

Variability of a Bacterial Surface Protein and Disease Expression in a Possible Mouse Model of Systemic Lyme Borreliosis

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Summary

During persistent infection of *scid* mice with *Borrelia turicatae*, an agent of relapsing fever and neuroborreliosis, there was variation in the surface proteins the bacteria expressed and in disease manifestations over time. Two serotypes, A and B, were isolated from the mice, cloned by limiting dilution, and further characterized. The only discernible difference between the two variants was in the size of the major surface protein they expressed: serotype A had a variable major protein (Vmp) of 23,000, and serotype B had a Vmp of 20,000. When other *scid* mice were inoculated with clonal populations of A and B, the infections were similar with respect to onset and degree of spirochetemia, involvement of the eye and heart, and occurrence of a peripheral vestibular disorder. However, there were differences between the serotypes in other respects: (a) serotype B but not A caused reddened and significantly enlarged joints, markedly impaired performance on a walking bar, and severe arthritis by histologic examination; (b) serotype A but not B invaded the central nervous system during early infection; and (c) serotype A penetrated monolayers of human umbilical vein endothelial cells more readily than did serotype B. The combination of arthritis, myocarditis, and neurologic disease resembled human Lyme borreliosis. The findings indicate that differences in disease expression are determined by variable surface proteins of the bacterium and that *scid* mouse infections with *B. turicatae* provide a model for the study of the pathogenesis of Lyme borreliosis and other persistent spirochetal diseases.

During Lyme borreliosis there is sequential involvement over weeks to years of different organs, including heart, joints, central and peripheral nervous systems, and eyes (1). Although viable bacteria clearly initiate disease, the host's immune response to the agent determines in part the severity of that disease (2-4). To further delineate the relative contributions of parasite and host in this pathogenesis equation an animal model would be helpful. Infections with *Borrelia burgdorferi*, the agent of Lyme borreliosis, have been established in laboratory animals, including mouse, rat, hamster, rabbit, and gerbil (5-9). In both immunocompetent and immunocompromised animals these infections have involved the joints and heart, but there has been little or no evidence of disease of the nervous system in these animal models. Infection of the brain by *B. burgdorferi* is inconsistent and short-lived, even in immunodeficient animals (5, 6, 10, 11).

A closely related group of spirochetes in the genus *Borrelia* cause relapsing fever (12). The two principal species that produce disease in North America are *B. hermsii* and *B. turicatae*

(13). The infection of both humans and experimental animals is characterized by periodic illness of the host and antigenic variation of the etiologic spirochete in the blood (14). A feature of relapsing fever that is less appreciated now than in the past is the involvement of the nervous system in human infections (15, 16). Several decades ago investigators first noted that certain strains of relapsing fever borrelias were neurotropic (17, 18). The specific phenomenon that these earlier investigators noted was the propensity of some but not all strains of borrelias to invade the nervous tissue and persist there for weeks to years (19, 20). Neurotropism was observed not only in laboratory animals but in the vector ticks as well (21). Relapsing fever borrelia strains have a large repertoire of proteins to display on their surface (22), and therefore it was possible that the neurotropic potential within a strain was conferred by one or more of these variable proteins.

The original goal of the study was to establish persistent infection by a *Borrelia* species of the brain of a laboratory animal. This aim was achieved, but as notable were the findings

that some variants of *B. turicatae* are neurotropic and that there were differences in disease expression with different variants. Most surprising was the similarity of the disease we observed in infected mice to systemic Lyme borreliosis of humans.

Materials and Methods

Strains and Culture Conditions. *B. turicatae* was isolated by injecting Swiss mice with tissues from *Ornithodoros turicata* collected in a cave near Ozona, TX (23). *B. hermsii* strain HS1 (35209; American Type Culture Collection, Rockville, MD) and *Spirochaeta aurantia* strain M1 have been described previously (24, 25). Borrelias were cultured in BSK II medium with 12% rabbit serum (26), and *S. aurantia* was grown in YT medium (25). When tissue samples were cultured, rifampin at 50 µg/ml and phosphomycin at 100 µg/ml were present in the medium. Intrastrain borrelia variants with different variable major proteins (Vmp's)¹ were designated by convention "serotypes" and, to distinguish the Vmp's from those of *B. hermsii*, they were also designated by letters instead of numbers (22). Serotypes were cloned by limiting dilution in BALB/c mice (Harlan Laboratories, Indianapolis, IN) irradiated with 650 rad from a ¹³⁷Cs gamma source (24). Plasma samples from infected mice were either frozen with 10% DMSO at -80°C or used to start broth cultures, which at cell densities of 10⁸/ml were aliquoted and similarly frozen until use. Spirochetes were counted in a Petroff-Hauser chamber under phase contrast microscopy (24).

Protein and Nucleic Acid Analysis. Whole cells from harvested cultures were subjected to SDS-PAGE with 12.5 or 14% acrylamide (22). Surface-exposed proteins of intact borrelias were cleaved with proteinase K (Boehringer Mannheim Corp., Indianapolis, IN) and examined by PAGE as described (27). Total DNA was obtained from the borrelias, digested with restriction enzymes, and subjected to agarose gel electrophoresis as described (28).

Monolayer Penetration Assay. Spirochetes in late-log phase cultures were harvested by centrifugation (5 min at 7,000 g) and after washing, adjusted to 10⁹ cells/ml in M199 medium (Gibco Laboratories, Grand Island, NY) containing 20% FCS. Human umbilical vein endothelial cells were isolated from freshly delivered umbilical cords by the method of Jaffe et al. (29). The cells were maintained in an atmosphere of 5% CO₂ in air at 37°C in M199 medium, 100 µg/ml of heparin, and 50 ng/ml of endothelial cell growth supplement (Gibco Laboratories). Confirmation of endothelial cell character and penetration assays were conducted as previously described (30). Briefly, 2.5 × 10⁴ endothelial cells were seeded onto 3-µm pore size, 6.5-mm diameter sterile polycarbonate membrane culture plate inserts (Nucleopore, Pleasanton, CA). Chambers were placed in 24-well plates containing 1 ml/well M199 with FCS. After incubation for 48 h, the monolayers were confluent, and high trans-endothelial resistance was confirmed; aliquots of 10⁸ spirochetes in 0.2 ml medium were added to the upper portions of the chambers. At different times of incubation, aliquots from beneath the filter were removed, and spirochetes were directly counted as described above. The assay was performed three times.

Mouse Infections. 4-6-wk-old female BALB/c mice (Harlan Laboratories) or male CB-17 *scid* mice (Charles River Laboratories, Wilmington, MA) were inoculated intraperitoneally or intracerebrally with borrelia suspensions in 300 and 60 µl of PBS, respectively. Control mice received PBS alone. *scid* mice were maintained in a germ-free environment before and after infection. For evalua-

tion of spirochetemia, tail vein blood was mixed with an equal volume of PBS and examined by phase contrast microscopy. The mice were examined daily, and presence of these signs were specifically noted: ruffled fur, eye discharge, reddened joints, head tilt, spinning in the air when lifted off the ground by the tail, and walking in circles (31). Abnormal signs were confirmed by an observer who did not know whether the mouse was infected or not. The sizes of the tibiotarsal joints and metatarsal regions at their greatest diameter were measured with a vernier caliper. Mouse sera were examined for anti-borrelia antibodies by indirect immunofluorescence as previously described (22).

Beam Walking Test. The mice were trained on a stationary horizontal beam 48 and 24 h before test trials (32). The Plexiglas beam measured 120 (length) × 1.2 (thickness) × 0.7 (width) cm, had platforms at both ends, and was at a 50 cm height. Test trials consisted in three complete crossings of the beam. For every crossing two determinations were made: traversing time in seconds and the number of times one of the hind feet slipped off the beam.

Tissue and Fluid Collection. Mice were killed with methoxyflurane. Sodium citrate was used as an anticoagulant for blood collection by heart puncture. Total body perfusion with 30 ml PBS was performed as described (33). After the skull was opened under sterile conditions, brain tissue was removed and rinsed twice with 1 ml of PBS in sterile 2-ml microfuge tubes (Sarstedt Inc., Newton, NC). To the brain tissue sample was added twice its volume of BSK II medium. With plungers of sterile 1-ml plastic syringes (Becton Dickinson & Co., Mountain View, CA) the brain tissue was homogenized in the tubes and then suspended in medium by briefly vortexing. Blood and brain suspensions were centrifuged for 5 s at 7,000 g. If red blood cells were observed microscopically in the brain suspension or as a layer in the brain pellet, the sample was discarded. Eyes and tibiotarsal joints were removed, rinsed with sterile PBS, and then added en block to culture tubes (34). Samples from uninfected mice were processed as negative controls for cross contamination between mice during procedures. All cultures were examined for a 3-wk period.

Histopathological Studies. Internal organs including brain, heart, and testis and different joints of mice were removed after total body perfusion with buffer. Tissues were immersion fixed in 10% neutral buffered formalin. The bones were decalcified in 5% nitric acid after fixation. Hematoxylin and eosin-stained slides were prepared by standard histologic methods. The slides were read by a veterinary pathologist (R. Crawley) without prior knowledge of which animals were infected and, if infected, with which serotype. The amount of inflammation in cardiac and synovial tissues was graded as none, mild, moderate, or severe. The criteria for grading arthritis were the following: mild, focal inflammation of synovial membrane with minimal exudation into joint space or periarticular tissues; moderate, coalescing areas of inflammation in the synovial membranes with moderate exudation into the joint space or periarticular tissues; and severe, inflammation of synovial membrane involving the entire joint with marked exudation into the joint space and periarticular tissues. The criteria for grading carditis were the following: mild, multifocal but nonconfluent leukocytic accumulation in myocardial or pericardial tissues; moderate, multifocal leukocytic accumulations that were confluent in some areas; and severe, largely confluent or transmural areas of inflammation.

Results

Isolation of Serotypes A and B. The present study began as an attempt to find an animal model of spirochete infection in which persistent infection of the brain occurred. We had

¹ Abbreviations used in this paper: CI, confidence interval; Vmp, variable major protein.

shown previously that *B. hermsii* invades the central nervous system of mice (33). However, after a preliminary study showed that *B. hermsii* infections of the brain of BALB/c mice did not last beyond 2 wk, we considered other *Borrelia* species. Previous reports had indicated that among the relapsing fever *Borrelia* species, *B. turicatae* was often associated with neurologic disease in humans (16). The Ozona strain of this species was implicated in an outbreak of relapsing fever, which was first thought to be Lyme disease on the basis of cranial neuritis (23). Using BALB/c mice we first documented the invasion of the brain by the Ozona strain of *B. turicatae*. Four mice were inoculated intraperitoneally with 10^3 borrelias on day 0. Borrelias were microscopically detectable in or cultivable from the blood in two mice examined on day 10 but not in mice examined on days 24 and 31. In contrast, the perfused brain contained cultivable *B. turicatae* on days 24 and 31 as well as 10. With the exception of ruffled fur when spirochetemic, the infected mice did not appear to be ill.

Different results were obtained with two *scid* mice who were given the same inoculum and similarly examined for blood and brain infection at various times. The mice had spirochetemia at all examinations and, in addition to ruffled fur, had a self-limited mucopurulent discharge from the eyes

early in the infection. The PAGE profiles from brain and plasma isolates obtained from one mouse examined on day 7 and from the other mouse examined on days 50 and 100 are shown in Fig. 1, left. Isolates from the plasma and brain of the mouse examined on day 7 were identical to one another but differed from isolates taken from the mouse examined on days 50 and 100. The differences between isolates were in major proteins with apparent M_r of 20,000–35,000 (35 K). Day 7 brain and plasma isolates had major proteins of 33, 22, and 21 K not present in the other isolates. Later isolates uniquely had either or both proteins of 23 and 20 K. Days 50 and 100 blood populations from the second mouse produced both proteins, albeit less of the 23 K protein by day 100 (Fig. 1). In contrast, the brain isolate from day 100 detectable produced only the 23 K Vmp protein.

Characterization of Serotypes A and B This experiment indicated that even in immunodeficient mice variation of major proteins of a relapsing fever borrelia occurred over time. Furthermore, populations of borrelias in the brain and blood of the same animal differed, the implication being that some isolates are more neurotropic than others. If this was the case, then one would expect in a prospective study that the variant expressing the 23K protein would more likely infect the brain than the variant population expressing predominantly the 20 K protein. For evaluation of this hypothesis clonal populations of the two variants were needed. These were obtained by cloning by limiting dilution in irradiated mice. First passage cultures of the clonal populations were characterized with respect to profiles of their total proteins and restriction enzyme digests, growth rate, infectivity, and in vitro invasiveness. The phenotypes that are described below for the two clones were stable after further passage in both broth medium and *scid* mice.

Fig. 1, right, shows the total cellular proteins of the two

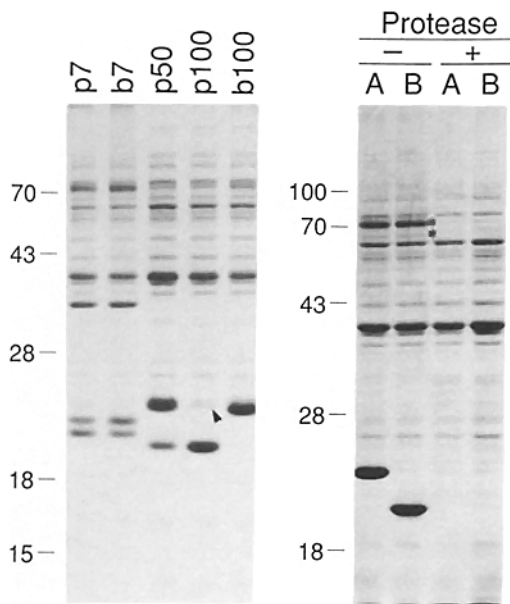


Figure 1. Coomassie blue-stained proteins in whole cell lysates of isolates of *B. turicatae*. The migrations of molecular weight standards ($\times 1,000$) are shown on the left. (Left) 14% acrylamide gel of brain (b) and plasma (p) isolates from days 7, 50, and 100 of *scid* mice inoculated with *B. turicatae*. The p7 and b7 isolates were from one mouse and the p50, p100, and b100 isolates from a second mouse. The arrow indicates a faint protein of 23 K that is more prominent in the p50 and b100 lanes. (Right) 12.5% acrylamide gel of cloned isolates from b100 and p100 populations, now designated serotypes A and B, respectively. In the two rightmost lanes of this gel the cells had first been treated with proteinase K. The Vmp proteins of 23 and 20 K in A and B populations were cleaved by the protease. Two other proteins that were cleaved in situ by the protease and were the same in cultures from both populations are indicated by asterisks. The upper band is BSA from the medium.

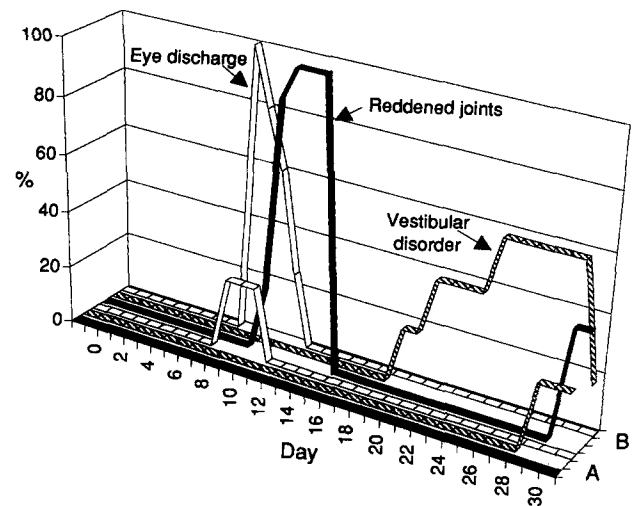


Figure 2. Disease manifestations in *scid* mice inoculated with either serotype A (foremost) or serotype B (hindmost) of *B. turicatae*. The day of examination is shown at the bottom, and the percentage of mice examined with either mucopurulent eye discharge (white ribbon), joint reddening (black ribbon), or vestibular disorder (cross-hatched ribbon) are shown in the y-axis.

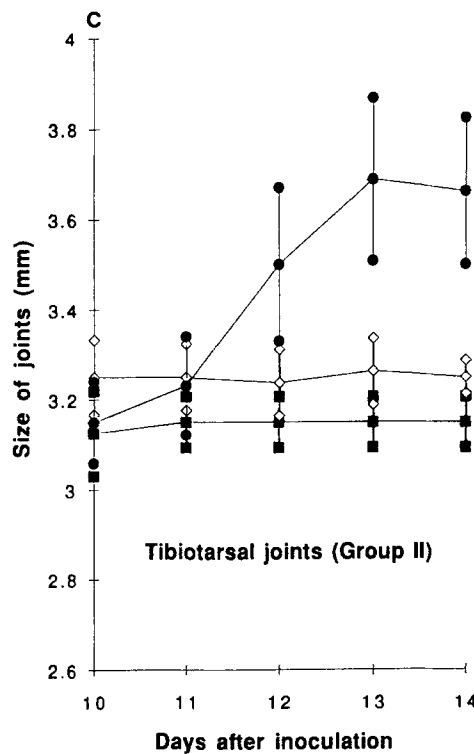
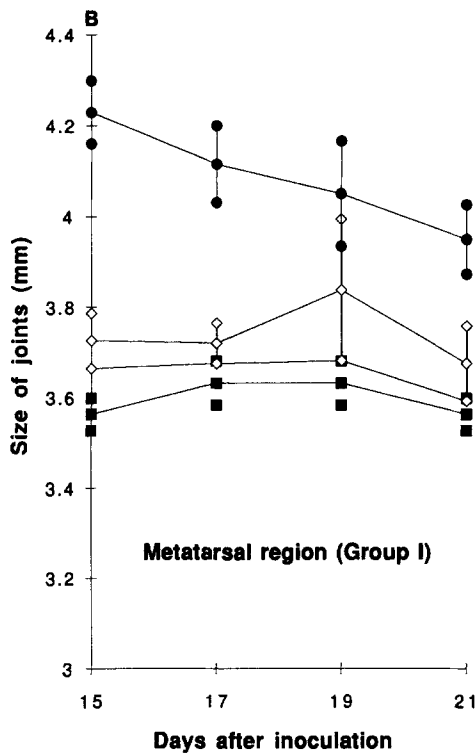
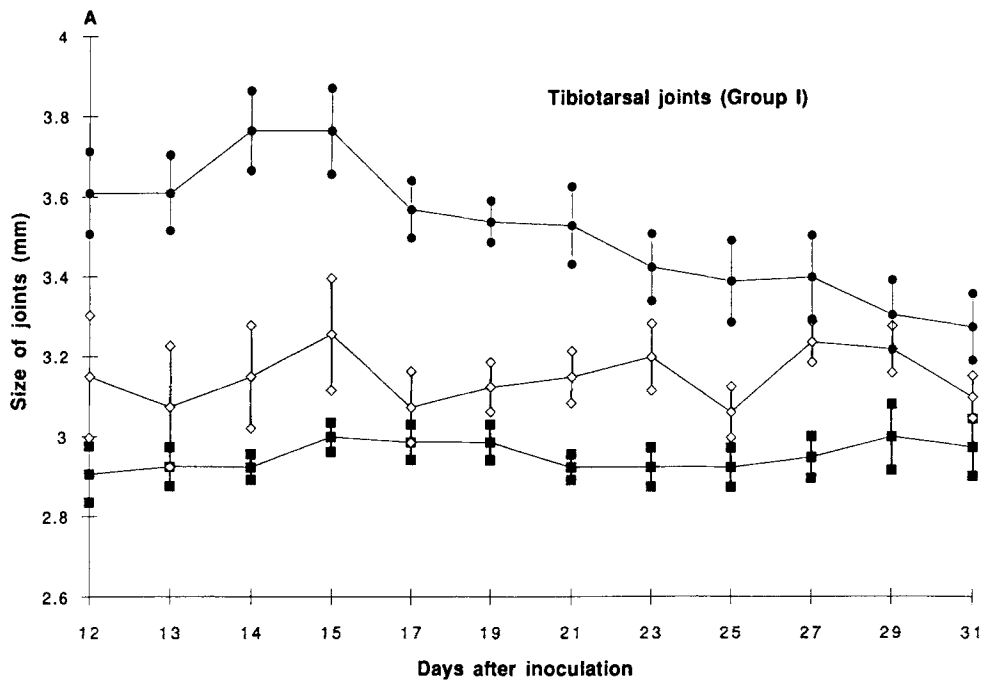


Figure 3. Mean diameters with 95% CI of tibiotarsal joints and metatarsal regions of *scid* mice by days after inoculation with PBS (solid squares), serotype A (open diamonds), or serotype B (solid circles) of *B. turicatae*. The two groups (I and II) of mice that were examined are described in the text.

clonal populations. The variant expressing the 23 K protein was designated serotype A, and this variant-specific protein was called VmpA. The corresponding designations for the other variant population were serotype B and VmpB. Previous studies with *B. burgdorferi* and *B. hermsii* indicated that serotypic specificity is largely determined by major proteins of 20–35 K that are surface exposed (35, 36). When intact, viable

cells were treated with proteinase K, VmpA and VmpB were cleaved from the cells (37), as shown in Fig. 1, and also in gels with a higher concentration of acrylamide. Two proteins common to serotypes A and B were also affected by the in situ protease treatment: one of 69 K, which corresponds to the bovine albumin in the medium, and one of 65 K (Fig. 1). The latter protein is probably homologous

to minor proteins of about the same size of *B. burgdorferi* and *B. hermsii* that are likewise cleaved in situ by proteases (38, 39). There was no detectable difference in the restriction fragment profiles of BglII, PstI, and HindIII digests of total DNA of the two serotypes (data not shown), an indication that the two serotypes were substantially isogenic.

The polymorphic character of Vmp proteins was demonstrated in two immunocompetent BALB/c mice inoculated with 10^4 cells of clonal populations of serotype A or B. The tail vein blood was cultured at the time of the peak spirochetemia and relapse spirochetemia, and the isolates were characterized by PAGE (data not shown). In the relapse population of the mouse inoculated with serotype A, VmpA was replaced by Vmp's of 37 and 21 K. In the mouse inoculated with serotype B the relapse population expressed a Vmp of 22 K instead of VmpB.

Serotypes A and B were examined with respect to growth rate in culture and infectivity of mice. The mean growth rates at 34°C for three determinations (95% confidence intervals [95% CI]) for serotypes A and B were 6.9 (5.9–7.9) h and 7.1 (6.9–7.3) h, respectively. When groups of three irradiated BALB/c mice each were inoculated intraperitoneally with an estimated 10^2 , 10^1 , 10^0 , and 10^{-1} spirochetes of serotype A or B, the number of infected mice in each group, in order of descending inoculum size, were 3, 3, 2, and 0 for serotype A and 3, 3, 1, and 0 for serotype B. This indicated that either serotype can produce infection with as few as one or two cells, as Schuhardt found with another strain of *B. turicatae* (40). When *scid* mice were inoculated with 10^3 cells of either serotype A or B, spirochetes were detectable by daily phase contrast microscopy of tail vein blood by day 4. These experiments indicated that serotypes A and B had similar capacities to grow in culture and to establish infection in mice.

In an initial study of biological differences between the two serotypes, the ability of the spirochetes to pass through monolayers of human umbilical vein endothelial cells was assessed. The mean percentage (95% CI) of the number of borrelias added to the upper part of the chamber that were detected on the other side at 6 h was 10.0 (8.8–11.3) for serotype A, 4.6 (4.1–5.0) for serotype B and 0.7 (0.4–0.9) for the nonpathogenic spirochete *S. aurantia*.

Differences in Disease in Serotype A- and B-inoculated Mice. The previous findings suggested that *B. turicatae* serotypes A and B, while detectably identical in all respects except in the Vmp proteins and in vitro invasiveness, might differ in the diseases they produced. To assess this *scid* mice were infected with the clonal populations of serotypes A or B. Mice numbered 1–29 were divided into groups of 4, 12, and 13 mice, which were injected intraperitoneally with PBS, 10^3 serotype A cells, or 10^3 serotype B cells in PBS, respectively, on day 0. From each of the infected groups four to five mice were taken on days 4, 11, and 31, and their organs and tissues were sampled for culture. All mice were spirochetemic through the 31 d of study. There was no difference between A- and B-inoculated mice in the spirochete count in the blood at any point.

Fig. 2 summarizes disease expression in the mice. By day

8 the eight remaining mice inoculated with serotype A and the nine remaining mice inoculated with serotype B were listless and had ruffled fur. Mucopurulent discharge from the eyes beginning by day 8 and not lasting beyond day 13 was observed in mice infected with either serotype but more commonly in those infected with B. The joints were involved next. Tibiotarsal joint and metatarsal region reddening began on day 10, 11, or 12 in all serotype B-inoculated mice but not at all in control or serotype A-inoculated mice. The mean sizes of the right and left tibiotarsal joints for control, A-inoculated, and B-inoculated mice for days 12–31 are shown in Fig. 3 A. Measurements of the metatarsal regions of these animals for days 15–21 are given in Fig. 3 B. The joints of B-inoculated mice were significantly larger than those of either control or A-inoculated mice. The measurable joint enlargement lasted a few days longer than joint reddening evident on eye examination (Fig. 2). Joint involvement recurred on day 30 in two of five B-inoculated mice (Figs. 2 and 3).

To confirm an association between joint disorder and serotype B a second group (II) of *scid* mice (nos. 30–39) were inoculated with PBS ($n = 2$), 10^3 serotype A cells ($n = 4$), or 10^3 serotype B cells ($n = 4$). Joints were measured daily; results for days 10–14 are given in Fig. 3 C. Reddened joints on days 12–14 were again noted in B-inoculated mice but not in control or A-inoculated mice. Tibiotarsal joints of B-inoculated mice on days 12–14 were significantly larger than joints of control or A-inoculated mice. In group II as well as group I, the joints of A-inoculated mice were slightly larger than those of control mice. On day 14 the animals were killed for culture and histopathologic examination of joints and other organs (see below).

In group I mice signs of an equilibrium disorder began on day 19 or later for four of five B-inoculated mice and day 29 for one of four A-inoculated mice (Fig. 2). The manifestations were head tilt, walking in circles in one direction, and spinning in the air when lifted off the ground by the tail. When deprived of proprioceptive input from the ground, the affected animals attempted to achieve a supported position. This disorder, like those of eyes and joints, resolved after a few days. There was no evidence of hemiparesis, abnormal posture, or involvement of cranial nerves other than VIII during the period of examination.

Assessment of Functional Impairment. Traversing an elevated narrow beam assesses both locomotor performance and vestibular system integrity of rodents (41). After mice in group I had first been trained for walking the beam, they were inoculated with PBS, serotype A, or serotype B as described above. Traversing times and footslips were measured daily for days 1–15 and 29–31 and every other day for days 16–28. Fig. 4 shows in radar graphs the results with the A- and B-inoculated and control mice that were followed for 31 d. The results obtained with mice examined daily for 4 or 11 d after inoculation were the same for these time periods as those obtained with mice examined for 31 d (data not shown).

The mean number of foot slips for control mice in 312 trials was 0.2 per crossing. Only mice infected with serotype B had functional impairment by the criterion of number of

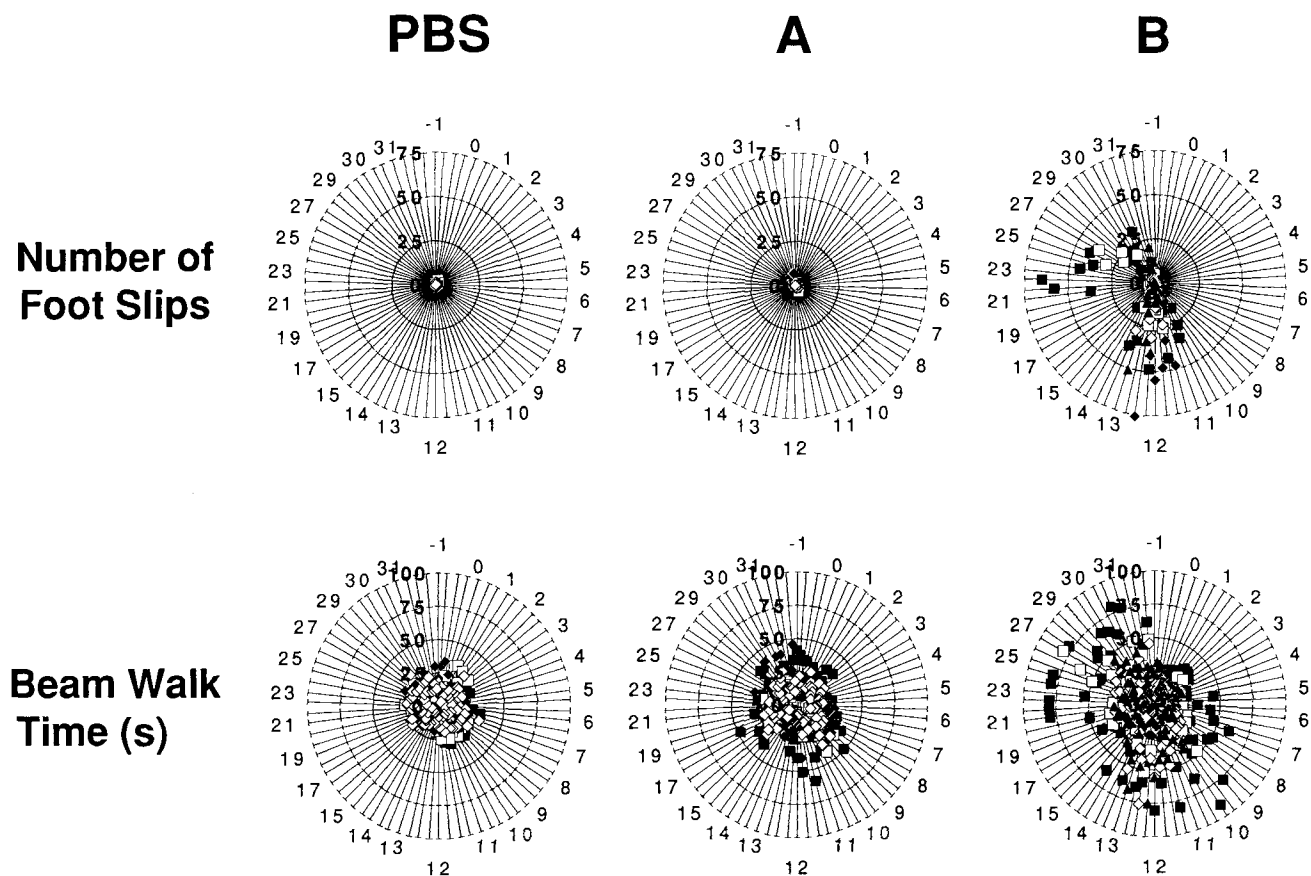


Figure 4. Radar graphs of functional impairment of *scid* mice inoculated with PBS, serotype A, or serotype B of *B. turicatae* by day after inoculation. The upper graphs show the number of footslips by day of examination. The lower graphs show the mean time in seconds to walk the elevated beam. The radius of the circles is the scale of either footslips or traversing times; the day of examination is given around the perimeter. Each symbol represents a different mouse.

footslips (Fig. 4). The impairment in B-inoculated mice was greatest during days 10–14 and 20–27, the respective periods of joint and vestibular disorders. On day 11, when B-inoculated but not A-inoculated mice had joint disease, the mean (95% CI) of foot slips was 0.1 (0–0.3) for A-inoculated mice and 16 (6–26) for B-inoculated mice.

Crossing times were more variable within groups (Fig. 4). The mean (SD) crossing time for uninfected mice in 312 trials was 16 (6) s. Crossing times in A-inoculated mice were longest between days 8 and 19, when minimal joint swelling occurred, and between days 29 and 31 when one mouse had vestibular disorder. Some A-inoculated mice had crossing times

Table 1. Inflammation of the Joints of *scid* Mice Infected with *B. turicatae* Serotypes A or B

	Serotype A				Serotype B			
	None*	Mild	Moderate	Severe	None	Mild	Moderate	Severe
Knees	4	0	0	0	0	0	3	1
Tibiotarsal	0	2	2	0	0	0	1	3
Metatarsophalangeal	0	4	0	0	1	2	1	0
Metacarpophalangeal	1	3	0	0	0	2	1	1
Elbow	3	1	0	0	0	4	0	0
Total	8	10	2	0	1	8	6	5

* See Materials and Methods for criteria.

>3 SD greater than the mean for controls. Crossing times were longer in B-inoculated mice; only mice in this group had crossing times of 75 s or more. On day 12 the mean crossing times (95% CI) for A- and B-inoculated mice were 17 (6–28) and 49 (32–66) s, respectively. As with foot slips, the greatest impairment in beam walking for B-inoculated mice corresponded to the periods of joint and later of vestibular disorders.

Histopathology. Blinded examinations were made on tissues of the eight group II mice killed on day 15 after inoculation and two uninfected *scid* mice of the same age. Most B-inoculated mice had reddened, swollen joints at this time, while A-inoculated mice had joints that were somewhat larger than controls but smaller than those of B-inoculated mice. Table 1 summarizes histologic findings in the joints of serotype A- and B-inoculated mice; control mice did not have any detectable inflammation of the joints or tendon sheaths. 8

of 20 examined joints of A-inoculated mice were normal compared with 1 of 20 in serotype B-inoculated mice ($p < 0.01$ by Fisher exact test). 11 of 20 joints of B-inoculated mice and 2 of 20 joints of A-inoculated mice had moderate to severe inflammation ($p < 0.01$). Only B-inoculated mice had severe arthritis. An example of severe arthritis in a mouse inoculated with serotype B is shown in Fig. 5. There is a mixed inflammation of the synovial lining of the joints and tendon sheaths with mononuclear and polymorphonuclear cells, and the synovial lining is thickened with several layers of cells.

Heart inflammation graded mild or moderate was observed in all infected mice but not in the controls. There was not a significant difference between serotypes A and B with respect to the degree of inflammation. The myocarditis was characterized by a predominantly mononuclear leukocytic infiltrate between myocardial fibers. The inflammation was

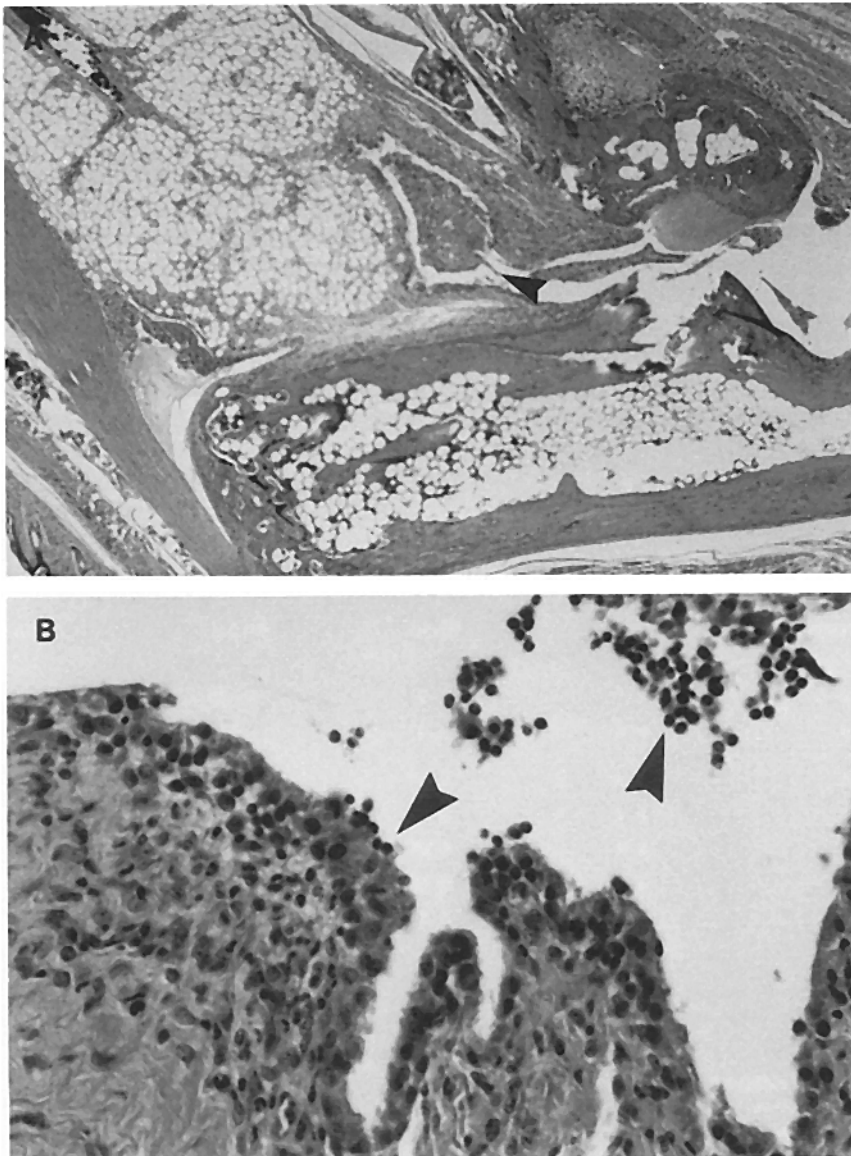


Figure 5. Histopathology of joints of *scid* mice examined 15 d after inoculation of *B. turicatae* serotype B. Paraffin sections were stained with hematoxylin-eosin. (A) Arrowhead points to generalized thickening of the synovial lining of a tarsal joint. Leukocyte and fibrinous exudate are also present in the joint space ($\times 44$). (B) Arrowheads point to thickened synovial membrane (bottom arrow) and exudation of mixed leukocytes into the knee joint space (top arrow) ($\times 440$).

greatest in the atria and around the base of the great vessels. There was also an epicarditis and pericarditis that was most marked over the atria and base of the heart.

Two serotype B-inoculated *scid* mice were killed when they had vestibular disorder; a mouse similarly infected but without this disorder was killed at the same time. When their brain and middle and inner ears were examined microscopically, no evidence of inflammation of these tissues was found. In neither these mice nor the group II mice described above were there abnormalities of the liver, bladder, pancreas, or spleen. The testes of both A- and B-inoculated mice contained numerous spirochetes in fluid obtained by puncture but no leukocytic infiltrate on histologic examination. These histologic studies demonstrated that serotype A and B differed only in the degree of inflammation they caused in the joints.

Serotype A Is Neurotropic during Early Infection. The finding in the *scid* mouse examined after 100 d of infection suggested that serotype A was more likely than serotype B to infect the brain. To further study this phenomenon 10^3 cells of the "b100" isolate (Fig. 1), in which only serotype A was detectable, or the "p100" isolate which was predominantly serotype B, were inoculated into five *scid* mice each on day 0. On day 8 the plasma and brain were cultured, and the first-passage culture harvests were subjected to PAGE for identification of the serotype. For this and subsequent experiments the blood was obtained first; only after exsanguination and total body perfusion with PBS was the brain taken (33). The results of this experiment, the first in this series, are given in Table 2. Plasma and brain cultures from the five mice in each group were positive. Mice inoculated with the b100 isolate had serotype A in both the plasma and brain. In contrast, the mice inoculated with the p100 isolate had both serotype A and B in the plasma but only serotype A in the brain, even though serotype A represented no more than 10% of

the inoculum in these mice (Fig. 1). In this and subsequent experiments there were, as estimated by number of days in culture to reach 10^6 cells/ml, 10–100-fold fewer borrelias per volume of brain tissue than in the same volume of plasma.

In experiment 2 of this part of the study cultures of the brain and plasma were taken from *scid* mice 5–29 (group I) inoculated with either serotype A or B and killed on days 4, 11, and 31. These times represented early infection (day 4), arthritis in B- but not A-inoculated mice (day 11), and vestibular disorder in A- and B-inoculated mice (day 31). Blood cultures were also obtained from the mice on days 18 and 22. Whereas mice inoculated with serotype A had positive plasma and brain cultures on days 4 and 11, B-inoculated mice had positive plasma cultures but negative brain cultures on these days (Table 2). The borrelias recovered in these cultures were the same serotypes used to inoculate the animals.

On day 31 all nine mice had positive brain as well as plasma cultures (Table 2). Whereas in A-inoculated mice only serotype A was detectable in blood and brain, B-inoculated mice had, besides serotype B, a new serotype in blood and brain on day 31. This new serotype, which was designated C, had a Vmp of 21 K (Fig. 6). The representation of serotype C was higher in brain cultures than in plasma cultures simultaneously obtained. Serotype C was detectable in the plasma cultures of only one of the five mice on day 22 and none of the mice on day 18 (data not shown). An additional 4 d of incubation was required before brain cultures of B-inoculated mice, in comparison to A-inoculated mice, reached a cell density of 10^6 /ml. This indicates that there were ~100-fold fewer borrelias in the brains of B-inoculated than A-inoculated mice examined on day 31.

Inasmuch as a new serotype, C, appeared in the blood and brains of mice inoculated with a clonal population of serotype B, it was possible that some *scid* mice were producing

Table 2. Cultures of Mice Infected with *B. turicatae* Serotypes A and B

Exp.	Serotype inoculated	Day examined	Culture results*		Serotypes in culture†	
			Plasma	Brain	Plasma	Brain
1	A + B [§]	8	5/5	5/5	A, B	A
	A	8	5/5	5/5	A	A
2	A	4	4/4	4/4	A	A
	B	4	4/4	0/4	B	–
	A	11	4/4	4/4	A	A
	B	11	4/4	0/4	B	–
	A	31	4/4	4/4	A	A
	B	31	5/5	5/5	B, C	B, C
3	A + B [§]	4	4/4	4/4	A, B	A

* Number of mice with positive cultures/number of mice cultured.

† As determined by electrophoretic migrations of Vmp proteins (see text).

§ A/B, 1:10 in experiment 1 (Fig. 1) and A/B, 1:1 in experiment 3.

|| $p = 0.01$ by Fisher exact test.

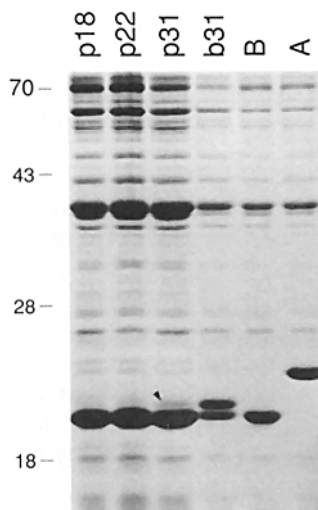


Figure 6. Coomassie blue-stained proteins of isolates from plasma (p) or brain (b) of a mouse sampled on days 18, 22, and 31 after inoculation with a clonal population of serotype B of *B. turicatae*. For comparison lysates of serotype B and A cells are in the rightmost two lanes. The samples from the plasma were purposefully overloaded with respect to the brain isolate. The arrow indicates a protein of 21 K in the lane of p31. This protein is in greater amounts in the b31 population. The acrylamide concentration was 12.5%. The migrations of molecular weight standards ($\times 1,000$) are shown on the left.

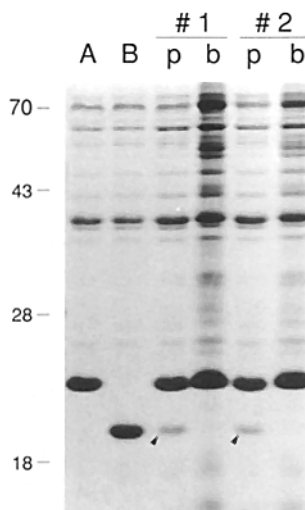


Figure 7. Coomassie blue-stained proteins of isolates from plasma (p) or brain (b) of two *scid* mice (nos. 1 and 2) inoculated with a 1:1 mixture of serotypes A and B of *B. turicatae*. For comparison lysates of serotype A and B cells are in the two leftmost lanes. The samples from the brain were purposefully overloaded with respect to the plasma isolates. The arrows indicate the presence of VmpB of 20 K in the plasma isolates. The acrylamide concentration was 12.5%. The migrations of molecular weight standards ($\times 1,000$) are shown on the left.

specific antibodies (42). To assess this possibility we examined sera from *scid* mice 4, 11, and 31 d after inoculation of serotype A or B cells by indirect immunofluorescence. None of the sera examined reacted with serotype A or B cells. In contrast, the sera from immunocompetent BALB/c mice inoculated with clonal populations of serotype A or B cells produced serotype-specific antibodies under the same conditions and in the same assay (data not shown). This was an indication that there was not antibody selection by the *scid* mice against the serotypes originally inoculated.

One explanation for the presence of serotype B in the brain by day 31 was that entry into the brain was facilitated by prior invasion of a neurotropic serotype. We evaluated this possibility by infecting mice with a mixture of clonal populations of serotype B and serotype A. For this experiment, number 3 in the series, four *scid* mice were infected with 5×10^2 each of serotype A and B cells on day 0. Plasma and brain were obtained and cultured on day 4. The results of cultures of two of the mice (nos. 1 and 2) are shown in Fig. 7; results with the other two mice were the same (Table 2). The lanes with lysates of brain cultures from mice nos. 1 and 2 were purposefully overloaded. Serotypes A and B were both present in the plasma, but only serotype A was detectable in the brain cultures. These findings confirmed that entry into the brain in early infection with *B. turicatae* was characteristic of serotype A but not B. Simultaneous inoculation with a neurotropic serotype did not facilitate early infection of the brain by serotype B.

To evaluate the ability of serotype B to proliferate and survive in the brain, 10^3 borrelias of serotype B were inoculated intracerebrally into three 4-wk-old *scid* mice. 8 d later plasma and perfused brain were obtained from the animals and cultured. Serotype B was found in both the plasma and brain of all three animals. This experiment showed that serotype B has the ability at least to survive in the brain, and, thus, its absence from the brains of mice infected peripherally is more likely the consequence of lower invasiveness. In this respect the culture findings were consistent with the results of the in vitro invasion assay.

Cultures of Joints and Eyes. Borrelias were recovered from all perfused tibiotarsal joints taken on day 14 from four A- and four B-inoculated mice. The serotype in the culture was the same as that used to inoculate the animals. These results suggest that serotypes A and B have equal ability to invade the joints. Eye cultures were positive from all A- and B-inoculated mice cultured on days 11 and 31. Inasmuch as the B-inoculated mice had negative brain cultures on day 11, the access route to the eye was likely the systemic circulation and not the subarachnoid space.

Discussion

Recent progress in our collective understanding of pathogenesis of infectious diseases has primarily been at the level of the microorganism or the interface between pathogen and eukaryotic cell. At one time animal infections were the principal experimental tool of investigators, but now generally less is known about the disease itself than about the molecular and cell biology of the etiologic agent. An impediment to reaching a fuller understanding of disease is the very complexity of the pathogenic process in an infected animal. Invasive or otherwise virulent microorganisms usually employ more than one strategy to enter and proliferate in the host and subsequently disseminate to new hosts. Moreover, the host responds to the pathogen in a variety of ways, specific as well as nonspecific. One way to sort out what is essential for virulence is to create mutants that are defective in one suspected virulence trait at a time and observe for the amount of disease in inoculated animals. This approach has led to the identification or confirmation of a number of virulence factors and the genes that encode them. An approach complementary to directed mutagenesis is the study of spontaneously occurring pathogen polymorphisms for their effects in animal hosts. Although this type of investigation often begins more empirically than directed mutagenesis, it has the advantage that manifestations may be qualitatively as well as quantitatively different between variants. When otherwise homogeneous populations have two or more alleles for a locus,

the different alleles may have been retained because in some circumstances proliferation of cells expressing the alternate allele is favored. The polymorphisms may only reflect a shift in the population from invaders to colonizers, but there may also be more profound differences in disease expression. The present study did not start with this second strategy in mind but ended showing its benefits.

The major findings were these: (a) clonal populations of two serotypes, A and B, of *B. turicatae* were similar in their ability to infect the blood of mice, to cause myocarditis and peripheral vestibular disorder, and to invade the eyes and testes; (b) serotype B but not A caused reddening and significant swelling of the joints, marked functional impairment on a walking bar, and severe arthritis by histologic examination; (c) serotype A but not B invaded the brain early in infection; and (d) serotype A penetrated monolayers of human umbilical vein endothelial cells more readily than did serotype B.

Invasion of the mouse brain by *B. hermsii* and *B. turicatae* was expected. Other investigators had reported infection of the brains of mice by relapsing fever borrelias (18, 43), and we recently established that *B. hermsii* invades the brain and cerebrospinal fluid of mice (33). Past investigators had suggested that certain strains of relapsing fever *Borrelia* species were neurotropic and that others were not (44). These observations were made on either different strains or uncloned populations of borrelias. In the present study we found that serotype A was neurotropic and serotype B was not during early infection. In three experiments, including two in which mixed infections were studied, both serotype A and B proliferated in the blood, heart, joints, and eye but only serotype A was found in the brain for at least 11 d of infection. Serotype A also infected the brain of immunocompetent BALB/c mice for at least 14 d (Kazragis, R., and A. G. Barbour, unpublished findings). These results were not attributable to a failure of serotype B to grow in the brain. Rather it appears to be the consequence of decreased entry of serotype B in comparison to A into the central nervous system. An *in vitro* correlate of this behavior was comparatively impaired penetration of an endothelial cell monolayer by serotype B. By day 31 serotype B was present in the brain in small numbers, but this was only after another serotype, C, had appeared. When simultaneous cultures of plasma and brain were compared, there was greater representation of C in the brain, a finding that suggests that serotype C is also neurotropic. Although *B. turicatae* invaded the brain early and persisted there for up to 100 d, there was no apparent inflammation of the brain by routine histopathologic examination.

Neurologic manifestations or complications are common in both relapsing fever and Lyme borreliosis (1, 12), but there have been, to our knowledge, no reports of neurologic disorders in experimental models of either relapsing fever or Lyme borreliosis. Evidence that the vestibular disorder resulted from a VIII cranial neuritis rather than from a central lesion or otitis were the following: the involvement appeared to be limited to the vestibular function and was unilateral, there was no evidence of involvement of either the inner ear or

the brain on pathologic examination, and it was limited in duration (31).

A specific antibody response to either serotype was not detected in the *scid* mice. Although a cell-mediated specific immune response can not be excluded, previous studies had shown that elimination of *B. turicatae* or *B. hermsii* from the blood is a T cell-independent immune response in mice (reference 45; A.G. Barbour, unpublished findings). It is likely that the resolution of the mucopurulent eye discharge, the arthritis, and the vestibular disorder is attributable to non-specific host responses. The *scid* mouse, as well as other laboratory animals with defined immune deficiencies, allows the aforementioned complexity of the animal-pathogen interaction to be reduced for experimental study.

An unexpected finding of the study was the marked arthritis in all mice infected with serotype B; A-inoculated mice had only mild to moderate inflammation of the joints that produced only minimal functional impairment on the walking bar. Arthritis had not been noted as a manifestation or complication of relapsing fever in humans or in experimental infections in animals. In our previous studies of *B. hermsii* we had never observed arthritis in mice, even in *scid* mice that were spirochetemic for weeks (unpublished findings). The character and distribution of the arthritis and synovitis were very similar to what has been observed in immunodeficient mice infected with *B. burgdorferi* (11). The severity of arthritis with *B. turicatae* appears to be the function of the Vmp. Although there is evidence that OspB of *B. burgdorferi* confers some degree of infectivity to that species (46), there has not been attribution of the severity of arthritis to a particular component in *B. burgdorferi*. An infectious but nonarthritogenic strain of *B. burgdorferi* has been characterized, but an association with a specific protein was not established (47).

The only discernible difference in components between serotypes A and B was their Vmp proteins. Although the population used to infect the first *scid* mice was not strictly clonal, the similarities between the serotype A and B populations with respect to protein and restriction enzyme fragment profiles indicate that the two populations were isogenic. The finding of four other size polymorphisms of the Vmp protein suggest that there is a repertoire of *vmp* genes and that variation of this protein in *B. turicatae* has a similar basis to that in the closely related species *B. hermsii*, which escapes immune clearance by multiphasic antigenic variation (35, 48). In *B. hermsii*, the Vmp proteins serve as the target for neutralizing antibody (22, 24), but another function for the protein has not been identified. The present study indicates that Vmp variability not only confers an ability to escape from the immune system but also a mechanism whereby the progeny of the founder population could sort out into different niches in the host. The differences in pathogen localization and organ involvement by *B. turicatae* in *scid* mice were evidence of this. Serotype A invaded the central nervous system earlier and penetrated endothelial cell monolayers better than did serotype B. Presumably this invasive capability demonstrated *in vitro* and *in vivo* was a function of VmpA. On the other

hand, VmpB was associated with significantly greater inflammation of the joints, notwithstanding the apparent equal ability of both serotypes to invade the joints. Vmp structure may be the proximate determinant of disease expression in this animal model.

A similar phenomenon was found during studies of the reovirus, another neurotropic pathogen. Field et al. (49) found that the viral hemagglutinin that determines serotype-specificity was also responsible for neurotropic potential and the type of nervous system disease that occurred. When a type 1 protein is expressed on the surface, it results in ependymal destruction and subsequent hydrocephalus. In contrast, if a type 3 protein is expressed, a necrotizing encephalitis with neuronal destruction but not ependymal damage occurs. Among bacteria the protein II family of proteins of *Neisseria gonorrhoeae* is the example that most closely resembles what we observed with *B. turicatae* (50). Protein II antigenic variation occurs during infection, and organisms expressing different protein II variants differ in their adherence to host cells, including phagocytic and epithelial cells.

We have up to this point discussed the results primarily within the context of borrelias and other infectious agents. Reviewing the disorders of the *B. turicatae* infected *scid* mice, we were struck by the similarity of their clinical presentation to what has been observed in humans with systemic Lyme borreliosis (1). Doubtless there are reasons to defer proposing

full equivalence between animal model and human disorder: the organisms are different species of *Borrelia*, and the mice were immunodeficient. Nevertheless, the *B. turicatae*-infected *scid* mice had more features of systemic Lyme borreliosis than have been observed to date in any other laboratory animals infected with *B. burgdorferi*, even those equivalently immunodeficient (51). In common with animals infected with *B. burgdorferi*, the *B. turicatae*-infected animals had myocarditis, and arthritis. Another similarity with Lyme borreliosis is the different stages and remitting course in the mice infected with *B. turicatae* (52). What the *B. turicatae* model uniquely offers is a neurologic disorder, consistent and persistent infection of the brain, and involvement of the eye. Of particular relevance for understanding the pathogenesis of the arthritis of Lyme borreliosis was the association of marked arthritis with a particular surface protein of the borrelia. One of the distinctions between Lyme borreliosis in North America and Europe is the infrequency of chronic arthritis in northern and eastern Europe (1, 53). This difference in disease expression may be the consequence of differences between the local etiologic agents (54). The invasion of and persistence in the brain by *B. turicatae* may also have relevance for studies of other chronic spirochetal diseases of the nervous system, syphilis, and the phenomenon of neurotropism in general.

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