate into synovial and periarticular tissue (10, 27). This was similar to what we observed in SCID mice infected with *B. turicatae*. When Hurtenbach et al. inoculated *B. burgdorferi* into subcutaneous chambers in SCID mice, tumor necrosis factor alpha, interleukin 1, and interleukin 6 levels in the chamber rose in a few hours but then decreased to undetectable levels within 24 h despite the persistence of spirochetes in the chamber (16). A short-lived cytokine increase may be the proximate cause of the temporary joint swelling observed in mice infected with *B. turicatae*.

The higher densities of serotype B cells in the blood were also associated with high mortality of infant mice. Presumably, this is also attributable to greater spirochete burden. Why serotypes A and B reach different numbers in the blood and tissues of adult and infant mice is not known. The spirochete levels in the blood are not determined by the infectivity; one spirochete is enough to start infection with either serotype (13). The two serotypes also had the same growth rates in the blood. Given the similar infectivities and growth rates of both serotypes, the differences in spirochete density in the blood could be attributable to other factors, such as differences in nutrient acquisition, susceptibility to intravascular substances, or the rate of migration and/or elimination from the circulation. Since there was no evidence for a specific immune response in these SCID mice, elimination from the blood was likely based on other factors. For instance, serotype A may have been cleared faster by the reticular endothelium system. Differences in the early vascular clearance of low- and high-passage *B. burgdorferi* isolates in rats have been documented (15). Alternatively, serotype A may have penetrated other tissues more frequently than serotype B.

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