Murine Model for Lymphocytic Tropism by *Borrelia burgdorferi*

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Received 19 October 2000/Returned for modification 30 November 2000/Accepted 7 December 2000

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 lymphonic 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 spirochosis 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 passed four times in vitro since being reisolated from a urinary bladder of an RML white mouse (27).

*Lympocytes.* Primary naïve 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) and centrifugation. The initial cultures used in this study had been passed four times in vitro since being reisolated from a urinary bladder of an RML white mouse (27).

*REFERENCES*

(Ly2)-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-Haussuer chamber, and the volume of the suspension was adjusted to approximately \(2 \times 10^6\) cells per ml of RPMI-serum.

**Lymphotropic spirochete enrichment and depletions.** Spirochetes with affinity for purified murine lymphocytes were enriched by coincubation with immunomagnetic bead-immobilized lymphocytes. Suspensions of *B. burgdorferi* containing approximately \(2 \times 10^7\) 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-ml sample was removed from each preparation for dark-field examination to confirm spirochetal adherence. Another 100-μl 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 lymphotrope. Additional cultures were prepared by successive rounds of enrichment and depletions 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 coincubation 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 RM1 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 \(10^8\) spirochetes from populations of lymphotropically enriched, lymphotropically 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 1,000 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-splenocyte 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 (Marblot 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). Blots were stained whole-cell *B. burgdorferi* extracts (Fig. 3). By day 5 postchallenge, all infected mice developed significant...
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. 1.** Attachment of *B. burgdorferi* to primary murine lymphocytes. Scanning electron microscopy of coincubation 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 μm.

**FIG. 2.** Attachment of spirochetes to immobilized murine lymphocytes. 35S-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.
rates of spirochetal motility from the injection site, or possibly
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of lymphotropism may reveal whether the phenotypic differ-
Further experimentation on the molecular and biological bases
contribute to binding under in vitro versus in vivo conditions.
lymphotropically depleted populations was delayed until 1 day
postchallenge. In vitro differences in constitutive adherence to
lymphotropically enriched populations. This finding indicates that 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 per-
sistent infections. Lymphocyte-associated spirochetes were
also recovered from both the stock infectious and depleted
populations. This finding indicates that constitutive lympho-
tropism, as assessed in vitro, is not required for successful
colonization of mice. These results also suggest that direct
interaction between spirochetes and lymphocytes during mamm-
alian infection is not an artifact of in vitro phenotypic seg-
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 differ-
ences 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 $10^7$ intrin-
sically labeled B. burgdorferi per ml of buffer in a 10:1 ratio with
bead-immobilized lymphocytes, we detected only minimal lev-
els of radioactivity in preparations containing immunomag-
netic beads lacking lymphocytes. Since B. burgdorferi was not
observed microscopically in any of the splenocyte and circulat-
ing mononuclear cell preparations, such samples probably con-
tained less than $10^4$ spirochetes per ml. Similarly, no cultures
were recovered from five preparations obtained by incubat-
ing 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 mononu-
clear cell preparations from which lymphocytes had been re-
moved 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 be-
tween the beads, cells, and bacteria, derived from the blood

<table>
<thead>
<tr>
<th>Prepn</th>
<th>No. of cultures recovered at indicated time point postchallenge</th>
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<tbody>
<tr>
<td>Stock Blood</td>
<td>0 0 2 3 2 0 1</td>
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<tr>
<td>Spleen</td>
<td></td>
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<tr>
<td>Enriched Blood</td>
<td>3 3 3 3 2 0 0</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
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<tr>
<td>Depleted Blood</td>
<td>0 0 2 2 3 0 0</td>
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Although microscopic examination of complexes formed be-
tween the beads, cells, and bacteria, derived from the blood

![FIG. 3. Humoral responses of mice to intradermal challenge with lymphotropically enriched B. burgdorferi.](http://iai.asm.org/)
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.

**ACKNOWLEDGMENTS**

We thank Tom Schwan and William Whitmire for critical review of the manuscript.

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**INFECT. IMMUN.**

Editor: D. L. Burns

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