Identification of the Protective 44-Kilodalton Recombinant Antigen of Ehrlichia risticii†

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Protective studies were conducted with mice by using recombinantly produced antigens, polyacrylamide gel electrophoresis-fractionated antigens, and a monoclonal antibody specific to the 28-kDa antigen of Ehrlichia risticii. Analysis of E. risticii-infected cell culture used as the challenge inoculum indicated an inverse relationship between the progression of cell culture infection and the infective capability of E. risticii for mice. A recombinant 44-kDa antigen was found to protect mice considerably against challenge infection, while the monoclonal antibody and fractionated antigens were not protective. A potentiation of protection was observed when the recombinant 44-kDa antigen was combined with the recombinant 70-kDa antigen and used for mouse immunization.

Potomac horse fever, which affects horses of all ages, is caused by Ehrlichia risticii (5, 10, 20). This pathogen is another example of obligate intracellular parasitism, and its significance is intensified by the ability of E. risticii to invade and multiply inside one of the main effector cells of the immune system, the macrophages (11).

The study of E. risticii so far has resulted in the identification of component antigens (9) and the development of diagnostic assays for serum antibodies (3, 17) and genomic DNA (3, 28). The phase of the study concerns protection against the disease. The large-scale growth of E. risticii is expensive, and vaccination with the whole organism may not be readily acceptable in nonendemic areas. Furthermore, the effect of long-term in vitro growth on the protective capability of E. risticii is not known. Studies are needed to identify antigenically defined immunoprotective components with potential for the development of a subunit vaccine and to facilitate further studies on the molecular pathogenesis of E. risticii.

As an intracellular parasite, E. risticii elicits intense humoral and cellular immune responses to an infection (4, 10, 11, 19). In spite of the efficiency of tetracycline (31) in treating clinical cases of rickettsial infection (32), the immune response is still considered necessary for the clearance of organisms in an rickettsial infection (25, 26). Protective immunity to Potomac horse fever in horses following initial infection with E. risticii (16) or E. senetssu (21) and in mice infected or immunized with E. risticii (8) has been demonstrated. Recently, in a mouse study, the protection against E. risticii by passive transfer of antibodies was demonstrated (12). Also, protection to various degrees has been demonstrated with the use of a commercially available vaccine to Potomac horse fever (22). In Potomac horse fever, the antibodies to several specific component antigens have been shown to appear at 4 to 5 weeks after infection (4), at which time they are refractile to challenge infection (6), suggesting that one or few of these component antigens might be involved in the protective immunity. The presence of immunodominant protective antigens in other rickettsiae has been demonstrated by using monoclonal antibodies (1, 2, 13) and recombinant antigens (14, 29).

We reported earlier the cloning of 44-, 55-, and 70-kDa antigen genes of E. risticii in lambda gt11 and a low protective capability of the affinity-purified 55-kDa recombinant antigen in mice (8). The 55-kDa recombinant antigen is a nonfusion full protein, whereas the 70- and 44-kDa recombinant antigens are fusion proteins representing about 50 and 73% of the native protein counterparts, respectively. In this paper we report the considerable protective capability of the recombinant 44-kDa antigen and the results of other methods used to evaluate the protective capabilities of different antigens. Also we report the relationship between the progression of E. risticii infection in cell culture and mice infectivity.

MATERIALS AND METHODS

E. risticii and E. risticii-infected cell culture inoculum for infection of mice. E. risticii isolated in our laboratory (5) was propagated in human histiocyte (HH) cells (ATCC U-937) in RPMI 1640 medium. For continuous cultures, to 20 ml of E. risticii-infected HH cells (more than 90%) 20 million naive HH cells in logarithmic phase were added and adsorbed for 1 h at 37°C. Eighty milliliters of prewarmed media was then added, and incubation was continued at 37°C in the presence of 5% CO₂ (7). The cell culture-propagated E. risticii (HH-ER) was standardized as an inoculum for mice infection. For this, the growth of E. risticii in HH cell culture was monitored via trypan blue exclusion and for percent infection by acridine orange staining (5) and by immunofluorescence assay with a monoclonal antibody (HybI) specific for the 28-kDa antigen (23). Each of these values were obtained from at least four replicates. E. risticii inoculum groups containing about 25, 50, and 75% infected cells were selected for standardization of challenge inoculum.

Preparation of antigens for immunization of mice. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (23) was performed to obtain fractionated antigens for mice immunization. About 2 mg of Renografin-purified E. risticii (7) was electrophoresed
by using a preparative comb, and the gel was cut into 26 slices. Each of the slices was crushed into small pieces and incubated overnight at 4°C with 1 ml of phosphate-buffered saline, pH 7.5, with 0.2 mM phenylmethylsulfonyl fluoride to allow the proteins to diffuse out. The supernatants from each of the fractions were collected after the larger pieces of polyacrylamide were allowed to settle down. A 50-μl aliquot from each fraction was analyzed by Western blotting (immunoblotting) (23) with a 1:100 dilution of rabbit E. risticii antiserum to identify the antigen(s) contained in them. The fractions containing antigens corresponding to 160 kDa; 110 kDa; 70 kDa; 55; 51, and 44 kDa; 51 and 44 kDa; and 33 and 28 kDa were obtained, and their protein contents were estimated by bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.) and used for the immunization of mice.

Recombinant antigens for immunization of mice were prepared from Escherichia coli Y1089 lysogens corresponding to the 44-, 55-, and 70-kDa antigens encoded by lambda gt11 clones (8). The lysogens were grown to an optical density at 600 nm of 0.5 at 32°C and the temperature was shifted rapidly to 42°C and incubated at that temperature for 20 minutes. Isopropylthiogalactoside (IPTG) was added to 10 mM, and incubation was continued at 37°C for 1 h. After being pelleted at 3,000 × g and resuspended to 1/50 volume in TEP buffer (100 mM Tris, pH 7.6, 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM iodoacetamide), the cells were freeze-thawed three times in liquid nitrogen and sonicated three times for 30 s. Following centrifugation at 8,000 × g for 10 min, the supernatants were collected and evaluated for the presence of the E. risticii antigen by Western blotting and were then stored frozen at −70°C.

**Immunization of mice.** Female Sprague-Dawley CF-1 mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) used were about 5 weeks of age at the start of immunization. For immunization of mice with SDS-PAGE-fractionated antigens, four mice in each group were immunized by the intraperitoneal injection of 50 μg of protein mixed with Freund's incomplete adjuvant. This adjuvant was used since the polyacrylamide pieces carried over were presumed to possess adjuvant properties. The second and third inoculations, administered 14 and 31 days later, respectively, contained 25 μg of the protein alone. The control groups comprised unimmunized mice, mice immunized with an extract processed as described above from a mock electrophoresis, mice immunized with a combination of all the fractions, and mice previously immunized with the whole E. risticii organism. These mice were challenge infected 41 days postimmunization (p.i.), and tail-bled serum samples were collected on days 21, 38, and 67 p.i.

Passive immunization of mice with the monoclonal antibody Hyb1 (23) was based on the procedure described by Anacker et al. for Rickettsia rickettsii (1); with few modifications. Mice were divided into four groups of three mice, and each mouse received 500 μl of the ascitic fluid intraperitoneally, containing the monoclonal antibody having an enzyme-linked immunosorbent assay (ELISA) titer of 10⁷. For the first group, ascitic fluid was preincubated with the E. risticii challenge inoculum for 30 min prior to injection. The second and third groups received the ascitic fluid 1 h before and 2 days after challenge infection, respectively, and the fourth group received ascitic fluid 1 h before and 2 days after the challenge infection. The controls included mice treated similarly with a control ascitic fluid (Bethesda Research Laboratories, Gaithersburg, Md.) and mice immunized with whole E. risticii.

Mice were immunized with the recombinant 44- and 70-kDa antigens. Further, to evaluate the protective capability of multiple antigens, 44- plus 70-kDa and 44- plus 55- plus 70-kDa recombinant antigen combinations were also included. Earlier, the low protective capability of the purified 55-kDa recombinant antigen was reported (8). Mice were immunized by injection with the crude lysates of the respective lysogens in E. coli Y1089; for the combination groups, equal volumes of the constituent lysates were pooled and administered. The first injection contained 267 μl of the lysate mixed with 133 μl of the Freund's complete adjuvant; the second injection, containing 135 μl of lysate mixed with Freund's incomplete adjuvant, was administered 20 days later. The third injection on day 34 contained 200 μl of the 10-fold-diluted lysates alone. Controls included unimmunized mice, those immunized with the adjuvant alone, and those immunized with lysate from E. coli BNN97 (a lambda gt11 lysogen; ATCC). For the positive control, mice were immunized with 100, 50 and 50 μg of protein equivalent of E. risticii by the above-described schedule. At the beginning of the experiment, there were at least six mice in each group, and a few mice injected with crude lysates of the recombinants died following the second immunization, probably because of high endotoxin levels. The mice were challenge infected on day 47 p.i., and the serum samples were collected on days 30, 41, and 65 p.i.

For the evaluation of antibody response of the mice immunized with PAGE-fractionated and recombinant antigens, ELISA (24) was performed by using Renografin-purified E. risticii whole organism as the antigen. The sera were at a dilution of 1:100 and peroxidase-labeled goat anti-mouse immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were used. The same dilution of the mouse sera was used for the Western blot analysis against transblotted E. risticii antigens (23).

**Challenge infection and evaluation of protection.** Mice immunized with PAGE-fractionated antigens and passively immunized with the monoclonal antibody were challenged infected with buffy coat cells from an E. risticii-infected horse according to the procedure described earlier (8).

In the subsequent experiment, mice immunized with the 44- and 70-kDa recombinant antigens and their combinations were challenge infected with cell culture-propagated E. risticii (HH-ER) by intraperitoneal inoculation. For standardization of this inoculum, mice were inoculated with 10⁴, 10⁵, and 10⁶ E. risticii-infected HH cells from each of the 25, 50, and 75% infective groups described earlier. The mice infected with the 25% infective group developed an intense sickness, and hence this infective group was selected for the challenge infection. The 50% lethal dose and 50% infective dose were calculated to be 10⁶ and 10⁵ cells, respectively, as determined by the Reed and Muench method (18). For challenge infection of mice, four 50% infective doses (4 × 10⁵ cells in 0.5 ml of cell culture suspension) were administered. The clinical signs observed included lethargy, squinty eyes, rough coat, hunched back, emaciation, moribund conditions, and death, similar to the conditions described in the literature (19). Infection in mice was evaluated by using a scoring system developed earlier in our laboratory for experimental infection of mice with E. risticii. This scoring on a scale of zero to five was based on the clinical signs described earlier. Normal and unremarkable conditions were scored as zero; lethargy and rough hair coat were scored as one; progressive signs of great lethargy rough hair coat, and a hunched back were scored as two; more pronounced versions of the above-described signs, eye pasting, and severe emaciation were scored as three; a moribund state
was scored as four; and death was scored as five. Clinical
signs judged to fall between any two stages were scored as
fractions of the corresponding scores. Blind scores were
averaged for each group and analyzed.

RESULTS

*E. risticii* infectivity. The kinetics of growth of *E. risticii* in
HH cells are presented in Fig. 1. The immunofluorescence
assay with monoclonal antibody HybI was consistently
superior to acridine orange staining in detecting the *E. risticii*
infection. However, because of the simplicity of the acridine
orange staining, it was used to demarcate the different
groups of infectivity. A good agreement in the relationship
between the viability of HH cells and the progression of
infection could also be observed. This evaluation provides a
semiquantitative profile of the growth characteristics of *E.
risticii* in cell culture. Under the conditions standardized,
cultures on days 4, 5, and 6 of infection were found to
contain approximately 25%, 50%, and 75% infected cells, re-
spectively.

Since our earlier observations (unpublished) had indicated
a low pathogenicity of cell culture-propagated *E. risticii* in
mice when a high percentage of infected cells were present,
it was thought that the viability of *E. risticii* infected cells
might be important. Hence, a comparative analysis of the
mouse pathogenicity of *E. risticii* at different stages of the
infective process in cell culture was undertaken. The results
(Fig. 2) indicated that the pathogenicity in mice was drasti-
cally reduced in very short periods of the cell culture
infective process. The 25% infective HH-ER was highly
pathogenic for mice, and the administration of $10^7$ cells
resulted in the death of all mice in the group. The initial peak
of sickness in the first 3 days seems to be the result of an
apparent general toxicity of the inoculum, as can be noticed
more prominently in Fig. 2F. Although inoculation of $10^7$
cells generally induced pronounced sickness, a general trend
of decreasing pathogenicity with the progression of infectiv-
ity could be observed from the results. These data helped to
standardize the *E. risticii* inoculum more efficiently.

Antibody response in mice to PAGE-fractionated and
recombinant *E. risticii* antigens. Mice immunized with the
110-, 70-, and 51- and 44-kDa PAGE-fractionated antigen
groups produced high ELISA antibody titers (Fig. 3a). The
Western blot analysis of these sera (Fig. 3b) revealed the
specificity of the response in all the groups, and their reactivity with 70-, 51-, 44-, and 28-kDa antigens was highly pronounced.

Antibody response of the mice immunized with 44- and 70-kDa recombinant antigens and the antigen combinations detected by ELISA and immunoblot is presented in Fig. 4. The antisera from mice immunized with the 44-kDa recombinant antigen exhibited high ELISA titers and an intense Western blot reactivity with the homologous antigen of *E. risticii*. This immunoblot reactivity was matched in the 41-day-p.i. sera from the 44- plus 70-kDa antigen group, while it was not observed for 30- or 41-day-p.i. sera from the 44- plus 55- plus 70-kDa antigen group. The reactivity with the specific antigens appeared to intensify following challenge infection in almost all the groups. Since crude *E. coli* extracts were used as inocula for the recombinant antigens, purified *E. risticii* organisms had to be used as the ELISA antigens for the evaluation of specific antibody response.

The use of whole *E. coli* cell extracts for immunization with recombinant antigens resulted in the production of antibodies to many *E. risticii* proteins (Fig. 4b). These cross-reactive epitopes are probably of a nonspecific origin as indicated by the presence of similar reactivities in the BNN97 cell group.

**Protective response of mice to immunization.** Mice immunized with the PAGE-fractionated antigens and passively immunized with the monoclonal antibody to the 28-kDa antigen of *E. risticii* were not protected from *E. risticii* challenge infection. Mice immunized with recombinant 44-kDa antigen and the antigen combinations containing this antigen showed a marked reduction in the severity of clinical signs. A comparison of the morbidity scores of mice immunized with the 44-kDa recombinant antigen and other groups is presented in Fig. 5. The protection observed in mice immunized with the 44-kDa antigen alone seemed to be potentiated by combining this antigen with 70-kDa recombinant antigen. Surprisingly, the intensity of sickness in mice immunized with the BNN97 cell control was reduced compared with that in the unimmunized mice, which followed the pattern observed in the infectivity titration studies (Fig. 2). The mice immunized with *E. risticii* were completely protected from any signs of sickness.

**DISCUSSION**

The establishment of protective immunity in horses following an initial exposure to *E. risticii* and the presence of
immunoprotective component antigens in other rickettsial species led us to search for the presence of a major protective antigen in *E. risticii*. The severity and extent of the prevalence of this recently identified disease underscored the necessity of such studies.

Among the monoclonal antibodies previously developed, Hyb1 was found to be specific to a 28-kDa antigen and was later utilized for the development of a unique immunodiagnostic assay (24). However, this monoclonal antibody did not protect the mice from challenge infection. The lack of protection with Hyb1 could be an indication that the epitope recognized by Hyb1 is not involved in protection directly or indirectly as a consequence of steric modification ensuing binding of the monoclonal antibody. An attempt was then made to study the protective response to PAGE-separated whole proteins of *E. risticii*. Significant antibody responses were revealed by ELISA and substantiated by specific Western blot reactivity. However, the lack of protection observed here could be the result of an irreversible denaturation of SDS-sensitive epitopes. It is interesting that the presence of antibodies to SDS-denatured proteins of *E. risticii* in the preinfected sera of horses and their subsequent susceptibility to *E. risticii* have been observed (4).

Production of recombinant *E. risticii* proteins will provide pure proteins in a relatively native state. In the earlier evaluation of the protective capability of 55-kDa recombinant antigen, we had utilized affinity purified protein (8). Since the affinity purification will also involve some form of a denaturation step, the experiments described in this paper were conducted by utilizing crude lysates of the recombinant products. Efficiency of specific immunization was demonstrated by ELISA and Western blot reactivities. However, the observed reactivity of these mouse sera with several *E. risticii* proteins could be the result of recognition of proteins homologous between *E. coli* and *E. risticii*. Such homology in the heat shock family of proteins has been noted in other rickettsiae (27, 30), and a similar phenomenon is highly probable because of the growth of *E. coli* at high temperatures.

Significant protection from clinical signs observed in mice immunized with the recombinant 44-kDa antigen and its combinations is encouraging. In fact, a potentiation of the protective response was observed in mice immunized with combinations of recombinant antigens. Further, our result is substantiated by the protective role for the 44-kDa antigen hypothesized by Rikihisa and coworkers (21). They based this finding on the reactivity of *E. sennetsu*-immunized horse sera with the 44-kDa antigen of *E. risticii* concomitant with protection from *E. risticii* challenge infection. In view of the extensive cross-reactivity between these two species in almost all the antigens (22a), our results provide evidence of the protective capability of the 44-kDa antigen. The lack of complete susceptibility of mice immunized with BNN97 might be due to a general heightened immune status. The enhanced release of several lymphokines under such circumstances and the reported inhibitory effect of gamma inter-feron on rickettsial intracellular parasites (15) might have compromised the infectivity of *E. risticii*.

In view of an earlier observation that *E. risticii*-infected cell culture was less effective in establishing infection in mice than were infected horse blood leukocytes (6, 19), we hypothesized the presence of a relationship between the progression of infection in cell culture and the mouse infectivity. Our results clearly demonstrate the presence of an inverse relationship between these two variables. It is possible that a visible indication of the saturation of host cell with the progeny of parasitics, accompanied by cell death, might be a reflection of the loss of viability of these organisms in general.

We have demonstrated the protective nature of the 44-kDa recombinant protein against *E. risticii* infection in mice. Future studies will be aimed at cloning the full gene for the 44-kDa antigen and determining the humoral and cell-mediated immune responses in elucidating the immunoprotective activity of this antigen.

### References


### FIG. 5. Morbidity scores of mice immunized with the recombinant antigens and their combinations, following their challenge infection with cell culture-propagated *E. risticii*. (A) Mouse groups immunized with the 44-kDa recombinant antigen and its combinations. (B) Mouse groups immunized with the 70-kDa antigen and the controls. The symbols refer to the immunizing agents. Each value represents the mean ± standard error of the mean scores.