Infection of Laboratory Mice with the Human Granulocytic Ehrlichiosis Agent Does Not Induce Antibodies to Diagnostically Significant Borrelia burgdorferi Antigens

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Laboratory diagnosis of Borrelia burgdorferi is routinely made by an enzyme-linked immunosorbent assay, with positive results confirmed by Western blot analysis. Concern has been raised that false-positive diagnoses may be made on the basis of serologic cross-reactivity with antibodies directed against other bacterial pathogens, in particular the agent of human granulocytic ehrlichiosis (HGE). The present study made use of a mouse model to ascertain the validity of these concerns. Two different strains of mice were inoculated with the HGE agent and assayed for production of polyclonal and monoclonal antibodies to antigens of both of these bacteria. Infection of mice with the HGE agent does not induce diagnostically significant B. burgdorferi serologic cross-reactions.

Many patients diagnosed with Lyme disease also have serologic evidence of infection by microbes other than Borrelia burgdorferi, especially the human granulocytic ehrlichiosis (HGE) agent and Babesia microti (7, 9, 11). These pathogens are transmitted by Ixodes scapularis in the northeastern United States and can share Peromyscus leucopus (white-footed mouse) as a reservoir host. An individual mouse and Ixodes sp. tick may harbor or cotransmit diverse microorganisms. Likewise, humans may be coinfected with one or more of the above-mentioned pathogens due to a bite from a multiply infected tick or due to sequential tick bites (10). In a hyperendemic focus for Lyme disease, up to 26% of I. scapularis ticks analyzed were coinfected with the HGE agent and B. burgdorferi (13). It is well recognized that B. burgdorferi or HGE agent infection will induce antibodies reactive with highly conserved protein antigens, such as heat shock proteins (HSPs), and that these may potentially be cross-reactive (3, 6, 14). However, these reactions are not considered diagnostic of Lyme disease when Western blot analyses are performed (2, 3, 6). Thus, it was surprising that in a study of HGE, 9 of 10 consecutive patients had Western blot-verified serologic evidence of concurrent B. burgdorferi infection in the absence of corroborating clinical evidence for Lyme disease. However, the probability of transmission of both agents by a single tick bite was estimated at only 0.00003 (15).

By inoculating inbred mice with the HGE agent, Hofmeister et al. (5) detected antibodies that cross-reacted with at least five B. burgdorferi antigens, including OspC, the predominant antigen expressed upon infection of the vertebrate host. Such findings suggested that the HGE agent may induce Western blot-demonstrated serologic reactions currently considered diagnostically significant Lyme disease despite the absence of infection by B. burgdorferi. To test this hypothesis, we conducted experimental infections of mice with the HGE agent and performed enzyme immunoassays for antibodies directed against recombinant B. burgdorferi antigens, including p41-G, OspC, OspE, and OspF. This approach was designed to directly assess if infection with HGE agent induces antibodies to diagnostically significant B. burgdorferi antigens under controlled circumstances that include the absence of tick bite and prior exposure.

To establish infection in a murine system, 16 laboratory-reared P. leucopus and 12 C3H/HeJ mice were inoculated with the HGE agent BDS strain obtained from an experimentally infected horse (courtesy of John Madigan, University of California, Davis). The inoculum was administered to the mice by intraperitoneal injection of 0.5 ml of whole acid-citrate-dextrose (ACD)-anticoagulated blood that was calculated to contain 107 infected neutrophils. In addition, eight P. leucopus and seven C3H/HeJ mice were mock infected with 0.5 ml of ACD-anticoagulated whole blood from a healthy horse that had no Ehrlichia equi or HGE agent antibodies. At 21 days postinoculation, blood was obtained from the mice by retro-orbital sampling, and plasma was examined for HGE agent antibodies by using an indirect fluorescent antibody method as previously described (1). EDTA-anticoagulated blood obtained at days 7, 14, 21, 28, 45, and 90 and tissues from mice obtained at necropsies performed on days 21, 45, and 90 were assayed for the presence of HGE agent DNA by PCR after a DNA preparation technique for blood was applied as previously described (4). Tissue samples were assayed by the same protocol except that DNA was extracted from tissues by using the QIAamp Tissue kit (Qiagen Inc., Santa Clarita, Calif.). Plasma samples were also obtained at 28 days postinoculation and were tested for polyvalent antibodies to B. burgdorferi whole-cell antigen and recombinant OspC, OspE, OspF, and p41-G antigens in enzyme immunoassays (ELISA), as previously described (8). A titer of $\geq 160$ was considered positive.

All animal protocols were approved by the Institutional An-
TABLE 1. Polyvalent antibody (immunoglobulin G and immunoglobulin M) titers in the 11 laboratory mice (n = 36) with any serologic reaction to recombinant B. burgdorferi antigens after experimental HGE agent or mock infection

<table>
<thead>
<tr>
<th>Mouse serum sample</th>
<th>Titer of antibody to antigen*</th>
<th>OspE</th>
<th>OspF</th>
<th>p41-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGE agent-infected</td>
<td>C1</td>
<td>640</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>–</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>C5</td>
<td>320</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C6</td>
<td>–</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>C8</td>
<td>–</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>C12</td>
<td>320</td>
<td>320</td>
<td>–</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>C20</td>
<td>–</td>
<td>–</td>
<td>320</td>
</tr>
<tr>
<td>P. leucopus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGE agent-infected</td>
<td>P11</td>
<td>–</td>
<td>160</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>P15</td>
<td>–</td>
<td>160</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>P16</td>
<td>–</td>
<td>–</td>
<td>2,560</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>P17</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
</tbody>
</table>

* No serologic responses to either B. burgdorferi whole-cell antigen or OspC recombinant antigen were detected in any mouse.

** Indicates that samples were interpreted as negative because they failed to react at the screening dilution of 1:160.

Animal Care and Use Committee of the University of Maryland School of Medicine and were maintained by the Program for Comparative Medicine at the University of Maryland School of Medicine.

By day 21, all infected animals developed HGE agent antibodies, and all mock-infected mice were seronegative in testing against this antigen. PCR assays of blood, bone marrow, or spleen proved that 10 of 16 P. leucopus mice and 8 of 12 C3H/HeJ mice had become infected by intraperitoneal inoculation. Plasma samples for analysis were available for 13 P. leucopus mice and 10 C3H/HeJ mice infected with HGE agent and for 7 P. leucopus mice and 6 C3H/HeJ mice that were mock infected. For P. leucopus, two HGE agent-infected mice and one mock-infected mouse were necropsied on day 21 and plasma was not obtained from one HGE agent-infected animal on day 28. For C3H/HeJ mice, three were necropsied on day 21 (two HGE agent-infected mice and one mock-infected mouse). Overall, a total of 36 animals were tested for B. burgdorferi antibodies.

The results of EIA for antibodies to native B. burgdorferi whole-cell antigen and to recombinant antigens OspC, OspE, OspF, and p41-G on day 28 plasma samples are shown in Table 1. All mice remained seronegative for antibodies to B. burgdorferi whole-cell antigen and recombinant OspC, a diagnostically important antigen. Of 36 samples, 2 contained OspE antibodies, 5 contained OspF antibodies, and 6 contained antibodies to p41-G, generally in low titers (Table 1). Antibodies to recombinant p41-G and OspF were detected in two mock-infected animals, a rate that was not different from that observed in HGE agent-infected mice, whether PCR-positive or PCR-negative (minimum P value of 0.3395, χ² test).

A major confounding factor in the serologic diagnosis of Lyme disease, whether in the context of HGE or not, is the relative lack of specificity of serologic tests that use conserved bacterial HSPs, including those of B. burgdorferi, are not included as part of the diagnostic criteria although they are known to develop in humans after development of HGE (2). Thus, we elected to examine only antibodies to proteins that are considered to have serodiagnostic significance for Lyme disease.

In the natural setting, mice and humans may be colonized by or infected with many different microorganisms, and so it is conceivable that other microbes could induce antibodies reactive with B. burgdorferi antigens. Although the appearance of B. burgdorferi antibodies in the context of HGE may or may not indicate concurrent infection (10, 15), neither should it be interpreted to indicate induction of B. burgdorferi antibodies by...
HGE agent antigens. Alternatively, serologic responses that indicate coinfection in humans may reflect immune response to unidentified antigens, nonspecific polyclonal activation, or immune restimulation after prior B. burgdorferi infection (10). One possible cause relates to the differential expression of bacterial antigens in the tick vector and host, as is known to occur with B. burgdorferi but which has not been investigated with the HGE agent (12). Previous studies that demonstrated such serologic reactions were carried out only in the context of natural infection, a situation likely to be confounded by a number of factors including tick transmission, the presence of multiple tick bites, and the possibility of concurrent or previous infection (15). Thus, it is unlikely that infection with the HGE agent contributes substantially to the serodiagnostic confusion. However, proof of coinfection still requires more convincing evidence than B. burgdorferi serologic reactions alone and should include a careful clinical evaluation for consistent signs and symptoms and the use of other laboratory diagnostic procedures, such as culture or PCR.

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REFERENCES