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Isolation, Cultivation, and Partial Characterization of the ELB Agent Associated with Cat Fleas

S. RADULOVIC,[1] J. A. HIGGINS,[1] D. C. JAWORSKI,[1] G. A. DASCH,[2] AND A. F. AZAD[1]*

Department of Microbiology and Immunology, School of Medicine, University of Baltimore, Baltimore, Maryland 21201-1559,[1] and Viral and Rickettsial Diseases Program, Naval Medical Research Institute, Bethesda, Maryland 20814[2]

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ELB rickettsiae from cat flea homogenates were recovered in tissue culture cells following sequential passage through laboratory rats and the yolk sacs of embryonated chicken eggs. Seven days after inoculation of ELB from the infected yolk sacs, Vero cells and L929 cells were observed to contain intracellular bacteria as demonstrated by Diff Quik and indirect immunofluorescence assay staining. The rickettsial and ELB identity of the cultured agent was confirmed by PCR detection of the 16S rRNA and citrate synthase genes and PCR-restriction fragment length polymorphism analysis of the 17-kDa conserved rickettsial antigen gene. The ELB rickettsiae induced plaques in Vero cells on day 11 postinfection. Rat anti-ELB serum reacted at 1:4,096 to cultured ELB and had lower reactivity to *Rickettsia typhi* Wilmington (1:1,024), *Rickettsia akari* Kaplan (1:512), and *Rickettsia australis* JC (1:64). Spotted fever group polyclonal sera also exhibited lower reactivity to ELB than to the homologous antigen. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the ELB isolate and two *R. typhi* strains were identical.

The ELB agent was initially recognized by electron microscopy as a rickettsia-like microorganism that was present in a colonized population of the cat flea, *Ctenocephalides felis* (1). This agent reacted with antisera to *Rickettsia typhi* and was present in tracheal matrix, muscle, midgut epithelial lining, hypodermis, ovaries, and epithelial sheath of testes in adults and 100% of newly emerged fleas. Because it could not be cultivated and appeared related to *R. typhi*, its genetic material was amplified by PCR with rickettsia-specific primers for the 17-kDa antigen and citrate synthase (3). This study confirmed that ELB was a rickettsia but that it differed from *R. typhi*, *Rickettsia prowazekii*, and *Rickettsia rickettsii* in its 17-kDa gene sequence. The 16S rRNA sequence of ELB agent also differed from those of *R. typhi* and *R. rickettsii* (16). In addition, ELB was found to be maintained in fleas by an unusual but highly efficient and stable transovarial transmission. ELB's possible importance in human public health was assessed in a series of epidemiological investigations. Cat fleas were collected from opossums in Los Angeles County, California (22), an urban and suburban area with transmission of murine typhus to humans (2, 18). Both *R. typhi* and ELB agent were found in fleas, and *R. typhi* was found in opossum tissues (22). In southern Texas where one-third of cases of murine typhus in the United States occur, Schriefer et al. (17) found that both cat fleas and opossum tissues had ELB agent. *R. typhi* was detected less frequently in those fleas. Subsequently, four murine typhus patients from Texas were confirmed by PCR to have been infected with *R. typhi* while the fifth was infected with ELB agent (16). These studies clearly implicate ELB agent as a potential cause of human murine-typhus-like illness. Its maintenance in a novel cat flea-opossum zoonosis in urban areas is a new concern for public health.

Because only limited quantities of ELB agent are available in the fleas, additional studies on its pathogenicity, biological properties, antibiotic susceptibility, and genetic and antigenic characteristics have been impeded. We describe here the successful cultivation of ELB agent in Vero cells and initial characterization of some of its antigenic, protein, and biological properties.

MATERIALS AND METHODS

Isolation of ELB agent from cat fleas. Since our repeated attempts to grow ELB rickettsiae in cell culture directly from infected flea homogenates were unsuccessful, Sprague-Dawley male rats (Charles River, Wilmington, Mass.; 200 to 250 g) were used to propagate the rickettsiae. Rats were inoculated intraperitoneally with 0.3 ml of a homogenate of fleas (30 fleas per ml) in brain heart infusion broth (Difco, Detroit, Mich.). The animals were euthanized on day 7, and their livers and spleens were collected. The pooled tissues were homogenized in 10 ml of brain heart infusion broth, and 0.4 ml of the homogenate as well as two 10-fold serial dilutions was inoculated into 7-day-old embryonated chicken eggs (Truslow Farms) and incubated at 34°C. No specific embryo deaths were observed up to 12 days postinoculation. Thirteen eggs receiving the two highest concentrations of homogenate were harvested, and a partially purified seed was prepared through a pre-bovine albumin step according to the method of Weiss et al. (20) and frozen in 25 ml of sucrose-phosphate-glutamate buffer (SPG). Two serial log dilutions of this seed sporadically killed 15% of the eggs 6 to 12 days postinoculation, and sparse typhus-like rickettsiae could be seen by acridine orange staining of smears of dead egg yolk sacs. Crude yolk sac seed made from these eggs did not kill specifically on further passage. Yolk sacs from the remaining surviving embryos of all three passages were processed by Renografin density centrifugation (20) to prepare a concentrated and purified ELB yolk sac seed for tissue culture inoculation. The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services publication (National Institutes of Health).

Cultivation, detection, and purification of ELB. Vero cells (African green monkey kidney cells, ATCC C 1008) were cultivated in Dulbecco modified Eagle medium (DMEM) containing 4 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS; Biofluids Inc., Rockville, Md.) at 37°C in 5% CO_2. The cells were trypsinized and placed into six-well plates (Nunc Inc., Naperville, Ill.) at a concentration of 2×10^5 cells per well. Monolayers of Vero cells were inoculated with 0.2 ml of ELB-yolk sac suspension diluted in SPG. After absorption for 2 h at 37°C, DMEM with 4% FBS and antibiotics (gentamicin, 5 μg/ml; penicillin, 10,000 U/ml; streptomycin, 5 μg/ml, and fungizone, 4 μg/ml) were added. The monolayers were incubated at 34°C in 5% CO_2 for 12 days. The intracellular growth of ELB was monitored by Diff Quik (Baxter HealthCorp., Miami, Fla.) and indirect immunofluorescence assay (IFA) staining (13) on days 7, 9, and 11. Briefly, the cells were scraped from the bottom of the plates with a cell scraper and spun at 1,500 × *g* for 10 min at 4°C. The supernatant was removed, and the cell suspension was examined by Diff Quik and IFA staining following fixation in cold acetone for 10 min at room temperature.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Maryland School of Medicine, 655 West Baltimore St., Baltimore, MD 21201.

To prepare host cell-free ELB, infected Vero cells were cultivated in 25 150-cm^2 plastic tissue culture flasks (Nunc Inc.) and harvested with cell scrapers on day 11 when 90 to 95% of the cells were infected. The cell suspensions were pooled and pelleted at 13,000 × *g* at 4°C for 30 min in a Sorvall-RC 5C Plus (Dupont, Newtown, Conn.) centrifuge. The pellet was resuspended in 10 ml of SPG and centrifuged again at 1,500 × *g* for 10 min at 4°C. The supernatant was pelleted again at 13,000 × *g* at 4°C. The pellets were combined, and the cells were disrupted with an Omni homogenizer (high speed, 20,000 rpm; 30 s), resuspended in 10 ml of SPG, and spun at 1,500 × *g* for 30 min at 4°C. The supernatants were pooled and spun at 13,000 × *g* for 20 min at 4°C. The pellet was resuspended in 5 ml of SPG and Renografin purified (20). Light and heavy bands were pooled, diluted in 10 ml of SPG, and pelleted at 13,000 × *g* for 30 min. The final pellets were resuspended in 1 ml of SPG and frozen at −80°C.

Infection of L929 and HUVEC with ELB. Murine fibroblast cells (L929, ATCC CLL 1.2) were kindly provided by D. H. Walker, University of Texas Medical Branch at Galveston, and were cultured in DMEM with 5% FBS. Primary culture of the human umbilical vein endothelial cells (HUVEC) was obtained from D. Silverman, University of Maryland, Baltimore, and was cultured in McCoy 5A medium (Cellgro) (Mediatech, Herndon, Va.) supplemented with 20% FBS and 30 μl of the endothelial cell growth supplement per ml (H-Neurext; Upstate Biotechnology, Inc., Lake Placid, N.Y.). The cells were incubated at 37°C in an atmosphere containing 5% CO_2 for 24 h prior to inoculation with purified ELB yolk sac seed.

Cytopathic effect and plaque formation induced by ELB. Vero and L929 cells were seeded into 24-well plates (Nunc Inc.) as described above. Serial 10-fold dilutions of host cell-free Renografin-purified ELB were made, and 0.2 ml was inoculated per well. The plates were incubated at 37°C for 1 h and overlaid with 1 ml of 0.5% agarose warmed to 37°C in the DMEM containing 5% FBS–cycloheximide–2 mM L-glutamine. The plates were incubated at 34°C. On day 7, the infected cells were fed by adding 1 ml of fresh medium to the top of the agarose layer. The monolayers were observed daily from day 4 until 21 days postinoculation by inverted phase-contrast microscopy for the development of cytopathic effect. Neutral red staining of living cells was performed by applying 0.01% neutral red in DMEM with 1% FBS to the top agarose layer 11 days postinfection. The monolayers were examined for plaques after subsequent incubation of the plates overnight.

Molecular detection of ELB. Detection of rickettsia-specific DNA sequences in ELB-infected cell cultures, infected and uninfected fleas (flea colonies from Louisiana State University, Baton Rouge, and Paravax, Fort Collins, Colo., respectively), and *R. typhi*-infected cells (Ethiopian AZ322 and Wilmington strains) by PCR amplification of the 17-kDa antigen gene, 16S rRNA, and citrate synthase genes was done as described previously (15, 17). Briefly, the infected cells from one 150-cm^2 flask were harvested and spun at 1,500 × *g* for 10 min. The pellet was resuspended in 1 ml of sterile distilled water and boiled for 10 min. PCR was done with 10 μl of the boiled suspension as template. The target DNA sequences amplified by PCR were detected by agarose gel electrophoresis and ethidium bromide staining. The PCR products of the 17-kDa antigen gene amplified from ELB-infected and *R. typhi* Ethiopian AZ322- and Wilmington-infected cells were endonuclease digested for 1 h at 37°C with *Alu*I according to the supplier's recommendations (Gibco BRL, Grand Island, N.Y.), electrophoresed in an 8% acrylamide gel with a ϕX174 *Hae*III digest standard (Gibco BRL), and visualized by ethidium bromide staining.

Preparation of immune sera to ELB. Rat anti-ELB polyclonal serum was produced in two adult male rats which were immunized with 3×10^2 PFU of ELB. Five weeks after immunization, the rats were bled, and the IFA titer of serum antibodies to ELB was determined. Fluorescein-labeled goat anti-rat immunoglobulin G (heavy plus light chain; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:50 in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 [pH 7.2]) was used as the secondary antibody. The ELB isolate reactivities with *Rickettsia akari* species-specific monoclonal antibody (MAb) (CDC 78-26-4 and 77-244-20), typhus group-positive sera (lot no. 88-004), and spotted fever group control sera (lot no. 88-008; gift from J. Olson, Centers for Disease Control and Prevention, Atlanta, Ga.) were determined in an IFA. Cross-reactive spotted fever group (SFG) *Rickettsia conori* MAb directed against 135-kDa antigen was a gift from D. H. Walker.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a slab gel by a modification of the method described by Laemmli (12) with a 7.5% separating gel. The purified ELB organisms, as well as Wilmington and Ethiopian strains of *R. typhi*, were solubilized at room temperature in sample buffer (0.25 M Tris-HCl [pH 6.8], 10% SDS, 0.5 M dithiothreitol, 0.5% bromphenol blue, 50% glycerol [5′ Prime-3′ Prime, Inc., Boulder, Colo.]) at a concentration of 2.0 mg of protein per ml. A 20-μl (40-μg) sample was loaded into each lane. As molecular mass standard, we used the 10-kDa protein ladder obtained from Gibco BRL.

Electrophoretically separated polypeptides were transferred from gels to 0.2-μm-pore-size nitrocellulose membrane (Gibco BRL) by electrophoresis at 365 mA for 1 h at 4°C. After nonspecific protein binding was blocked with 5% nonfat dry milk in 25 mM Tris-buffered saline, antigens were detected on the nitrocellulose paper by reaction with rat anti-ELB polyclonal sera. After being incubated with the primary antibody for 1 h at room temperature, the nitrocellulose sheet was incubated for 1 h with goat anti-rat immunoglobulin G (heavy plus light chain) horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Inc.) diluted 1:1,000 in 1% milk–Tris-buffered saline, washed three times in Tris-buffered saline, and reacted with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) as the substrate.

RESULTS

Repeated attempts to grow ELB rickettsiae in cell culture directly from infected flea homogenates were unsuccessful, and although cells could be infected as determined by PCR and direct staining, the organisms were gradually lost without significant replication. In contrast, ELB rickettsiae from similar homogenates were recovered following passage by infection of laboratory rats and passaging in yolk sacs of embryonated chicken eggs. From the yolk sac seed, we successfully propagated ELB agent in Vero and L929 cell lines. The antibiotic concentrations of gentamicin and penicillin-streptomycin which we used in primary isolation were sufficient to be bactericidal for other bacteria associated with fleas, yet allow the ELB organism to multiply intracellularly in host cells. Seven days after inoculation of the ELB-infected yolk sac suspension, Vero cells, L929 cells, and HUVEC were observed to contain intracellular bacteria which multiplied in the cytoplasm of the host cells, demonstrated by both Diff Quik and IFA staining. Immunofluorescence staining of the infected Vero cell monolayer on day 7 with rat anti-ELB sera revealed organisms with typical rickettsial morphology in the cytoplasm of the infected cells as well as outside of the host cells. In contrast, rat anti-ELB serum did not stain uninfected Vero cells examined by IFA. The organisms grew slowly, but by day 11 postinfection, from 90 to 95% of Vero cells were infected. The infectivity was even lower in L929 cells, with 65 to 70% of the cells harboring ELB by day 11 postinfection. The ELB rickettsiae induced round cytopathic foci and plaques on day 11 postinfection in Vero cells. At 21 days postinfection, neutral red staining of the cells showed very distinct macroscopic plaques forming a typical reticular pattern. Uninfected Vero cell monolayer did not show any cytopathic foci or plaques. Cytopathic foci and plaque formation were not observed in L929 cells. HUVEC did not support the growth of the ELB agent compared with Vero and L929 cells. The number of ELB organisms decreased significantly in HUVEC passages. The HUVEC mostly detached on day 7 postinfection with ELB. Heavily infected Vero cells showed injury and necrosis at later stages of intracellular infection. The cells between cytopathic foci underwent dramatic lysis when the foci fused, making them difficult to identify individually. The ELB agent adapted well in Vero and L929 cells, confirming that it is capable of entry, survival, and exit from the host cell.

IFA tests with a panel of polyclonal and monoclonal sera were used to confirm the identity of the cultured ELB agent (Table 1). Rat anti-ELB serum reacted at 1:4,096 to cultured ELB and had lower reactivity to *R. typhi* Wilmington (1:1,024), *R. akari* Kaplan (1:512), and *Rickettsia australis* JC (1/64) (not shown). Rat and guinea pig anti-*R. typhi* and human anti-typhus group sera reacted 8 to 16 times more strongly to *R. typhi* than cultivated ELB. Similarly, although MAb T65-1G2.2 (14) detects native and denatured surface protein antigens of *R. typhi* but not *R. prowazekii* or *Rickettsia canada*, it reacted weakly with ELB (1:8,192 homologous titer). Spotted fever group polyclonal sera also exhibited lower reactivity to ELB than to the homologous antigen, varying from relatively high reactivity to *R. akari* (one-fourth titer), to low reactivity with mouse anti-*R. australis* sera (1/64 titer), to negligible reactivity with human anti-spotted fever group sera. Similar levels of low or negligible cross-reactions of ELB agent with three MAbs to spotted fever rickettsiae were also obtained.

PCR assays utilizing rickettsia-specific 17-kDa antigen gene, citrate synthase gene, and 16S rRNA resulted in the amplifi-

TABLE 1. Reactivity of the ELB isolate with typing sera and MAbs by IFA

Typing serum or MAb	Reciprocal IFA titer	
	Homologous strain	ELB isolate
Sera		
Rat anti-*R. typhi* (Wilmington strain)	16,384	1,024
Guinea pig anti-*R. typhi* (Wilmington strain)	8,192	1,024
Rabbit anti-*R. akari* (Kaplan strain)	2,048	512
Mouse anti-*R. australis* (JC strain)	4,096	64
Human anti-spotted fever group rickettsia	16,384	64
Human anti-typhus group rickettsia	8,192	512
Human anti-*R. rickettsii*	4,096	<16
MAbs		
R. akari 78-26-4	8,192	128
R. akari 77-244-20	16,384	<16
R. typhi T65-1G2.2[a]	8,192	64
R. conori F2-53	81,920	128

[a] *R. typhi*-specific MAb directed against species-specific protein antigen (SPA) (14).

cation of these gene segments from ELB-infected fleas and Vero cells. The pattern of the *Alu*I restriction digest of the 17-kDa antigen gene product from infected Vero cells was typical of ELB-infected fleas but different from that of *R. typhi* (Fig. 1; citrate synthase and 16S rRNA results not shown). Thus, our PCR data confirmed the propagation of the rickettsial agent in vitro.

Coomassie blue-stained SDS-PAGE profiles of the ELB isolate and two *R. typhi* strains (Wilmington and Ethiopian AZ322) exhibited no obvious differences (Fig. 2). All three strains have a major 120-kDa antigen and immunoreactive components occupying the lanes between the 40- and 70-kDa regions of the gel. A band corresponding to 190-kDa antigen, previously described for spotted fever group rickettsiae and *R. canada*, was not observed for the ELB isolate.

Immunoreactivity of the ELB isolate, two *R. typhi* strains (Wilmington and Ethiopian AZ322), and *R. akari* Kaplan with rat anti-ELB serum is presented in Fig. 3. Strong lipopolysaccharide (LPS) and 50- to 60-kDa protein reactivity was observed for the two *R. typhi* strains and the ELB isolate. There was no cross-reactivity between rat anti-ELB serum and the LPS of *R. akari* Kaplan. On the other hand, rat anti-ELB serum recognized the 120-kDa protein of *R. akari* Kaplan.

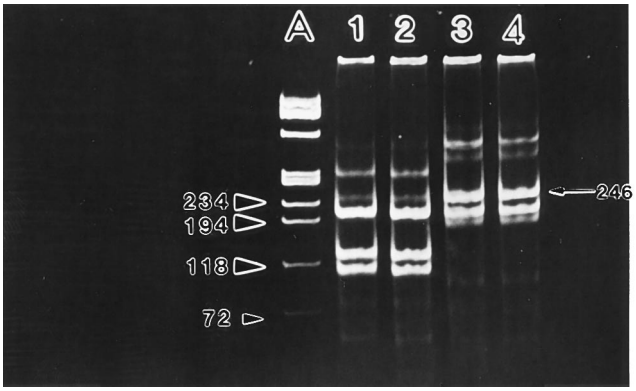

FIG. 1. Ethidium bromide-stained, polyacrylamide gel electrophoretogram of *Alu*I-digested, 17-kDa antigen gene PCR product amplified from in vitro-propagated ELB isolate. Lane A, ϕX174 marker (molecular mass [in kilodaltons] is indicated at either side of the figure); lane 1, ELB agent propagated in vitro; lane 2, ELB agent in fleas; lane 3, *R. typhi* (Wilmington strain); lane 4, *R. typhi* (Ethiopian AZ322 strain).

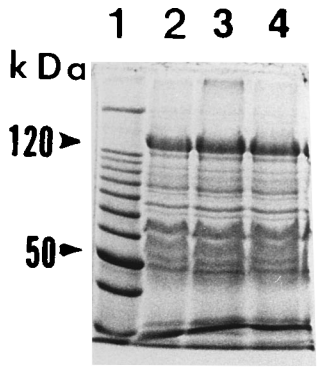

FIG. 2. ELB isolate separated by SDS-PAGE on a 7.5% polyacrylamide gel and Coomassie blue stained. Lane 1, 10-kDa molecular mass marker; lane 2, ELB isolate propagated in vitro (antigen); lane 3, *R. typhi* (Wilmington strain) (antigen); lane 4, *R. typhi* (Ethiopian AZ322 strain) (antigen).

DISCUSSION

Attempts to directly grow insect-associated intracellular bacteria in mammalian cell culture remain problematic. The primary problems faced by investigators are the recovery of a few physiologically intact intracellular microorganisms from chitinous tough arthropod tissues and the presence of diverse external and gut-associated bacterial and fungal contaminants associated with the arthropod. Surface sterilization prior to the dissection of internal tissues and the use of antibiotics in cell culture often reduce microbial contaminants to manageable levels. Nonetheless, recovery of the desired microorganisms may still be compromised because of other physiological problems. Although we were able to obtain initial recovery of ELB rickettsiae in Vero cells from flea homogenates, for unknown reasons the agent was gradually lost without significant growth. One could speculate that the long-term maintenance of ELB agent in the flea via transovarial transmission resulted in the loss of rickettsial infectivity for vertebrate hosts. Thus, several passages of ELB agent through vertebrate hosts may have then enhanced its infectivity for mammalian cells. We were able to isolate ELB from the flea homogenates following sequential passage from infected rats through embryonated chicken eggs. However, even with several passages in the yolk sacs of embryonated chicken eggs, the ELB agent still exhibited limited growth and was not lethal to the developing embryo.

Another possible explanation for the difficulty experienced

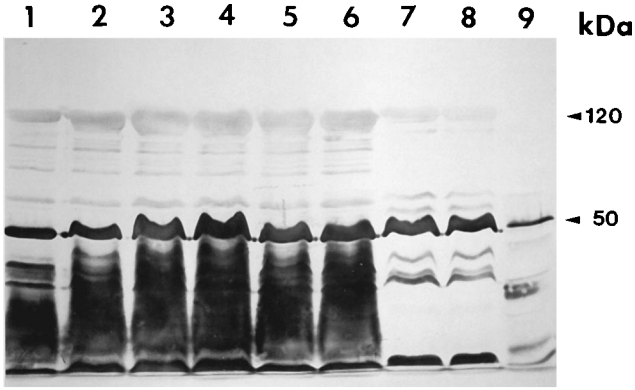

FIG. 3. Immunoblotting of the ELB isolate, *R. typhi* Wilmington, *R. typhi* Ethiopian AZ322, and *R. akari* Kaplan with rat anti-ELB serum. Lanes 1 and 2, *R. typhi* Ethiopian AZ322 (antigen); lanes 3 and 4, *R. typhi* Wilmington (antigen); lanes 5 and 6, ELB isolate propagated in vitro (antigen); lanes 7 and 8, *R. akari* Kaplan (antigen); lane 9, 10-kDa molecular mass marker.

in direct adaptation of ELB to cell culture is that ELB is exposed to immune factors (5–7, 10, 11) present in the flea homogenates that damage it sufficiently to prevent growth, but not infection, of tissue culture cells. It is very likely that similar factors are present in intact fleas and may influence the maintenance, transmission, and isolation of ELB rickettsiae in this host. Whether ELB also influences the activity of this system (10) is unknown.

Here we describe the adaptation of ELB agent to mammalian cell culture. The rickettsial identity of the cultured agent was confirmed by PCR amplification of its 17-kDa antigen, citrate synthase, and 16S rRNA genes. The *Alu*I digest of the 17-kDa PCR product was identical to that of ELB from cat fleas (3, 16, 17). ELB grown in culture more strongly resembled typhus than spotted fever group rickettsiae in its light microscopic morphology, growth pattern in Vero cells, and delayed formation of small plaques.

Our isolate of ELB has been provided to other investigators, who successfully used it for further in vitro culture; we have also been able to use our isolate to infect rats and cat fleas. After freezing and thawing of the isolate, we have not observed marked differences in its growth in Vero cells. Therefore, we are confident that our isolate retains its infectivity for both vertebrate and invertebrate cells.

Genetic and antigenic studies relating to the precise phylogenetic affinities of ELB agent remain inconclusive. The initial characterization of the sequence of a portion of the 17-kDa antigen gene and its reactivity with anti-*R. typhi* MAbs suggested that it resembled typhus rickettsiae (3). However, its 16S rRNA sequence more closely resembled those of *R. akari* and *R. australis*, a distinct clade of the spotted fever group (16, 19). However, the PAGE profile obtained here clearly resembled that of *R. typhi* and lacked the prominent larger rOmpA protein found in the spotted fever group and *R. canada* (8, 9). PCR amplification with rOmpA primers Rr.190.70p and Rr.190.602n was unsuccessful with ELB (unpublished data). This result is perhaps not surprising since these primers also do not amplify DNA from typhus rickettsiae, *R. akari*, or *R. australis* (4, 15). Further genetic analysis will be necessary to resolve whether ELB lacks rOmpA entirely and the precise relationship of these rickettsiae. Immunoblotting studies suggest that ELB contains an LPS which reacts to typhus group-reactive sera but not with antisera to the spotted fever group LPS (Fig. 3). This is consistent with the somewhat stronger IFA reactions obtained with typhus-reactive sera than with spotted fever sera (Table 1).

In summary, ELB has been adapted to growth in mammalian cell culture. This has permitted initial study of its antigenic and biological properties. The ability to grow this agent in greater quantities will permit detailed assessment of its pathogenic properties and susceptibility to antibiotics and examination of its genetic, physiological, and biochemical characteristics. Moreover, the unique serological properties of ELB are consistent with the conclusions reached by genetic analyses that ELB is a distinct species of rickettsia. This conclusion is perhaps not surprising since this group also contains the only other well-characterized insect-associated rickettsiae. It is likely that the recently described but presently uncultivated AB bacterium from ladybird beetles (19, 21) will have similar typhus-like properties.

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