Murine Model for Lymphocytic Tropism by *Borrelia burgdorferi*

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In vitro studies have demonstrated direct interactions between *Borrelia burgdorferi* and human B and T cells. However, largely because disseminated infections typically occur at very low density, little is known about associations between spirochetes and mammalian host cells in vivo. To assess whether spirochetes interact directly with lymphocytes in mammals, we developed a mouse model for lymphotropism. By repeatedly coincubating spirochetes with primary mouse lymphocytes that were immobilized by adherence to immunomagnetic beads, we were able to preferentially enrich cultures for or against bacteria with constitutive affinity for murine B and T cells. Populations of lymphotropically enriched, stock infectious, and lymphotropically depleted spirochetes were injected intradermally into mice. Lymphocytes were then purified from the blood and spleens of challenged mice and placed into spirochetal culture medium. Cultures of *B. burgdorferi* were obtained from primary lymphocyte preparations from mice challenged with each of the three populations of spirochetes. Recovery of lymphocyte-associated bacteria occurred within 1 h of challenge with enriched bacteria. Lymphocyte preparations from mice challenged with stock infectious and lymphotropically depleted bacteria produced cultures after 1 day postchallenge. All lymphocyte preparations were culture negative after 1 week. These results demonstrate that lymphotropic *B. burgdorferi* is infectious in mice and suggest that associations between spirochetes and lymphocytes occur in vivo. The results also suggest that factors involved in lymphocytic binding may be inducible in vivo. Thus, this system provides a model for studying the role of such interactions in mammalian infections.

Shortly after *Borrelia burgdorferi* was identified as the infectious agent of Lyme disease (3), lymphocytic involvement in both the responses to and pathological effects of this spirochetosis was recognized in patients and experimental animals (1, 4–7, 12–21, 23–25, 28–30, 32, 36). Immune responses have been well studied in Lyme disease (5, 28). In contrast, although numerous studies have reported pathological findings involving lymphocytes, comparatively little is known about the nature of the relationship between the spirochetes and mammalian lymphocytes that lead to manifestations such as lymphopenia (28), inflammatory lymphocytic infiltrations and aggregates (6, 7, 12–17, 19, 23, 25, 29), lymphocytoma (7, 24), pseudo-lymphoma (20), and malignant lymphoma (1, 4, 18, 21, 30). Previous work has shown that *B. burgdorferi* cells and components, including major surface lipoproteins and extracellular membrane vesicles or blebs, are potent lymphocytic mitogens, inducing polyclonal B-cell proliferation and immunoglobulin M secretion (22, 26, 31, 33–36). More recently, direct adherence, invasion, and killing of both primary and cultured human B and T cells by *B. burgdorferi* were demonstrated in vitro (10). In that study, spirochetes preferentially targeted human B and T cells in primary mixed mononuclear cell preparations. Both the avidity of spirochetes for lymphocytes and the susceptibility of B and T cells to invasion and killing by the spirochetes were phenotypically selectable. However, significant lymphocytic killing was observed only in coinoculation mixtures containing more than one spirochete per lymphocyte, a ratio far exceeding that believed to occur in natural infections (10).

In a preliminary study to determine whether an animal model for direct spirochetal interactions with lymphocytes could be

developed, we found that *B. burgdorferi* could adhere to purified primary murine lymphocytes in vitro (9). In this study, we used such adherence to cultivate populations of spirochetes exhibiting and lacking constitutive affinity for mouse B and T cells in vitro. These enriched and depleted populations, along with stock infectious spirochetes, were then used to experimentally challenge mice. Over a period of 3 weeks, splenic and circulating lymphocytes were recovered from challenged and uninfected mice and used as sources of inoculum for *B. burgdorferi* cultures. The results provide evidence of in vivo lymphotropism in mice.

(Portions of this study were summarized in an abstract for the VIII International Conference on Lyme Borreliosis and Other Emerging Tick-Borne Diseases, June 20 to 24, 1999, Munich, Germany.)

MATERIALS AND METHODS

Bacteria. Cultures of *B. burgdorferi* sensu stricto Sh-2-82, a tick isolate from Shelter Island, N.Y. (27), and derivatives of this strain described herein were maintained in BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) at 36°C as previously described (2). For intrinsic radiolabeling, late-log-phase cultures were diluted 1:100 into BSK-H medium containing 2 mCi of [³⁵S]methionine-cysteine mixture (New England Nuclear, Boston, Mass.) per liter and incubated as above. Radiolabeled spirochetes were recovered by centrifugation at 1,500 × g for 5 min and washed by repeated cycles of gentle resuspension in Hanks' buffered salt solution (HBSS; Life Technologies, Inc., Gaithersburg, Md.) and centrifugation. The initial cultures used in this study had been passaged four times in vitro since being reisolated from a urinary bladder of an RML white mouse (27).

Lymphocytes. Primary naive mouse lymphocytes were purified from fresh heparinized visceral blood essentially as previously described (9). Care was taken to avoid microbial contamination during all procedures. Erythrocytes were removed by centrifugation of the blood through lymphocyte separation medium (ICN Biomedicals, Aurora, Ohio) according to the manufacturer's instructions. The suspended leukocytes were transferred to an equal volume of RPMI medium containing 10% certified fetal calf serum (RPMI-serum) (Life Technologies), washed twice by gentle centrifugation for 3 min at 800 × g, and resuspended in 2 ml of HBSS. Murine B cells (B220) and CD4 (L3T4) and CD8

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(Lyt2)-positive T cells were purified from the mixed mononuclear cells by incubation with pooled immunomagnetic beads specific for these markers (DynaL, Inc., Lake Success, N.Y.) as instructed by the manufacturer except that HBSS was substituted for phosphate-buffered saline. After initial recovery of bead-immobilized lymphocytes, RPMI-serum was used for resuspension and washing steps. The concentration of immobilized lymphocytes recovered was estimated by microscopic examination in a Petroff-Hausser chamber, and the volume of the suspension was adjusted to approximately 2×10^6 cells per ml of RPMI-serum.

Lymphotropic spirochete enrichment and depletion. Spirochetes with affinity for purified murine lymphocytes were enriched by coinubation with immunomagnetic bead-immobilized lymphocytes. Suspensions of *B. burgdorferi* containing approximately 2×10^7 spirochetes per ml of BSK-H medium and suspensions of bead-immobilized lymphocytes were cooled in a water bath over a 30-min period to 4°C. One-milliliter aliquots of each suspension were mixed and maintained at 4°C for 1 h. Resulting association complexes of beads, lymphocytes, and adherent and intracellular spirochetes were concentrated with a magnet and washed twice with fresh changes of 10 ml of cold BSK-H medium. Following the washes, 3 ml of BSK-H medium was added. A 20- μ l sample was removed from each preparation for dark-field examination to confirm spirochetal adherence. Another 100- μ l sample was removed for scanning electron microscopy as previously described (9). The remaining portion of each mixture was incubated at 36°C. Spirochetes remaining in suspension after removal of bead-immobilized lymphocytes, and associated bacteria were also cultivated and termed lymphotrophically depleted. Four successive rounds of enrichment and depletion were performed before experimental infections were attempted.

Lymphotropic quantitation. Associations between *B. burgdorferi* and primary murine lymphocytes were quantified using intrinsically radiolabeled spirochetes. Triplicate 1-h coinubation mixtures containing 10:1 ratios of 35 S-labeled spirochetes and bead-immobilized lymphocytes, as above, were harvested with magnets, washed repeatedly with HBSS, and measured by liquid scintillation. Bacterial binding specificity was assessed using pooled immunomagnetic beads lacking attached lymphocytes.

Experimental challenge. Intradermal experimental challenge was performed in RML white mice (11). Seven groups of three weanling (4- to 5-week-old) mice were injected intradermally on the back with 0.1 ml of BSK-H medium containing approximately 10^6 spirochetes from populations of lymphotrophically enriched, lymphotrophically depleted, and stock infectious *B. burgdorferi*. Some experiments also included mice, which were sham challenged with 0.1 ml of BSK-H medium alone. Each group of mice was sacrificed under anesthesia at time points ranging from 1 h to 21 days postchallenge. Visceral blood and spleens were collected aseptically for subsequent recovery of lymphocytes and plasma. Blood was added to an equal volume of RPMI-serum containing 40 U of heparin per ml. Lymphocytes were prepared using Ficoll and immunomagnetic beads as described above, and the plasma layer was retained from the Ficoll gradient for later immunoblot analysis. Spleens were dissected and macerated into heparinized RPMI-serum with sterile scalpel blades. The resulting cell aggregates were further dissociated by repeated passage through 20-gauge hypodermic needles. Resulting crude splenocyte preparations were washed twice by centrifugation for 3 min at $800 \times g$ followed by resuspension in 2 ml of HBSS. Lymphocytes consisting of B cells and CD4- and CD8-positive T cells were purified from the visceral blood leukocyte and splenocyte preparations by incubation with immunomagnetic beads as described above. Resulting bead-lymphocyte complexes were examined by dark-field microscopy and transferred to fresh BSK-H medium for culture of *B. burgdorferi*. Cultures were examined after 10 to 12 days of incubation by dark-field microscopy for evidence of spirochetal growth. Cultures in which no growth occurred were reexamined after 3 weeks of incubation and then discarded.

Immunoblot analysis. Plasma samples from each mouse were retained, diluted to a final ratio of 1:100 in phosphate-buffered saline, and used to probe *B. burgdorferi* Marblot strips (MarDx Diagnostics, Inc., Carlsbad, Calif.) according to the manufacturer's instructions. The strips were then labeled with a 1:1,000 dilution of goat anti-mouse immunoglobulin G (heavy and light chain)-horseradish peroxidase conjugate (Sigma) and visualized by chemiluminescence (Amersham Pharmacia, Piscataway, N.J.).

RESULTS

To enrich for populations of *B. burgdorferi* exhibiting or lacking murine lymphotropic activity, low-passage spirochetes were coinubated aseptically with immunomagnetic bead-immobilized primary mouse B cells and CD4- and CD8-positive T cells. Spirochetes that adhered to or penetrated the lympho-

cytes and those that did not were separated via the magnetic beads, and each fraction was transferred to BSK-H medium for culture. Dark-field microscopic examination of the resulting complexes formed between immobilized lymphocytes and adherent spirochetes showed numerous lymphocytes that had one to several bacteria attached. There were no noticeable differences in the levels of spirochetal aggregation among lymphocyte-associated and nonadherent bacteria (data not shown). Whereas mixtures in which beads were preincubated with mouse lymphocytes consistently produced actively growing spirochetal cultures, no cultures were obtained from five mixtures containing spirochetes and beads preincubated in RPMI-serum alone. Scanning electron microscopic examination (Fig. 1) showed that lymphocytes were intimately bound to the immunomagnetic beads. Spirochetes were attached to lymphocyte cell surfaces or to filopodia extending from the cells, but not directly to the immunomagnetic beads.

Attachment of intrinsically radiolabeled spirochetes to primary mouse lymphocytes was also assessed after four rounds of enrichment and depletion. The percentage of lymphotrophically enriched spirochetes that adhered to immobilized lymphocytes was nearly twice the value obtained for stock bacteria (Fig. 2). In contrast, the percentage of lymphotrophically depleted spirochetes that bound to lymphocytes was only marginally greater than the level of binding to immunomagnetic beads alone.

As in the in vitro adherence assays, four rounds of successive enrichment or depletion of lymphotropic spirochetes were conducted before mice were challenged by intradermal injection. At various time points after challenge, spleens and visceral blood were removed from cohorts of three mice. Using immunomagnetic beads, lymphocytes were purified, examined by dark-field microscopy, and placed into spirochetal growth medium for culture. No spirochetes were observed by microscopic examination of such mixtures. However, cultures were obtained from many preparations. Table 1 shows the results of the culture experiments. In total, cultures of lymphocyte-associated *B. burgdorferi* were recovered from the blood or spleens (or both) from 32 of 63 mice. Such recovery occurred with each population of spirochetes. Stock, enriched, and depleted spirochetes were cultured from 9 of 21, 16 of 21, and 7 of 21 animals, respectively. In each case the ability to culture spirochetes from the lymphocyte preparations was transient, varying with respect to the rate at which lymphocyte-associated spirochetes were first recovered. Cultures were first recovered from stock and depleted populations 1 day after intradermal challenge. In contrast, cultures were recovered from animals challenged with enriched populations of spirochetes within 1 h. At the 1- and 4-h time points, the difference in recovery of cultures from all three animals challenged with enriched spirochetes, versus none of those challenged with stock or depleted populations, was statistically significant ($P < 0.001$ by Student's *t* test). Cultures were not obtained at 7 days postchallenge. At 21 days, however, stock infectious and enriched spirochetes were isolated in association with circulating and splenic lymphocytes, respectively.

Plasma collected from the experimentally infected and control mice was used to probe immunoblot strips containing electrophoresed whole-cell *B. burgdorferi* extracts (Fig. 3). By day 5 postchallenge, all infected mice developed significant

FIG. 1. Attachment of *B. burgdorferi* to primary murine lymphocytes. Scanning electron microscopy of coinubation mixtures containing *B. burgdorferi* and immunomagnetic bead-purified lymphocytes (L) showed that spirochetes (S) adhere to immobilized cells but not to the antibody-coated beads (B). Examination of paired stereomicrographs showed that attachment occurred at variable locations along the axis of the spirochete. However, adherence was observed most frequently in association with the terminal ends of filopodia extending from the surface of immobilized lymphocytes. Scale bar, 0.5 μ m.

humoral responses to *B. burgdorferi* antigens. No bands were detected in any sham-challenged plasma samples.

DISCUSSION

These findings demonstrate that *B. burgdorferi* can adhere to mouse B and T cells and that spirochetes with affinity for murine lymphocytes can establish disseminated infections in mice. Furthermore, the results showed that *B. burgdorferi* can exhibit lymphotropism in vivo and can be recovered in intimate and stable association with lymphocytes purified from infected mice. Such results are consistent with in vitro studies of direct interactions between *B. burgdorferi* and mammalian lymphocytes (8–10), with previous findings of pathological changes involving lymphocytes in infected mammals, and with cultivation of spirochetes from blood and spleen samples (1, 4, 6, 7, 12–21, 23–25, 27, 29, 30, 32, 36). However, extensive review of literature revealed no previous reports demonstrating recovery of viable spirochetes in stable association with specific cells from infected mammals.

Previous in vitro work had shown that phenotypically selectable populations of spirochetes could preferentially target, adhere to, and invade primary human B and T cells in mixed mononuclear cell preparations (10). Although the avidity with which the spirochetes attacked the lymphocytes was inversely correlated with continued passage in culture, whether or not the lymphotropic proportion of bacteria in any given culture was infectious in mammals remained unclear. Subsequent ex-

FIG. 2. Attachment of spirochetes to immobilized murine lymphocytes. 35 S-labeled spirochetes were incubated with murine lymphocytes attached to immunomagnetic beads or with the beads alone. After a 1-h incubation at 4°C, the beads were retained, repeatedly washed, and measured for radioactivity. In these experiments the background emission, which averaged 26 cpm (standard deviation, ± 2.3 cpm), was subtracted from each of the raw values obtained. The error bars represent 2 standard deviations. Spirochetes that were enriched in vitro for adherence to murine lymphocytes exhibited significantly greater affinity for the immobilized lymphocytes than the stock infectious and lymphotropically depleted populations. Binding by the depleted spirochetes to immobilized lymphocytes was only marginally greater than the binding to beads alone.

TABLE 1. Recovery of spirochetal cultures from cohorts of three challenged mice

Prepn	No. of cultures recovered at indicated time point postchallenge						
	1 h	4 h	1 day	2 days	3 days	7 days	21 days
Stock	0	0	2	3	2	0	1
Blood							
Spleen	0	0	2	1	2	0	0
Enriched	3	3	3	3	2	0	0
Blood							
Spleen	1	3	3	3	2	0	1
Depleted	0	0	2	2	3	0	0
Blood							
Spleen	0	0	2	1	2	0	0

periments showed that primary murine lymphocytes, purified by adherence to immunomagnetic beads, could also be targeted in vitro (9). In this study we found that spirochetes that adhered to the bead-immobilized lymphocytes remained viable and could be selectively cultured after magnetic separation from nonadherent bacteria. This enabled enrichment of spirochetes exhibiting considerable constitutive avidity for lymphocytes. Such spirochetes were able to form stable interactions with the cells in less than 1 h at low temperature. Conversely, the system also allowed segregation of spirochetes with little or no constitutive lymphotropic activity. Both lymphocyte-binding and nonbinding phenotypes were clearly present in the original stock infectious population.

Intradermal challenge with 10^6 lymphotropically enriched *B. burgdorferi* resulted in disseminated infections in most if not all experimental animals. Immunoblot analysis demonstrated clear seroconversion by 5 days postchallenge. And all animals challenged with lymphotropically enriched spirochetes were culture positive at time points ranging from 1 h to 3 days. Recovery of isolates from splenic and circulating lymphocyte preparations at 21 days suggests that lymphotropic and stock spirochetes, respectively, were both capable of inducing persistent infections. Lymphocyte-associated spirochetes were also recovered from both the stock infectious and depleted populations. This finding indicates that constitutive lymphotropism, as assessed in vitro, is not required for successful colonization of mice. These results also suggest that direct interaction between spirochetes and lymphocytes during mammalian infection is not an artifact of in vitro phenotypic segregation but may be an integral step in the infectious process. Recovery of lymphocyte associated spirochetes from stock and lymphotropically depleted populations was delayed until 1 day postchallenge. In vitro differences in constitutive adherence to immobilized murine lymphocytes suggests that this delay may reflect a period of induction of one or more lymphotropic factors in vivo or differences in the nature of factors that contribute to binding under in vitro versus in vivo conditions. Further experimentation on the molecular and biological bases of lymphotropism may reveal whether the phenotypic differences observed in vitro and under selective pressure in vivo result from de novo expression of lymphotropic factors, varying rates of spirochetal motility from the injection site, or possibly variation in expression of factors directing alternate tropisms.

Recovery of viable spirochetes in association with immuno-

magnetic bead-purified B and T cells suggests that intimate interactions between the bacteria and cells occur in vivo. Such associations remained intact through several centrifugations, washings, and magnetic separations, suggesting a physically stable interaction. Although these experiments could not rule out the possibility that spirochetes might have been bound directly to the immunomagnetic beads or to other murine cells, contributing to the cultures obtained, we believe that those possibilities are unlikely. In this study, which used 10^7 intrinsically labeled *B. burgdorferi* per ml of buffer in a 10:1 ratio with bead-immobilized lymphocytes, we detected only minimal levels of radioactivity in preparations containing immunomagnetic beads lacking lymphocytes. Since *B. burgdorferi* was not observed microscopically in any of the splenocyte and circulating mononuclear cell preparations, such samples probably contained less than 10^4 spirochetes per ml. Similarly, no cultures were recovered from five preparations obtained by incubating cultured spirochetes with immunomagnetic beads alone. Furthermore, erythrocytes are largely removed from the blood samples by gradient centrifugation. Also, a previous study found that *B. burgdorferi* did not bind to residual murine mononuclear cell preparations from which lymphocytes had been removed by equivalent immunomagnetic bead preparations (9). Further studies may identify the specific factors that mediate spirochete-lymphocyte binding, and hence define and confirm the specific cell type(s) involved in these in vivo interactions.

Although microscopic examination of complexes formed between the beads, cells, and bacteria, derived from the blood

FIG. 3. Humoral responses of mice to intradermal challenge with lymphotropically enriched *B. burgdorferi*. Mice were injected with approximately 10^6 spirochetes in 0.1 ml of BSK-H medium or with BSK-H medium alone. Plasma samples were retained from visceral blood and used at 1:100 dilution for probing immunoblot strips containing electrophoresed *B. burgdorferi* proteins. By day 5 postchallenge, all animals injected with spirochetes, lanes 1 to 3 at each time point, exhibited a significant antibody response to the organisms. Plasma retrieved from sham-challenged control animals (C) did not contain detectable anti-*B. burgdorferi* antibodies. The apparent molecular masses of major bands are labeled in kilodaltons.

and spleens of infected mice, failed to reveal any spirochetes, the same preparations produced active spirochetal cultures. It is presumed that the concentration of spirochetes recovered in association with the lymphocytes was below a threshold needed for direct microscopic observation, roughly between 10^3 and 10^4 bacteria per ml. Thus, we were unable to determine whether the spirochetes were adherent to cell surfaces, intracellular, or both. Once removed from the host and cooled during experimental purification procedures, spirochetes and lymphocytes maintained a stable association. However, the duration of interactions between spirochetes and lymphocytes in vivo remains in question. The transient nature of recovery of lymphocyte-associated spirochetes suggests a temporary or perhaps cyclic interaction.

As parasitic bacteria with obligate alternate acarid and mammalian hosts, *B. burgdorferi* spirochetes likely encounter lymphocytes during multiple phases of the infectious cycle. Thus, development of physical interactions between the spirochete and lymphocytes hypothetically could influence several phases of the cycle, such as spirochetal activity within the tick during feeding, colonization and dissemination within mammalian hosts, immune recognition of and response to the infection, and eventual transmission of the spirochetes back to feeding ticks. Use of this murine model to follow associations between spirochetes and mammalian lymphocytes in vivo may help us understand whether these or other possible factors contribute to the virulence of *B. burgdorferi* or elicit pathogenic consequences in Lyme disease.

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