Passive Transfer of Antibody to Ehrlichia risticii Protects Mice from Ehrlichiosis

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Mice that recovered from Ehrlichia risticii infection were immune to a challenge dose of 100 50% lethal doses. Immune or normal mouse serum was passively transferred to mice challenged with E. risticii. Clinical signs of ehrlichiosis were completely prevented in 22 of 24 recipients of immune serum, and the onset of signs of illness was delayed in the remaining two mice compared with the onset of illness in 24 of 24 recipients of nonimmune serum. Purified immunoglobulin G (IgG) was used to passively protect mice from infection with E. risticii. All 15 mice that received IgG from normal serum but none of the 15 mice that received IgG from immune serum developed clinical signs of illness. Antibodies in immune mouse serum immunoprecipitated [35S]methionine metabolically labeled E. risticii proteins with apparent molecular masses ranging from 14 to 90 kDa. The major antigens recognized by dilute immune serum in immunoblot analysis had molecular masses of 62, 53, 40, 33, 27, and 25 kDa, and the 62- and 27-kDa antigens were prominent in immunoprecipitations with dilute antibody. Antigens with molecular masses of 62, 53, 40, 33, and 27 kDa are likely surface exposed, as determined by immunoprecipitation of 125I-labeled organisms with immune mouse serum.

Ehrlichia risticii, a rickettsial pathogen, is the etiological agent of equine monocytic ehrlichiosis (synonym, Potomac horse fever), a systemic disease of equids (5, 10, 18). The disease is characterized by leukopenia, biphasic fever, anorexia, mild to profuse diarrhea, colic, edema of the legs and abdomen, and laminitis, with a case fatality rate as high as 30% (6, 12, 27). E. risticii causes a monocyte-associated bacteremia and is consistently found in monocytes, macrophages, and glandular epithelial cells in the intestinal tracts, primarily in the large colon, of infected equids (16, 19). Immunity develops in horses that have recovered from E. risticii infection, with resistance to reinfestation for at least 20 months (14). Mechanisms of protective immunity to E. risticii are unknown. However, involvement of antibody is indicated by the fact that serum from ponies immunized with an E. risticii bacterin protected mice, which develop dose-dependent morbidity and mortality (11, 17), against challenge (25a). Antibody directed towards surface-exposed antigens of other rickettsial organisms has been used to block the initial attachment and penetration steps in infection (8) and to enhance phagocytosis, phagosome-lysosome fusion, and the subsequent destruction of the organism (3, 7).

In this study, we tested the hypothesis that antibody can mediate immunity to E. risticii and identified antigens that may be important in the protective immune response, using the mouse model.

MATERIALS AND METHODS

E. risticii source. E. risticii ATCC HRC-IL (10) was propagated in a mouse macrophage (P388D1) cell line and used for all experiments. The percentage of P388D1 cells infected with E. risticii was determined by indirect fluorescent antibody assay as previously described (21) with immune mouse serum and a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody for detection. The inoculum of E. risticii to infect mice was prepared by washing infected P388D1 cells three times in phosphate-buffered saline (PBS). The percent infected P388D1 cells was determined, and the cells were resuspended to a known number of infected cells per milliliter of PBS.

Animals. Eight-week-old female Sprague-Dawley non-Swiss albino mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were used in all experiments. Mice were monitored for signs of E. risticii infection (squinted eyes, huddling, lethargy, rough coats, and diarrhea [11]) twice daily. Observations of mice in all experiments were confirmed by a second observer unaware of the treatment groups.

Determination of 50% lethal doses (LD50). Mice were assigned to six groups of seven mice. Each group was inoculated with log10 dilutions of E. risticii-infected P388D1 cells (10¹ to 10⁹). Eight control mice were inoculated with 9 x 10⁷ uninfected P388D1 cells. Mice were observed twice daily for clinical signs of infection.

Demonstration of protective immunity. Mice which had recovered from E. risticii infection in the LD50 determination were inoculated with 10⁵ E. risticii-infected P388D1 cells to boost their immune response. Serum from these mice and from control mice inoculated with uninfected cells was obtained, and the antibody titers were determined. All mice were then challenged with 100 LD50 and monitored for clinical signs of E. risticii infection.

Experiment 1: passive transfer of immune serum. Two groups of nine mice each were inoculated intraperitoneally with 10⁸ E. risticii-infected cells. At the time of inoculation and 3 days postinoculation (p.i.), the mice also received 80 μl (day 0) and 100 μl (day 3) of either immune mouse serum (titer of 4,000) or normal mouse serum with no detectable titer of antibody to E. risticii. The mice were monitored for clinical signs of E. risticii infection.

Experiment 2: purification and passive transfer of IgG. Immunoglobulin G (IgG) was purified from immune mouse serum by affinity chromatography on a protein G column.
from Jackson Immuno Research Laboratories, Inc. The purity of the eluted immunoglobulin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The immunoglobulin was dialyzed against PBS and concentrated to 8 mg/ml. Antibody titers of the sera and purified IgG preparations were determined by indirect fluorescent-antibody assay. Sixty mice, assigned to four groups of fifteen, were inoculated intraperitoneally with $10^5$ *E. risticii*-infected P388D1 cells. At the time of infection and 3 days p.i., the groups were also inoculated intraperitoneally with 100 μl of either immune mouse serum, IgG from immune mouse serum, normal mouse serum, or IgG from normal mouse serum. The mice were monitored twice daily for clinical signs of illness.

SDS-PAGE. Samples were electrophoresed on 7.5-to-17.5% continuous-gradient polyacrylamide gels under reducing conditions. Gels containing 125I-labeled proteins were fixed in 10% acetic acid-40% methanol, vacuum dried, and exposed to Kodak XAR-2 X-ray film with an intensifying screen at −70°C. Gels containing [35S]methionine-labeled proteins were processed identically, but before being dried, they were processed for fluorography with En3Hance (Du Pont Co., Boston, Mass.). Gels containing purified IgG were fixed in 10% acetic acid-40% methanol and stained with 0.2% Coomassie blue.

Cycloheximide inhibition of P388D1 protein synthesis. P388D1 cells were grown in a 24-well plate, and cells in eight of the wells were subsequently infected with *E. risticii*. Cycloheximide (10 μg/ml) was added to four infected and four uninfected wells. Cycloheximide (10 μg/ml) and tetracycline (20 μg/ml) were added to the other four infected wells. Tetracycline alone (20 μg/ml) was added to four uninfected wells. The plate was incubated at 37°C for 1 h, 60 μCi/ml of [35S]methionine was added to each well, and the cells were incubated for 48 h at 37°C. The cells were harvested and washed in PBS, the number of counts per minute per microliter of sample precipitated with trichloroacetic acid was determined, and a sample was electrophoresed in polyacrylamide gels.

[35S]methionine radiolabeling of *E. risticii*. For large-scale metabolic labeling, cycloheximide (10 μg/ml) was added to a T150 flask of infected P388D1 cells, which were incubated at 37°C for 1 h before the addition of [35S]methionine (60 μCi/ml). After incubation for an additional 48 h, the cells were harvested and washed in PBS. 125I surface radiolabeling of *E. risticii*. *E. risticii* was purified from two T150 flasks with a confluent monolayer of P388D1 cells that were approximately 70% infected. The cells were centrifuged at 500 × g for 10 min, resuspended in PBS three times, and then disrupted by sonication. The suspension was centrifuged at 500 × g for 10 min to remove debris and then 5,000 × g for 10 min to pellet the organism. Morphologically intact *E. risticii* cells were demonstrated by transmission electron micrographs. The pelletted organism was resuspended in a minimal volume of PBS and radiolabeled with 1.0 mCi of 125I by the surface preferential lactoperoxidase technique (22). Free iodine was removed by G-50 column chromatography and dialysis against PBS. Material from two flasks of uninfected P388D1 cells was identically processed and radiolabeled as a control.

Immunoprecipitation. The [35S]methionine-labeled cells and 125I-labeled isolated organism were disrupted by suspension in 50 mM Tris (pH 8) buffer containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 M N-α-p-tosyl-l-lysyl chloromethyl ketone, 10 mM dithiothreitol, and 1% Nonidet P-40 (lysis buffer). The antigen was sonicated, incubated at 37°C for 2 h, sonicated again, and centrifuged at 130,000 × g for 1 h. Samples with 250,000 cpm of trichloroacetic acid-p precipitable 35S- or 125I-radiolabeled organism were added to 5 μl of mouse serum (undiluted or diluted either 1:10 or 1:100 in PBS) and immunoprecipitated as described previously (9). Antigens eluted from the protein G-Sepharose were boiled in SDS-PAGE sample buffer and applied to polyacrylamide gels.

Immunoblot. The cells from two infected and two uninfected T150 flasks were harvested, washed in PBS, and resuspended in lysis buffer. Samples were prepared as for immunoprecipitation, boiled in SDS-PAGE sample buffer, and electrophoresed on 7.5-to-17.5% continuous-gradient polyacrylamide gels under reducing conditions. The proteins were electrophoretically transferred to nitrocellulose, and detected as described previously (2) with immune mouse serum (1:500), rabbit anti-mouse second antibody, and 125I-radiolabeled protein A.

RESULTS

LD50 determination. To provide a basis for challenge doses used in subsequent experiments, LD50 was determined by infecting mice with log dilutions of *E. risticii*-infected cells ($10^7$ to $10^3$). Infected mice demonstrated dose-dependent morbidity and mortality (data not shown). The LD50 was calculated by the method of Reed and Muench (15) to be 5 × $10^7$ *E. risticii*-infected cells.

Passive transfer of immunity. To validate the use of the mouse model to study the mechanism of immunity, we confirmed that mice, like horses, develop a protective immune response to *E. risticii*. Mice previously inoculated with either *E. risticii*-infected or uninfected P388D1 cells were subsequently challenged with 100 LD50 of infected cells. Prechallenge titers of serum antibody to *E. risticii* were 2,000 to 4,000 in the *E. risticii*-immunized mice, while no antibody was detected in the mice inoculated with uninfected P388D1 cells. All control mice (inoculated with uninfected P388D1 cells) were sick by 5 days postchallenge, and six of eight subsequently died. In contrast, none of the 16 mice previously immunized by *E. risticii* infection displayed signs of illness up to 5 months postchallenge when the mice were sacrificed.

To determine the protective capability of serum, immune and nonimmune sera were tested in a passive protection assay. Serum from immune mice protected naive mice from a challenge of $10^3$ infected cells. All mice in experiment 1 that received normal serum were sick by 11 days p.i., whereas none that received immune serum developed disease (Table 1). To determine whether the protective capability of immune serum resided in the IgG fraction, purified immunoglobulin was passively transferred to mice that were subsequently challenged with $10^3$ infected cells. Mice in experiment 2 were injected with $10^5$ *E. risticii*-infected cells and inoculated either with immune or nonimmune serum or with IgG purified from immune or nonimmune serum. All mice that received normal serum or IgG from normal serum were sick by 10 days p.i., and six subsequently died (Table 2). In contrast, none of the 15 mice that received IgG from immune serum developed illness. Thirteen of the fifteen mice that received immune serum remained healthy. Two of the fifteen became ill on day 16 p.i., and one subsequently died (Table 2).
TABLE 1. Protection of mice against E. risticii infection by passive transfer of immune serum

<table>
<thead>
<tr>
<th>Serum transferred</th>
<th>Titer</th>
<th>No. sick/no. challenged</th>
<th>Days to illness (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmune</td>
<td>0°</td>
<td>9/9</td>
<td>11 ± 0°</td>
</tr>
<tr>
<td>Immune</td>
<td>4,000</td>
<td>0/9</td>
<td>0—</td>
</tr>
</tbody>
</table>

* Mice were inoculated with immune or nonimmune mouse serum and were challenged with 10^6 E. risticii-infected cells.
  
* Titer is specific-antibody titer of transferred serum to E. risticii, as determined by indirect fluorescent-antibody assay, and is expressed as the reciprocal of the highest dilution giving a positive result.
  
* Negative at lowest dilution (1:10) tested.
  
* All mice developed illness on day 11 postinfection.
  
* —, mice never developed signs of illness.

Analysis of radiolabeled E. risticii antigens. When used at 10 μg/ml, cycloheximide, an inhibitor of protein synthesis in eukaryotes (1), eliminates incorporation of radiolabel into host cell proteins (Fig. 1, lanes 1 through 4). Cycloheximide does not directly inhibit prokaryotic protein synthesis; therefore, the incorporation of radiolabel is due exclusively to incorporation into E. risticii polypeptides (Fig. 1, lanes 7 and 8). E. risticii polypeptides labeled with [35S]methionine migrated with apparent molecular sizes of <14 to >150 kDa. Specificity of uptake was confirmed by abrogation with tetracycline, an inhibitor of bacterial protein synthesis (25) (Fig. 1, lanes 5 and 6).

Immune mouse serum specifically bound E. risticii polypeptides (apparent molecular masses of 87, 50, 46, 44, and 33 kDa), including two prominent bands at 62 and 27 kDa (Fig. 2, lane 2). These two antigens were bound by immune mouse serum at 1:10 (Fig. 3, lane 2), and the 62-kDa antigen was precipitated when a 1:100 dilution of serum was used (Fig. 3, lane 3). These antigens are also two of the five predominant antigens (62, 53, 33, 27, and 25 kDa) recognized in immunoblot analysis (Fig. 4, lane 1) with a 1:500 dilution of immune serum. Immunoprecipitation of the 125I-labeled organism with immune serum revealed six polypeptides with apparent molecular masses of 70, 53, 40, and 33 kDa, including two prominent bands at 62 and 27 kDa (Fig. 5, lane 1). Immune serum did not precipitate any 125I-labeled antigens from labeled uninfected cells (Fig. 5, lane 3). Normal mouse serum did not precipitate any 125I-labeled P388D1 or E. risticii antigens (Fig. 5, lanes 2 and 4).

**DISCUSSION**

IgG to E. risticii antigens protected mice from ehrlichiosis. It is not clear whether cellular infection was completely prevented or merely contained below the threshold of clinical disease. We hypothesize that antigens which induce the protective antibody response are surface exposed, and we have identified polypeptides that are likely candidates. Surface-exposed antigens may be important for the attachment and penetration stages in E. risticii infection of the host cell. Once E. risticii infects a monocyte or macrophage, it specifically inhibits phagolysosome fusion (26). Because this inhibition is specific to phagosomes containing E. risticii and is abrogated in the presence of tetracycline, it is presumably due to either a secreted or a surface membrane protein of E. risticii (26). Specific antibody to surface antigens of other pathogens in the family Rickettsiaceae blocks host cell
attachment and penetration, thereby preventing infection (8), and can increase phagocytosis and phagolysosome fusion, which results in the destruction of the organism (3, 7).

We have identified polypeptides (70, 62, 53, 40, 33, and 27 kDa), likely surface exposed, that are recognized by the antibody that protects mice from E. risticii infection. These proteins are specifically E. risticii since they are metabolically radiolabeled in the presence of cycloheximide. The 62- and 27-kDa antigens are immunodominant in immunoprecipitations of both [35S]methionine- and [125I]-labeled E. risticii proteins, while the 33-kDa antigen is the most prominent band in immunoblots.

Antigenic differences, defined by incomplete protection against heterologous challenge, occur among strains in other Ehrlichia species (13, 24). It is likely that strains defined by antigenic diversity occur with E. risticii. Using a strain of E. risticii isolated in Maryland, Shankarappa and Dutta (23) demonstrated a profile of [125I]-labeled whole organism that identifies surface antigens with apparent molecular masses of 110, 86, 55, 44, 36, 33, and 28 kDa. Antigens from E. risticii isolated in Ohio and recognized by horse serum ranged in apparent molecular mass from 24 to 160 kDa, with predominant antigens with molecular masses of 110, 70, 55, 44, 33, and 28 kDa (4, 20). Differences in identified antigens may reflect differences in strains, cultivation conditions, methods of antigen preparation, or sources of antibodies. Monoclonal antibodies can be used to identify antigenic differences and similarities among E. risticii isolates, including the Illinois strain (HRC-IL) antigens identified here with protective serum.

A long-term goal is to determine the mechanisms of immunity to E. risticii infection induced by either vaccination or recovery from infection. Antibodies to specific surface antigens would provide the opportunity to examine the ability of antigens to induce neutralization and, in combination with in vitro assays, the mechanism of neutralization. The mouse model, which provides an in vivo correlate of immunity, can be used to identify antigen-specific responses required for protection.

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