INFECTION AND IMMUNITY, Mar. 1991, p. 1162–1169
0019-9567/91/031162-08$02.00/0

Vol. 59, No. 3

Molecular Cloning and Analysis of Recombinant Major Antigens of *Ehrlichia risticii*†

SUKANTA K. DUTTA,* BASAVARAJU SHANKARAPPA,‡ AND BONNIE L. MATTINGLY-NAPIER

Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland 20742

Received 6 August 1990/Accepted 30 November 1990

The genome of *Ehrlichia risticii*, the etiologic agent of Potomac horse fever, was cloned in the λgt11 expression vector. The efficiency of recombinant phage production with different restriction fragments of *E. risticii* DNA was generally between 20 and 95%. The antigen-positive frequency, detected by immunoscreening with *E. risticii* antibodies, was between 8 and 40 per 10^4 recombinants. Four (70, 55, 51, and 44 kDa) major antigens of *E. risticii* were identified from the recombinant phages by using recombinant antigen-selected monospecific antibodies. Characterization of three (70, 55, and 44 kDa) of these recombinant antigens indicated that the 70- and 44-kDa polypeptides were β-galactosidase fusion products that were dependent on isopropylthiogalactoside induction for expression; they contained about 50 and 73%, respectively, of the native polypeptides. The 55-kDa antigen was a nonfusion protein expressed independently of isopropylthiogalactoside induction; it was a complete protein with a molecular weight identical to that of its native counterpart. The cloned *E. risticii* DNAs from of the recombinants expressing 70-, 55-, and 44-kDa proteins were 3.5, 3.9, and 4.8 kb, respectively, in size, and they were unique. The insert DNAs hybridized to multiple restriction fragments of the genomic DNA, the sum of the sizes of which was much greater than that of the corresponding insert. Mice immunized with the affinity-purified 55-kDa recombinant antigen produced a high titer of antibody in serum as measured by an enzyme-linked immunosorbent assay and gave a monospecific reaction by Western immunoblotting. Challenge infection of these immunized mice showed low protection from clinical infection.

Ehrlichia risticii is the etiologic agent of Potomac horse fever, a recently recognized disease of horses (5, 9, 17). Eighteen component antigens of the organism have been identified (8), of which nine (110, 86, 70, 55, 51, 49, 44, 33, and 28 kDa) are major antigens and apparent surface components (22). Western immunoblot profiles of *E. risticii* antisera obtained sequentially from experimentally infected horses (6) indicated that six major antigens (70, 55, 51, 44, 33, and 28 kDa) were recognized early in the antibody response and that the remaining antigens were recognized later (4). Transient immunodepression in horses (4) and reduced immune responsiveness along with a lymphoid depletion in mice (16) resulting from experimental infections with *E. risticii* have been observed. *E. risticii* is reported to be antigenically closely related to *Ehrlichia sennetsu*, a human pathogen, and to a lesser degree to *Ehrlichia canis* but unrelated to *Ehrlichia equi* (19). Because of these complexities and a need to understand the pathogenesis and identify the immunogens of *E. risticii*, it is important to elucidate the functional and immunogenic roles of the component proteins and the nature of the corresponding genes for this organism.

The λgt11 expression vector system has been used for cloning and expression of antigens detected with specific antibodies (28) for a number of pathogens (1, 15). In this system, genes are expressed as β-galactosidase fusion proteins under the control of the *lacZ* promoter (28).

In this paper we describe the cloning of the *E. risticii* genome in λgt11 and characterization of three expressed major antigens, including immunogenicity of one of the antigens in mice, and the corresponding insert DNAs of the recombinants.

MATERIALS AND METHODS

Construction of *E. risticii*-λgt11 recombinants. *E. risticii* DNA, extracted from purified organisms (7) as described previously (8), was subjected to partial (0.5 U of restriction enzyme, incubated for 15 min) or complete (10 U of restriction enzyme, incubated for 1 h) digestions with a battery of restriction enzymes, singly or in combinations of two, to obtain a variety of restricted fragments (8). After the size distribution was determined in 0.8% agarose gel, the restricted DNA fragments were inserted into λgt11 by using conversion adaptors as described by Stover et al. (25). Single-stranded oligonucleotides (New England BioLabs) of different lengths, when mixed in equimolar quantities, formed duplex adaptors of three different lengths, so that gene fusions at all three reading frames were possible. The conversion adaptors carried an *Eco*RI cohesive end at one terminus for ligation to the λgt11 arm and a restriction enzyme-specified cohesive end at the opposite terminus for ligation to restriction DNA fragments. The restricted DNA fragments were ligated to the conversion adaptors with 10 U of T4 DNA ligase at 16°C for 1 h. The unligated adaptors were removed by selectively precipitating adaptor-modified insert DNA with graded (full-strength and half-strength) polyethylene glycol solution (full strength is 1.5 M NaCl–13% polyethylene glycol 8000). The adaptor-modified insert DNA was phosphorylated with 5 U of T4 polynucleotide kinase at 37°C for 30 min and ligated to λgt11 vector DNA (Promega Biotec) with 2.5 U of T4 DNA ligase at 16°C for 2.5 h, and then the ligation mixture was packaged in a packaging mix (Promega Biotec) at 22°C for 2 h.

* Corresponding author.

† Scientific article A6129, contribution 8294, of the Maryland Agriculture Experiment Station.

‡ Present address: Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15261.

Immunoscreening of recombinants for antigen expression. The recombinants were screened with adsorbed *E. risticii* antisera by the procedure of Oaks et al. (15) with some modifications. Antisera obtained from rabbits hyperimmunized with purified *E. risticii* (8) and horse antisera collected at 6 to 8 weeks postinoculation from horses experimentally infected with *E. risticii* (6) were absorbed with *Escherichia coli* Y1090 and λgt11 as described previously (15) to reduce the background signals. These absorbed antisera contained high titers of antibodies as detected by serologic tests (4), and Western immunoblotting conducted with these sera (1:100 dilution for rabbit anti-*E. risticii* serum and 1:20 dilution for horse anti-*E. risticii* serum) detected all of the known *E. risticii* component antigens (4, 8).

The packaged λgt11 phages were incubated with *E. coli* Y1090 in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (400 μg/ml), and the plaques were lifted onto isopropylthiogalactoside (IPTG)-saturated nitrocellulose filters as described by Maniatis et al. (12). The filters were blocked in casein solution (2% casein in 10 mM Tris [pH 7.5]–120 mM NaCl) and incubated with absorbed rabbit anti-*E. risticii* serum, diluted to 1:100 in casein solution, for 3 h at room temperature. This was followed by treatment with alkaline phosphatase-labeled second antibody and substrate solution as described previously (4) to identify the immunoreactive plaques. The specificity of immunoreactivity was also determined by using horse anti-*E. risticii* serum diluted to 1:20. The antigen-positive recombinants were purified by single-plaque isolation and stored in 0.1 M NaCl–10 mM Tris (pH 7.9)–10 mM $MgSO_4$.

Recombinant antigen-selected monospecific antibody preparation. Preparation of monospecific antibody by affinity purification was performed as described previously (11). Briefly, recombinant phages mixed with *E. coli* Y1090 culture to give approximately 3×10^5 PFU were plated on Luria-Bertani (LB) agar plates. When distinct plaques developed after incubation, the plates were overlaid with IPTG-saturated nitrocellulose filters for 2 h at 37°C; then the filters were inverted and incubated for an additional 2 h. The filters were washed in Tris-saline, blocked in casein solution, incubated with adsorbed rabbit anti-*E. risticii* serum (1:100) for 3 to 5 h at room temperature, and then washed. The antibody was eluted in glycine buffer (0.2 M glycine, 0.15 M NaCl [pH 2.8]) and immediately neutralized with Tris-base (1.32 M) to pH 7. The monospecific antibodies were used at a dilution of 1:3 for Western immunoblot analysis.

Polyacrylamide gel electrophoresis and Western immunoblotting. The electrophoresis and immunoblotting procedures described previously (8) were followed.

Generation of recombinant lysogens and preparation and purification of lysates. Recombinant phages were lysogenized in *E. coli* Y1089 and Y1090 by the procedure of Stover (23a). A 5-μl sample of the phage suspension was spotted onto a lawn of *E. coli* Y1089 or Y1090 in 0.8% agarose in LB broth. After incubation overnight at 32°C, the area showing a clear plaque was touched with a sterile loop and streaked onto a fresh LB plate and grown at 32°C. Individual colonies were replica plated; one plate was maintained at 32°C, and the other was maintained at 42°C. The colonies that grew at 32°C but failed to grow at 42°C were purified by two more colony purifications. The lysogen colonies were tested for the production of immunoreactive proteins by using monospecific antibodies.

Lysates were prepared according to the protocol of the manufacturer (Promega Biotec). Single colonies of Y1089 recombinant lysogens were grown in LB medium at 32°C to an optical density at 600 nm of 0.5; then the temperature of the culture was increased rapidly to 42°C, and the culture was incubated for 20 min. IPTG (10 mM) was added to appropriate cultures, and incubation was continued at 37°C for 1 h. The cells were harvested by centrifugation at 2,600 × *g* for 5 min at 24°C, suspended to 1/50 of the original volume with 100 mM Tris-HCl (pH 7.6)–10 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride, and then immediately frozen in liquid nitrogen and stored at −70°C. The frozen cells were thawed, sonicated, and centrifuged at 8,000 × *g* for 10 min at 4°C. The supernatant was precipitated with 50% ammonium sulfate; the precipitate, dissolved and dialyzed with phosphate-buffered saline containing phenylmethylsulfonyl fluoride, was made up to 1/50 of the original culture volume. The lysates were used for Western immunoblot analysis with rabbit anti-*E. risticii* serum, monospecific antibodies, and rabbit anti-β-galactosidase serum.

Affinity chromatography purification of the lysates on Affi-Gel-10 (Bio-Rad) was carried out according to the manufacturer's recommended procedure by mixing 2.5 ml of gel with 3 ml of monospecific antibody previously dialyzed exhaustively against 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5). The coupling was carried out for 6 h at 4°C, and then the unbound sites were blocked by the addition of 0.3 ml of 1 M ethanolamine (pH 8) and incubated for an additional 1 h at 4°C. A 3-ml sample of the lysate, corresponding to the monospecific antibody, was passed slowly through the column several times for 2 h to facilitate antigen-antibody binding. The column was washed with 10 volumes of 1 M NaCl followed by 10 volumes of HEPES buffer. The protein was eluted with 12 ml of 6 M sodium thiocyanate and then dialyzed against phosphate-buffered saline. Immunoaffinity purification of lysates with ProtoSorb lacZ, designed for the purification of β-galactosidase fusion protein, was carried out as recommended by the manufacturer (Promega Biotec).

Preparation of *E. risticii* insert DNA from the recombinants and subcloning. Recombinant phages were obtained from Y1090 lysogens by polyethylene glycol precipitation and purified over a glycerol step gradient (23). They were treated with DNase (1 μg/ml), and then DNA was extracted by a standard method (12).

The DNAs from the recombinants encoding the 55- and 70-kDa antigens were digested with *Eco*RI to excise the inserts. In the case of the recombinant encoding the 44-kDa antigen, it was not possible to cut the insert DNA with *Eco*RI. Since the conversion adaptors contained an *Xmn*I site, the DNA extracted from it was digested with *Xmn*I. The fragments from the digests were separated by gel electrophoresis on 1% agarose, and the location of insert DNA was determined by Southern blot hybridization with ^{32}P-labeled *E. risticii* genomic DNA. In the case of the 44-kDa recombinant, it was not possible to separate the insert DNA from the comigrating λ fragment. The insert DNAs were either electroeluted (12) or purified with Geneclean (Bio 101, La Jolla, Calif.) according to the instructions of the manufacturer.

These DNA inserts were subcloned into pBluescript SKII$^+$ phagemids (Stratagene, La Jolla, Calif.). For this, the phagemid, digested with *Eco*RI or *Sma*I as appropriate, was ligated to insert DNAs by standard techniques (12). The *E. coli* XL1-blue competent cells (Stratagene) were transformed with the ligated DNAs as described previously (20). Plasmid minipreparations were made from the transformants; after Southern hybridization with ^{32}P-labeled *E. risticii* DNA, clones containing specific inserts were se-

lected. Restriction maps of the DNA inserts were generated after digestion with 15 6-base enzymes.

Preparation of probe and Southern blot hybridization. Probes were prepared with the insert DNAs and the total genomic *E. risticii* DNA. A 1-μg sample of each DNA was labeled with [α-^{32}P]dCTP with a nick translation kit by the protocol of the manufacturer (BRL) to a specific activity of 10^8 cpm/μg. The unincorporated nucleotides were removed by desalting over Bio-Gel P-6DG (Bio-Rad) equilibrated with 50 mM Tris–1 mM EDTA (pH 7.8) (26).

For Southern blotting, 1 μg each of the insert DNA and total *E. risticii* DNA were digested with appropriate restriction enzymes and electrophoresed on an 0.8% agarose gel. Further Southern blotting to Zeta-Probe membrane was conducted as recommended by the manufacturer of the membrane (Bio-Rad). The gels were depurinated in 0.25 M HCl, denatured with 0.5 M NaOH–1.5 M NaCl for 30 min, and then neutralized in a solution of 3 M NaCl–0.5 M Tris-HCl (pH 7.4). The DNA was capillary transferred overnight to a Zeta-Probe membrane with 10× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate). The membranes were washed in 2× SSC, vacuum dried at 80°C for 20 min, and then incubated with a prehybridization solution (1 mM EDTA, 0.5 M NaH_2PO_4 [pH 7.2], 7% sodium dodecyl sulfate) at 65°C for 30 min. The prehybridization solution was replaced, each of the DNA probes (10^6 cpm/ml) was added, and hybridizations were allowed to proceed overnight at 65°C. The membranes were washed at 65°C two times for 30 min each with washing buffer (1 mM EDTA, 40 mM NaH_2PO_4 [pH 7.2], 5% sodium dodecyl sulfate) and twice with the same washing buffer with 1% sodium dodecyl sulfate. The membranes were exposed to X-Omat film for overnight. The molecular weights of bands in Southern blots were estimated by hybridizing the kilobase ladder DNA (BRL), run in adjacent lanes of each gel, with the labeled kilobase ladder DNA.

Immunization and challenge infection of mice. Since the 55-kDa protein was a complete protein, apparently identical to the native counterpart, the immunogenicity of this antigen in mice was evaluated.

Two groups of six mice were immunized with affinity-purified 55-kDa antigen. They were injected intraperitoneally with 50 μg of protein equivalent, alone or mixed with Freund's complete adjuvant. This was followed by a second intraperitoneal injection of 25 μg of antigen, either alone or mixed with Freund incomplete adjuvant, as appropriate, on day 14 postimmunization. Six mice were immunized similarly with adjuvant alone. Six mice each, previously infected with buffy-coat cells from *E. risticii*-infected horses (6) or with *E. risticii*-infected human histiocyte cell line U937 (American Type Culture Collection, Rockville, Md.) and demonstrating high *E. risticii* antibody titers, were included as positive controls. Six unimmunized mice were used as a negative control. Serum samples from these immunized mice were collected on days 14 and 26 postimmunization, and the presence of antibodies was evaluated by enzyme-linked immunosorbent assay (ELISA) (7) and Western blotting. The mice were challenge infected on day 26 postimmunization by the intraperitoneal route with 25 50% infective doses of buffy coat cells from *E. risticii*-infected horses. Mice were scored for sickness independently by three individuals. Mice showing signs ranging from dullness and rough coat to moribund condition were considered sick.

TABLE 1. Efficiency of recombinant production and the frequency of antigen expression by the λgt11 recombinants constructed with DNA fragments obtained by digestion with different restriction enzymes

Restriction endonuclease[a]	% Recombinant production	Antigen positive frequency per 10^4	Major *E. risticii* antigens[b] (kDa)
*Dde*I (P)	95	30	51, 44
*Hpa*II (P)	90	34	70,* 51
*Hin*fI (P)	80	8	None
*Hpa*II-*Hin*pI (C)	20	20	55,* 44*
*Sau*3A (P)	90	40	44
*Taq*I (P)	95	20	44
*Taq*I (C)	90	30	51, 44

[a] C, Complete digestion; P, partial digestion.
[b] Identified by Western immunoblotting. The recombinant antigens that were characterized further are indicated by asterisks.

RESULTS

Production of *E. risticii* recombinants expressing antigens. The recombinant efficiency was generally above 80%, and antigen-positive frequencies of the recombinants were in the range of 8 to 40 per 10^4 (Table 1). The immunoreactivity of the proteins expressed by the recombinants in each of the libraries ranged from weak to strong signals.

Identification of recombinant antigens. The identity of the recombinant antigens was established by Western immunoblot analysis with corresponding monospecific antibody. Usually one and sometimes two of the same antigens from a library of a particular restriction enzyme were detected consistently on replicate screening. Several major antigens expressed by the recombinants from the libraries were identified (Table 1). The expression of the recombinants obtained from *Hin*fI digestion could not be confirmed for any major antigen by Western immunoblotting. Several of the expressing recombinants from different libraries, on further cloning, lost their immunoreactivity. Three recombinant antigens of 70 kDa (*Hpa*II partial digestion), 55 kDa (*Hpa*II-*Hin*pI complete digestion), and 44 kDa (*Hpa*II-*Hin*pI complete digestion) were expressed strongly; they were characterized further (Fig. 1 and 2).

Characteristics of recombinant antigens. Among the three recombinant antigens, the 55-kDa antigen was a nonfusion protein expressed independently of IPTG induction (Table 2; Fig. 1). This recombinant antigen was purified on an Affi-Gel column, whereas it did not bind to a lacZ column, confirming its nonfusion nature. The molecular mass of the 55-kDa recombinant antigen was identical to that of its native counterpart, indicating expression of complete protein. The 70- and 44-kDa recombinant antigens were both fusion proteins. They migrated in the gel in conjunction with β-galactosidase, and their expression was dependent on IPTG induction (Table 2; Fig. 2). The mobility of these two fusion proteins in a 7% polyacrylamide gel showed their sizes to be approximately 150 and 147 kDa, respectively, indicating the presence of about 50 and 73%, respectively, of their respective native *E. risticii* polypeptides. Affinity-purified antigens showed characteristic reactivity in Western blotting. None of the recombinant antigens cross-reacted with each other with heterologous monospecific antibodies.

Characteristics of cloned *E. risticii* DNA. The sizes of the DNA inserts of the recombinants expressing the 70-, 55-, and 44-kDa antigens were 3.5, 3.9, and 4.8 kb, respectively (Table 2; Fig. 3). The inserts hybridized with the genomic *E. risticii* DNA and with the homologous inserts, whereas the

TABLE 2. Description of the λgt11 recombinants

Antigen encoded by recombinant (kDa)	Size of recombinant protein (kDa)	Nature of recombinant protein		Size of insert (kb)	Hybridization with *E. risticii* DNA fragments	
		Galactosidase fusion	IPTG[a] dependence		*Eco*RI (kb)	*Hin*dIII (kb)
44	147 (73[b])	Yes	Yes	4.8	6.5, 5.3, 0.6	13.5
55	55 (100)	No	No	3.9	8.0, 4.3, 3.2	8, 2, 1.7, 1.4
70	150 (50)	Yes	Yes	3.5	9.8, 5.6, 3.8	13.5

[a] IPTG, Isopropylthiogalactoside.
[b] Number in parentheses indicates the estimated percentage of the native *E. risticii* protein encoded by the recombinant.

heterologous inserts did not cross-hybridize, indicating the specificity and specific identity of each clone DNA (Fig. 3).

To localize the gene fragments in the specific restriction segments of the *E. risticii* genome, the genomic DNA was digested overnight with *Eco*RI and *Hin*dIII, and the Southern blots were hybridized with labeled insert DNAs. The 44-kDa insert fragment hybridized to three *Eco*RI fragments of 6.5, 5.3, and 0.6 kb, with a low signal intensity to a few intermediatory bands, and to a single *Hin*dIII fragment 13.5 kb in size (Table 2; Fig. 4). Similarly, the 55-kDa insert hybridized to 8-, 4.3-, and 3.2-kb *Eco*RI fragments and 8-, 2-, 1.7-, and 1.4-kb *Hin*dIII fragments, whereas the 70-kDa insert hybridized to 9.8-, 5.6-, and 3.8-kb *Eco*RI fragments and a 13.5-kb *Hin*dIII fragment (Table 2; Fig. 4).

The pBluescript plasmids containing the subcloned inserts were designated pB44-10, pB55-6, and pB70-1 for clones expressing 44-, 55-, and 70-kDa antigens, respectively. The restriction maps for the inserts indicated the presence of single to multiple sites for different 6-base restriction enzymes (Fig. 5). It should be noted that with the 55-kDa recombinant, the pattern of distribution of *Hin*dIII sites in pB55-6 closely corresponds to the hybridization pattern observed in the Southern hybridization of *Hin*dIII-digested *E. risticii* DNA (Fig. 4).

Antibody response to immunization and protection against challenge infection in mice. A high titer of antibody was produced in mice immunized with the 55-kDa recombinant antigen; the response was higher in mice receiving the antigen mixed with adjuvant, as demonstrated by ELISA reactivity with the purified 55-kDa recombinant antigen. The time course of the ELISA antibody responses of the immunized mice to purified 55-kDa antigen and to the whole organism is presented in Fig. 6. Mouse anti-55-kDa antigen sera did not show reactivity with the whole organism in the ELISA. However, in Western blots of *E. risticii* antigens, the sera demonstrated monospecificity of the antibody response by reacting with the 55-kDa antigen. Similarly, the sera of positive control mice infected with *E. risticii* did not produce an appreciable ELISA response to purified 55-kDa

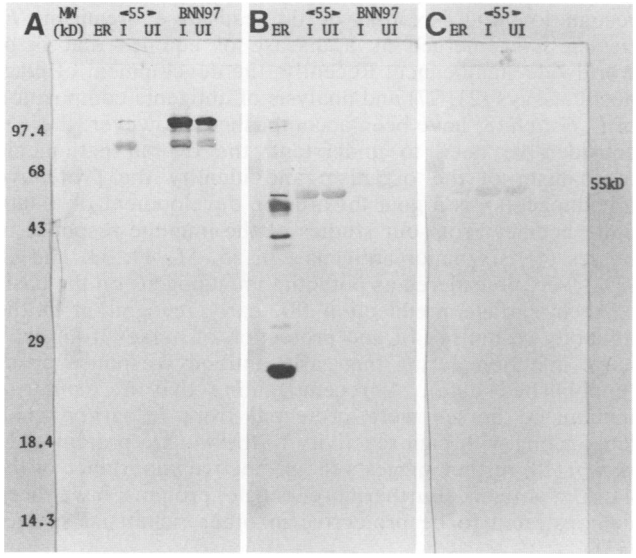

FIG. 1. Western blot analysis of the 55-kDa recombinant antigen, which is a nonfusion, complete protein. Shown are Western blots of *E. risticii* (ER), 55-kDa recombinant antigen (IPTG induced [I] and uninduced [UI]), and BNN 97 (λgt11 lysogen in *E. coli*) treated with rabbit anti-β-galactosidase serum (A), rabbit anti-*E. risticii* serum (B), and 55-kDa antigen-selected monospecific antibody (C). β-Galactosidase and its proteolytic degradation product (A), *E. risticii* antigens and the 55-kDa recombinant antigen (B), and the 55-kDa *E. risticii* antigen and the 55-kDa recombinant antigen (C) are shown.

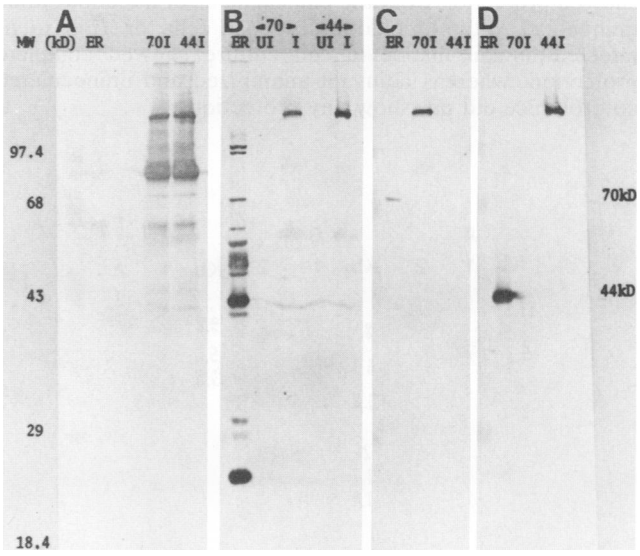

FIG. 2. Western blot analysis of 70- and 44-kDa recombinant antigens, which are β-galactosidase fusion proteins. Shown are Western blots of *E. risticii* (ER) and recombinant 70- and 44-kDa antigens (IPTG induced [I] and uninduced [UI]) treated with anti-β-galactosidase serum (A), anti-*E. risticii* serum (B), 70-kDa antigen-selected monospecific antibody (C), and 44-kDa antigen-selected monospecific antibody (D). β-Galactosidase and its proteolytic degradation product (A), *E. risticii* antigens and 70- and 44-kDa fusion proteins (B), *E. risticii* 70-kDa antigen and 70-kDa recombinant antigen (C), and *E. risticii* 44-kDa antigen and 44-kDa recombinant antigen (D) are shown.

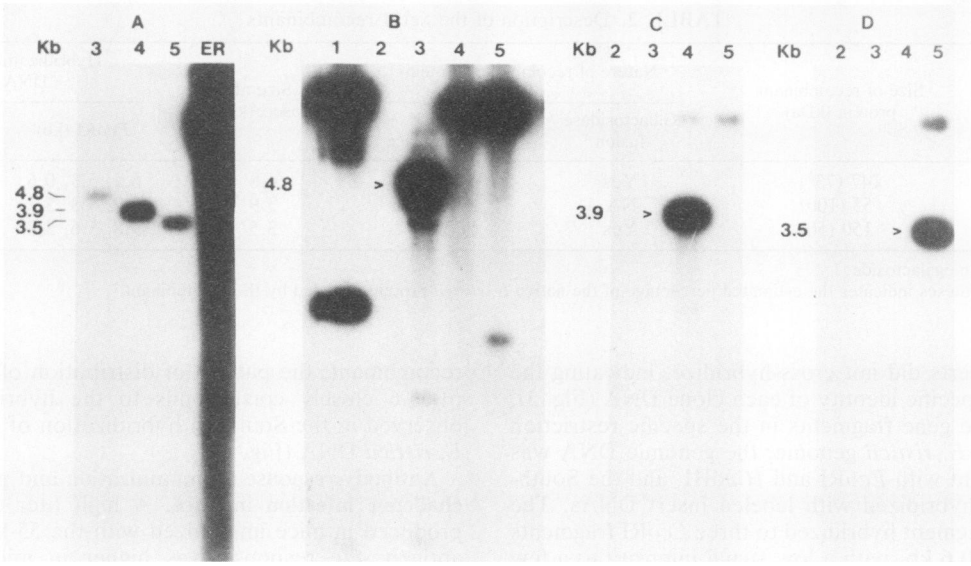

FIG. 3. Autoradiography of Southern blots of *E. risticii* (ER) DNA digested with *Eco*RI and insert DNAs of recombinant 44-kDa (lanes 3), 55-kDa (lanes 4), and 70-kDa (lanes 5) phages hybridized with ^{32}P-labeled total genomic *E. risticii* DNA (A), 44-kDa insert DNA (B), 55-kDa insert DNA (C), and 70-kDa insert DNA (D). Also shown are λ DNA digested with *Hin*dIII (lanes 1) and kilobase markers (lanes 2). The insert DNAs hybridized with the homologous probes (arrow) but not with the heterologous probes. A λgt11 *Xmn*I fragment, which comigrated and coeluted with the 44-kDa insert, produced intense hybridization signals with λgt11 fragments in all lanes (B).

recombinant antigen. However, after challenge infection of these mice, there was a noticeable ELISA antibody response to the 55-kDa recombinant antigen (Fig. 6).

Two of the six mice (33%) immunized with the 55-kDa recombinant antigen either alone or mixed with adjuvant showed protection from the clinical signs of infection. Mice immunized with infected buffy coat cells or *E. risticii*-infected human histiocyte cell culture showed complete protection, whereas adjuvant-immunized and uninoculated control mice did not show any protection.

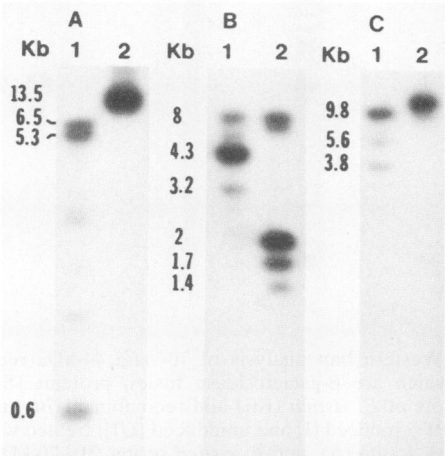

FIG. 4. Southern blot analysis of *E. risticii* genomic DNA digested with *Eco*RI (lanes 1) and *Hin*dIII (lanes 2) hybridized with ^{32}P-labeled inserts from 44-kDa (A), 55-kDa (B), and 70-kDa (C) recombinant phages. *E. risticii* genomic DNA was digested overnight with respective restriction enzymes, and approximately 2 μg of DNA was run on each lane.

DISCUSSION

In view of the complex nature and the multitudes of component proteins associated with an obligate intracellular parasite such as *E. risticii*, studies aimed at understanding the pathogenesis and identification of protective immunogen(s) are demanding. Potomac horse fever, since its recent recognition and isolation of the causative organism, *E. risticii*, has emerged as a disease of equines that is of worldwide significance. Recently, the development of diagnostic assays (21, 27) and analysis of antigenic composition of *E. risticii* (8) have been accomplished. However, there is considerable need to understand the overall pathogenic mechanism of the organism and identify the protective immunogen(s) as a goal toward the development of a subunit vaccine. From our studies of the immune response in horses (4), six major antigens (70, 55, 51, 44, 33, and 28 kDa) were considered as potential immunogens on the basis of their surface predilection (8), early recognition in the antibody response (4), and protection of horses from challenge infection during this early antibody response phase (unpublished data). A recent finding that *E. sennetsu*-immunized horses were protected from *E. risticii* challenge along with high reactivity to the 44-kDa protein of *E. risticii* (18) further suggests the protective importance of the 44-kDa antigen. Furthermore, single proteins have been demonstrated to be protective in other rickettsial species (13).

To evaluate the protective capabilities and the respective roles in the immune response of these individual proteins, it is necessary to obtain them in a relatively pure and native form. λgt11 has been a powerful vector for the expression of cloned proteins (28). Since the targeted proteins were highly antigenic, immunoscreening of λgt11 clones was successful in identifying and isolating the genes for four of the major antigens. Although several 4-base enzymes were used to eliminate underrepresentation of any particular genes, the

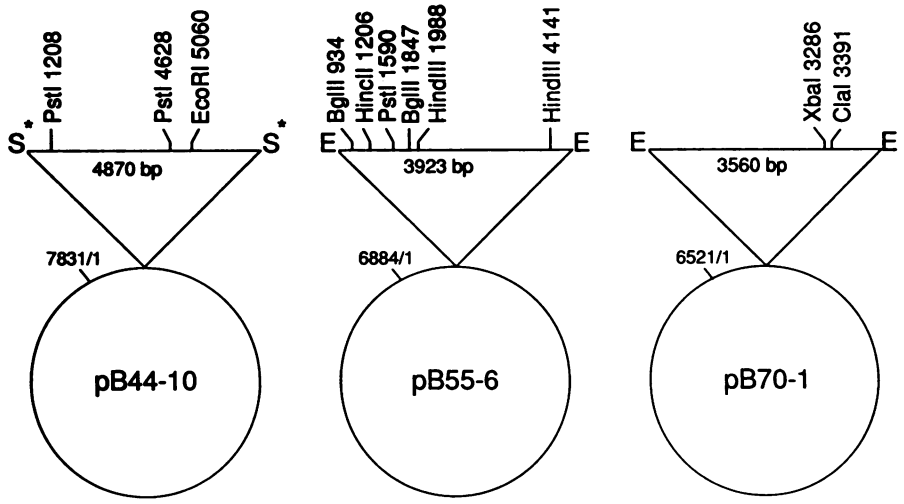

FIG. 5. Restriction maps of the three *E. risticii* recombinants subcloned into pBluescript SKII$^+$ plasmids. The numbers 44, 55, and 70 in the plasmid designations correspond to the antigens expressed by the recombinants. S* represents the lost *Sma*I site in the vector after ligation of the 44-kDa insert. E in the other plasmids corresponds to the *Eco*RI site, where the 55- and 70-kDa inserts have been cloned.

clones obtained seemed to be limited to few of the major antigens. Several of the expressing recombinants lost their immunoreactivity after attempts at plaque purification. We can speculate that this lack of stability and the reactivities could be the result of toxicity of the expressed proteins to the host cell (10), as has been observed for *Rickettsia rickettsii* (2), and thus might have resulted in the deficiency of clones for other major antigens.

It is interesting that the 55-kDa recombinant protein is a complete protein of identical in size to its native form and independent of IPTG induction (Fig. 1). This indicates that the protein could have been produced in the ehrlichae without any modifications. Further, the lack of dependency on the *lac* promoter for synthesis indicates the inclusion of a functional ehrlichial promoter along with the structural gene in this clone. Alternately, another λ promotor like *lom* (3) may be contributing to the synthesis of this protein. Since the DNA requirement for a protein of 55 kDa is about 1.4 kb and the insert size is 3.9 kb, nucleotide sequence information will help in understanding this phenomenon. β-Galactosidase fusion of the 70- and 44-kDa recombinants expressing 50 and 73%, respectively, of the native protein equivalents indicates that considerable sequences of these genes are present. The estimated sizes of the genes expressing the 70- and 44-kDa proteins are 1.9 and 1.1 kb, respectively, whereas the insert sizes are 3.5 and 4.8 kb, respectively.

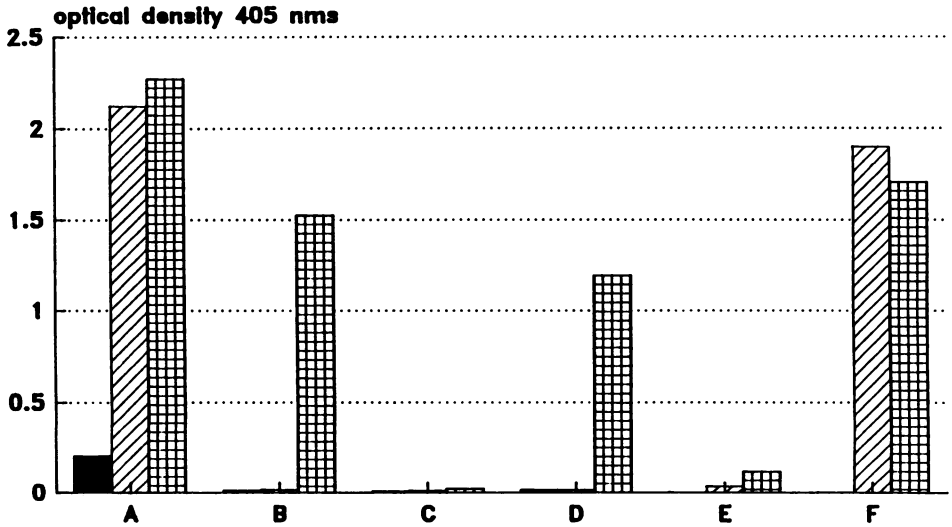

FIG. 6. ELISA antibody response of sera from mice immunized with the affinity-purified 55-kDa recombinant antigen, measured against the affinity-purified 55-kDa recombinant antigen (A) and *E. risticii* (B); sera from adjuvant-immunized mice measured against affinity-purified 55-kDa recombinant antigen (C) and *E. risticii* (D); and sera from mice immunized with *E. risticii* measured against affinity-purified 55-kDa recombinant antigen (E) and *E. risticii* (F). Symbols: ■, 14 days postimmunization; ▨, 26 days postimmunization; ▩, 54 days postimmunization. Mice were challenged on day 26 postimmunization. The 14-day postimmunization sera were not collected from mice immunized with *E. risticii*.

After sequence studies, full-length genes of these proteins can be isolated and characterized.

Lack of antigenic cross-reactivity by the respective monospecific antibodies and lack of cross-hybridization of the inserts and distinctly different patterns of hybridizations with genomic *E. risticii* DNA suggest the specific identity of each of the clones. It is interesting to note that the sum of sizes of the genomic DNA restricted fragments hybridizing with each of the inserts is much greater than can be accounted for by the insert size alone (Fig. 4). It is improbable that this could be due to incomplete digestion, since 3 to 5 times the needed amounts of enzymes were used in overnight digestions. However, methylation of DNA could have caused poor cutting with the restriction enzymes. Other possibilities are the presence of repeated sequences within the genes, as in the case of *R. rickettsii* (2) and *Rickettsia tsutsugamushi* (24), which detect similar sequences in other genes, or the presence of multiple copies of the genes or the flanking sequences, as has been suggested for *R. tsutsugamushi* (14). These can be verified by cloning and sequencing the complete genes.

Low ELISA reactivity of mouse anti-55-kDa antigen serum to the whole organism, as compared with that of the purified 55-kDa recombinant antigen, may be due to the relatively lower proportion of 55-kDa antigen as compared with total ehrlichia antigen available in the solid phase. Similarly, low reactivity of mouse anti-*E. risticii* serum to the purified 55-kDa recombinant antigen may conversely be due to a relatively low abundance of antibodies to the 55-kDa antigen as compared with that of antibodies against other ehrlichial antigens in the sera. Since Western blotting concentrates the individual protein onto a region on the nitrocellulose membrane, the specific reactivity of 55-kDa antigen antisera to the 55-kDa antigen of *E. risticii* was recognized. It may also be possible that the 55-kDa antigen might not be exposed on the surface of the organism and hence not available for binding in ELISA when intact organisms are used as the antigen. The 55-kDa antigen, since it protected only 33% of the mice, is probably not a major protective antigen.

Since these three *E. risticii* antigens were recognized by all experimentally infected horses and the recombinant forms were reactive with rabbit and horse antisera in Western blot analyses, studies are being undertaken to assess their protective capability in mice, singly and in combinations. Also, with the efforts to obtain clones for the other major antigens, it is hoped that we will be able to identify the protein(s) responsible for eliciting a protective immune response.

REFERENCES

1. **Allen, G. P., and M. R. Yeargen.** 1987. Use of λgt11 and monoclonal antibodies to map the genes for the six major glycoproteins of equine herpesvirus 1. J. Virol. **61**:2454–2461.
2. **Anderson, B. E., G. A. McDonald, D. C. Jones, and R. L. Regnery.** 1990. A protective protein antigen of *Rickettsia rickettsii* has randomly repeated, near identical sequences. Infect. Immun. **58**:2760–2769.
3. **Chirala, S. S.** 1986. The nucleotide sequences of the lac operon and phage junction in λgt11. Nucleic Acids Res. **14**:5935.
4. **Dutta, S. K., B. L. Mattingly, and B. Shankarappa.** 1989. Antibody response to *E. risticii* and antibody reactivity to component antigens in horses with induced Potomac horse fever. Infect. Immun. **57**:2959–2962.
5. **Dutta, S. K., A. C. Myrup, R. M. Rice, M. G. Robl, and R. C. Hammond.** 1985. Experimental reproduction of Potomac horse fever in horses with a newly isolated *Ehrlichia* organism. J. Clin. Microbiol. **22**:265–269.
6. **Dutta, S. K., B. E. Penney, A. C. Myrup, M. G. Robl, and R. M. Rice.** 1988. Disease features in horses with induced equine monocytic ehrlichiosis (Potomac horse fever). Am. J. Vet. Res. **49**:1747–1751.
7. **Dutta, S. K., R. M. Rice, T. D. Hughes, P. K. Savage, and A. C. Myrup.** 1987. Detection of serum antibodies against *E. risticii* in Potomac horse fever by enzyme-linked immunosorbent assay. Vet. Immunol. Immunopathol. **14**:86–92.
8. **Dutta, S. K., B. Shankarappa, S. R. Thaker, and B. L. Mattingly-Napier.** 1990. DNA restriction endonuclease cleavage pattern and protein antigen profile of *E. risticii*. Vet. Microbiol. **25**:29–38.
9. **Holland, C. J., M. Ristic, A. L. Cole, P. Johnson, G. Baker, and T. Guetz.** 1985. Isolation, experimental transmission and characterization of causative agent of Potomac horse fever. Science **227**:522–554.
10. **Helfman, D. M., and S. H. Hughes.** Use of antibodies to screen cDNA expression libraries prepared in plasmid vectors. Methods Enzymol. **152**:451–457.
11. **Lyon, J. A., R. H. Geller, J. G. Haynes, J. G. Chuley, and J. L. Weber.** 1986. Epitope map and processing scheme for the 195,000-dalton surface glycoprotein of *Plasmodium falciparum* merozoites deduced from cloned overlapping segments of this gene. Proc. Natl. Acad. Sci. USA **83**:2989–2993.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **McDonald, G. A., R. L. Anacker, and K. I. Garjian.** 1987. Cloned gene of *Rickettsia rickettsii* surface antigen: candidate vaccine for Rocky Mountain spotted fever. Science **235**:83–85.
14. **Oaks, E. V., R. M. Rice, D. L. Kelly, and C. K. Stover.** 1989. Antigenic and genetic relatedness of eight *Rickettsia tsutsugamushi* antigens. Infect. Immun. **57**:3116–3122.
15. **Oaks, E. V., C. K. Stover, and R. M. Rice.** 1987. Molecular cloning and expression of *Rickettsia tsutsugamushi* genes for two major protein antigens in *Escherichia coli*. Infect. Immun. **55**:1156–1162.
16. **Rikihisa, Y., G. C. Johnson, and C. J. Burger.** 1987. Reduced immune responsiveness and lymphoid depletion in mice infected with *Ehrlichia risticii*. Infect. Immun. **55**:513–517.
17. **Rikihisa, Y., and B. D. Perry.** 1985. Causative ehrlichial organisms in Potomac horse fever. Infect. Immun. **49**:513–517.
18. **Rikihisa, Y., C. I. Pretzman, G. C. Johnson, S. M. Reed, S. Yamamto, and F. Andrews.** 1988. Clinical, histopathological, and immunological responses of ponies to *Ehrlichia sennetsu* and subsequent *Ehrlichia risticii* challenge. Infect. Immun. **56**:290–296.
19. **Ristic, M.** 1986. Pertinent characteristics of leukocytic rickettsiae of humans and animals, p. 182–187. *In* L. Leiv (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
20. **Seidman, C. E.** 1988. Introduction of plasmid DNA into cells, p. 1.8.1. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
21. **Shankarappa, B., and S. K. Dutta.** 1989. Monoclonal antibody-mediated immunodiagnostic competitive enzyme-linked immunosorbent assay for equine monocytic ehrlichiosis. J. Clin. Microbiol. **27**:24–28.
22. **Shankarappa, B., S. K. Dutta, J. Sanusi, and B. L. Mattingly.** 1989. Production and characterization of monoclonal antibodies to *E. risticii*. Am. J. Vet. Res. **50**:1145–1149.
23. **Silhavy, T., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 137–138. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

23a. **Stover, C. K.** Personal communication.

24. **Stover, C. K., D. P. Marana, J. M. Carter, B. A. Roe, E. Mardis, and E. V. Oaks.** 1990. The 56-kilodalton major protein antigen of *Rickettsia tsutsugamushi*: molecular cloning and sequence analysis of the *sta-56* gene and precise identification of a strain-specific epitope. Infect. Immun. **58**:2076–2084.
25. **Stover, C. K., M. H. Vodkin, and E. V. Oaks.** 1987. Use of

conversion adaptors to clone antigen genes in λ gt11. Anal. Biochem. **163**:398–407.

26. **Struhl, K.** 1988. Separation of radio-active labeled DNA from unincorporated dNTP precursor by column chromatography, p. 3.4.7. *In* F. M. Ausubel, et al. (ed.), Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
27. **Thaker, S. R., S. K. Dutta, S. Adhya, and B. L. Mattingly-Napier.** 1990. Molecular cloning of *Ehrlichia risticii* and the development of gene probes for the diagnosis of Potomac horse fever. J. Clin. Microbiol. **28**:1963–1967.
28. **Young, R. A., and R. W. Davis.** 1983. Yeast RNA polymerase II genes: isolation with antibody probes. Science **222**:778–782.