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Molecular Cloning and Expression of *Rickettsia tsutsugamushi* Genes for Two Major Protein Antigens in *Escherichia coli*

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Several polypeptide antigens of *Rickettsia tsutsugamushi* are recognized by human or primate convalescent sera and may be important protective immunogens. Molecular cloning and expression of the genes encoding the 110K (110 kilodalton) and 56K polypeptide antigens of *R. tsutsugamushi* Karp were accomplished in the λgt11 expression vector system. Southern blot analysis with the cloned fragments for the 56K polypeptide antigen (0.7 kilobases) and the 110K polypeptide antigen (5.4 kilobases) confirmed that the insert DNA was rickettsial and not host cell in origin. Expression of a complete 110K polypeptide was shown to be independent of isopropyl-β-D-thiogalactopyranoside induction, suggesting that an intact rickettsial promoter was operational. Epitopes of the 56K polypeptide were expressed as *lac* promoter-dependent β-galactosidase fusion proteins. Polyclonal antibody, affinity purified against the recombinant 110K and 56K polypeptides, reacted with polypeptides of similar size in the Kato and Gilliam strains of *R. tsutsugamushi*. Group-reactive, but not strain-specific, monoclonal antibodies against the 56K polypeptide reacted with the cloned portion of the 56K polypeptide. Western blot analysis demonstrated that the cloned 56K Karp antigen gene product is recognized by human convalescent serum.

The scrub typhus group of rickettsiae contains one species (*Rickettsia tsutsugamushi*) consisting of a multitude of strains which exhibit serologically distinct epitopes as well as many cross-reactive epitopes. These obligate intracellular bacteria, which are transmitted to humans by infected mites, are the causative agents of scrub typhus fever. The disease is endemic in many areas of the Orient, including Japan, Southeast Asia, and the Philippines. Structurally, scrub typhus rickettsiae resemble gram-negative bacteria, in that distinct outer and inner membranes are present (15). Components of the envelope, such as proteins, lipids, and carbohydrates, are largely undefined owing to the technical difficulties in obtaining large quantities of scrub typhus rickettsiae for analysis. Recently, several different proteins, with a variety of molecular weights, have been identified as *R. tsutsugamushi* structural components. A subset of these proteins is recognized by sera obtained from immunized animals (3, 6, 7, 17) and infected humans (unpublished observations). Although analytical approaches can be used to study scrub typhus protein antigens, it still remains technically and economically unfeasible to produce large quantities of purified *R. tsutsugamushi* for the isolation of individual antigens for vaccine purposes. Expression of rickettsial antigens via recombinant DNA technology should overcome this problem to a large degree.

This report describes the cloning of *R. tsutsugamushi* genes for two protein antigens in the λgt11 expression vector system. This recombinant system, which has been used successfully for cloning several antigens of other pathogens which are difficult to grow (19, 22), allowed us to minimize potential problems with the expression of rickettsial genes and antigen instability. We report the successful cloning of the 110 kilodalton (110K) and 56K polypeptide antigens of *R. tsutsugamushi* Karp; we demonstrate with antibody, affinity purified against the recombinant antigens, that cross-reactive antigens of similar molecular weights are present in the Kato and Gilliam strains as well.

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MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and media. Yolk sac seeds of *R. tsutsugamushi* Karp, Kato, and Gilliam were used to infect mouse L-929 cells. The passage histories of these rickettsial seeds, which were derived from plaque-purified organisms, are as follows: Karp strain, passaged 52 times in embryonated chicken eggs, 3 times in mouse L-929 cells, and 6 times in embryonated chicken eggs (E52-L3-E6); Kato strain, E162-L3-E8; and Gilliam strain, E164-L3-E8. These strains are part of the Walter Reed Army Institute of Research collection. In addition, a non-plaque-purified Karp seed (passage history, E107) with no known passage in L cells, was propagated in primary chicken embryo cells for Southern blot experiments. This seed was kindly provided by B. Hanson of the University of Maryland School of Medicine, Baltimore. Gradient-purified *Rickettsia conorii* (Moroccan strain) was also used in Southern blot experiments.

The *Escherichia coli* strains Y1089 (21) and Y1090 (21) were used for growing and screening λgt11 and λgt11 clones. The *E. coli* strains were grown in L broth (LB) or on L agar containing ampicillin at 100 μg/ml (Sigma Chemical Co., St. Louis, Mo.).

Mouse L-929 cells were grown in an antibiotic-free minimal essential medium (MA Bioproducts, Walkersville, Md.) containing Earle salts, 2 mM glutamine, and 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Growth and purification of *R. tsutsugamushi*. Subconfluent monolayers of mouse L-929 cells in 150-cm^2 tissue culture flasks were infected with yolk sac suspensions of *R. tsutsugamushi* diluted in sucrose-phosphate-glutamate buffer (1). After incubation for 1 h at ambient temperature, the monolayers were washed with Hanks balanced salt solution (MA Bioproducts) and incubated at 34°C in minimal essential medium containing 2% fetal calf serum in an atmosphere of 5% CO_2 and 95% air. The infected monolay-

ers were harvested when greater than 70% of the cells were infected. *R. tsutsugamushi*-infected monolayers of L-929 cells were scraped into cold Tris-EDTA-saline buffer (TES buffer; 25 mM Tris, 10 mM EDTA, 150 mM NaCl [pH 8.0]), centrifuged at 2,600 × *g* for 5 min, and resuspended in TES buffer. This suspension was maintained on ice and blended at high speed for 2 min in a Sorvall Omni mixer (Dupont Co., Newtown, Conn.) The blended suspension was subjected to low-speed centrifugation (360 × *g* at 4°C) to pellet most of the unwanted host cell nuclei. This step was repeated twice on the resulting supernatant. The nucleus-depleted lysate was concentrated by centrifugation (16,000 × *g*), resuspended in TES buffer, loaded onto 12 Renografin (Squibb Diagnostics, New Brunswick, N.J.) step gradients (19, 30, and 50% Renografin in TES buffer), and centrifuged for 2 h at 83,000 × *g*. The rickettsiae at the interface of the 30 and 50% Renografin layers were collected, diluted five-fold, and pelleted at 20,000 × *g* for 20 min. Rickettsiae at the top of the 30% interface contained a substantial amount of host cell material, while those at 50% interface were relatively free of host cell material as determined by light microscopy and polyacrylamide gel electrophoresis of these preparations.

DNA manipulation and isolation. *R. tsutsugamushi* chromosomal DNA was prepared from the purified bacteria by sodium dodecyl sulfate lysis, proteinase K digestion, RNase A (Sigma) treatment, phenol-chloroform (International Biotechnologies, Inc., New Haven, Conn.) extraction, and ethanol precipitation as described by Silhavy et al. (14). Phage DNA was prepared as described by Silhavy et al. (14).

Restriction enzyme digests, agarose gel electrophoresis, and Southern blot hybridizations were all carried out as described by Maniatis et al. (11). Probes were labeled by nick translation by using a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and [α-^{32}P]dCTP (New England Nuclear Corp., Boston, Mass.). Unincorporated nucleotides were removed on a 5-ml column of Sephadex G-50 (Pharmacia, Inc., Piscataway, N.J.) in TE buffer (50 mM Tris, 1 mM EDTA [pH 7.8]).

Construction of phage libraries and subclones. *R. tsutsugamushi* Karp DNA (10 μg) was partially cleaved with a mixture of restriction enzymes yielding blunt ends by the following method. Equal unit amounts of the enzymes *Aat*I, *Alu*I, *Dra*I, *Eco*RV, *Pvu*II, and *Rsa*I (International Biotechnologies) were mixed immediately prior to digestion of DNA. The DNA was then digested with 0.3 U of the enzyme mixture per μg of DNA to obtain blunt-ended restriction fragments of approximately 0.2 to 8 kilobases (kb). *Eco*RI sites were methylated with 50 U of *Eco*RI methylase (New England BioLabs, Inc., Beverly, Mass.) at 37°C for 30 min. Before linker ligation, the DNA was extracted with phenol-chloroform-isoamyl alcohol (24:23:1) and concentrated by ethanol precipitation. The DNA was suspended in ligation buffer (50 mM Tris [pH 7.8], 10 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM spermidine, 0.7 mM ATP) and split into three separate linker ligation reactions for the addition of either 8-mer, 10-mer, or 12-mer phosphorylated *Eco*RI linker (final concentration, 80 μg/ml; Pharmacia). The ligation was performed by overnight incubation with 10 U of T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 4°C. The separate linker ligation reactions were pooled, and NaCl was added to 100 mM. Excess linkers were digested with 500 U of *Eco*RI and separated from ligated insert-linker DNA by gel filtration on a 5-ml column of Sepharose CL-4B (Pharmacia) in TE buffer plus 0.3 M NaCl. This procedure was repeated to exhaustively remove any remaining excess linker oligonucleotides. The linker-ended insert DNA was concentrated by ethanol precipitation and suspended in TE buffer. An estimated 0.2 μg of linker-ended DNA was ligated to 1 μg of dephosphorylated *Eco*RI-digested λgt11 DNA (Promega Biotec, Madison, Wis.) in a 5-μl ligation reaction. After incubation at 16°C for 4 h with 1 U of T4 DNA ligase (New England Biolabs), the ligation mixture was added directly to a λ-packaging extract (Promega Biotec) and incubated for 2 h at 22°C. This mixture was gently vortexed with 0.5 ml of SM buffer (0.1M NaCl, 10 mM Tris [pH 7.9], 10 mM $MgSO_4$) (11) and 25 μl of chloroform prior to screening or storage at 4°C.

For Southern blot experiments, probes were prepared from purified recombinant phage DNA prepared from high-titered phage lysates. Recombinant λgt11 DNA (50 μg) was digested with *Eco*RI and subjected to agarose gel electrophoresis (0.7% SeaPlaque, low-melting-temperature agarose; FMC Corp., Marine Colloids Div., Rockland, Maine). The desired insert fragments were excised from the gel, and electrophoresed to remove any contaminating vector DNA. The cloned insert restriction fragments were again cut from the gel, melted, and diluted in a low-salt buffer (0.25 M NaCl in 10 mM Tris [pH 7.2], 1 mM EDTA). Agarose was removed from the restriction fragments on Elutip-d columns (Schleicher & Schuell, Inc., Keene, N.H.). Restriction fragments were eluted from the column with 0.3 ml of high-salt buffer (1 M NaCl in 10 mM Tris [pH 7.2], and 1 mM EDTA). The DNA was recovered with ethanol precipitation, washed with 70% ethanol, dried, and suspended in TE buffer. These purified fragments were used as probes for Southern blot hybridization analysis of *R. tsutsugamushi* genomic DNA digested with *Hin*dIII.

Preparation of antisera. Two rabbits repeatedly immunized with a yolk sac seed of *R. tsutsugamushi* Karp by intravenous inoculation were used as a source of antisera for screening recombinant libraries and for Western blot analysis. Before use as a screening reagent, the sera from both rabbits were pooled and absorbed with *E. coli* and λgt11 to reduce background signals. For each 5 ml of pooled sera, the following procedure was used. A culture of *E. coli* Y1090 (4 liters) and a 2-liter culture of *E. coli* BNN97 (20) (induced at 42°C to generate λgt11 phage and subsequently grown at 37°C in the presence of 10 mM isopropyl-β-D-thiogalactoside [IPTG] until lysis occurred) were collected by centrifugation. The pellets were stored at −80°C. The induced BNN97 culture broth was treated with polyethylene glycol overnight at 4°C (11) to precipitate λgt11 phage. The phage were collected by centrifugation at 11,000 × *g* for 20 min and stored at −80°C. The bacterial pellets (Y1090 and BNN97) were suspended in 20 ml of Tris buffer (10 mM Tris [pH 7.4]) containing 50 μM phenylmethylsulfonyl fluoride (Sigma), pooled, and sonicated for three 1-min cycles on ice. The phage preparation was suspended in 5 ml of Tris buffer and sonicated separately. The sonicated bacteria were mixed with Triton X-100 (2%) at 37°C for 1 h, and the resulting lysate was centrifuged at 3,000 × *g* for 10 min. The 3,000 × *g* pellet was suspended with 5 ml of pooled sera, incubated at 25°C for 1 h with occasional mixing, and finally centrifuged at 20,000 × *g* for 20 min. The supernatant, containing partially absorbed sera, was stored frozen until the next step. The 3,000 × *g* supernatant, containing membranes, disrupted bacteria, and solubilized proteins, was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet from the 100,000 × *g* centrifugation was suspended with the sonicated λgt11 phage and mixed with the partially absorbed sera for 3 h at 25°C. This preparation was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was collected and stored frozen.

Sera absorbed by this procedure had a significantly reduced amount of reactivity with *E. coli* proteins when monitored by Western blot analysis and plaque-screening procedures.

Monoclonal antibodies (MAb) used in this study have been previously described (4, 7).

Screening λgt11 libraries with *R. tsutsugamushi* antisera. The packaged λgt11 phage (0.1 ml) were mixed with a stationary-phase Y1090 culture (0.2 ml) for 15 min at room temperature and then with preheated (48°C) LB soft agarose (0.7% agarose) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 400 μg/ml) and poured on an 90-mm LB plate. After incubation at 42°C for 3 h, these plates were overlaid with nitrocellulose disks (BA85; Schleicher & Schuell) which had been previously saturated in 10 mM IPTG. Incubation was continued at 37°C for 2 h before the filter was removed and a second IPTG-treated filter was overlaid for an additional 1-h incubation. Duplicate filters prepared in this manner were used so that false-positives could be identified in the screening process. The nitrocellulose filters were washed in three changes of Tris-saline buffer (10 mM Tris, 150 mM NaCl [pH 7.2]), followed by incubation with 2% casein (Sigma) in Tris-saline buffer for 30 min. The absorbed (described above) rabbit antiserum pool (diluted pool 1:200 in 2% casein), which recognized at least eight distinct *R. tsutsugamushi* Karp polypeptides by Western blot analysis, was then incubated with the filter for 3 h. After removal of the antibody, the filters were washed for 10 min with Tris-saline buffer, followed by two 10-min washes with Tris-saline buffer containing 0.05% Triton X-100 and then another Tris-saline wash. Protein A (Pharmacia), iodinated by the chloramine T method (8), was added to the filters for 1 h. The filters were washed again as described above, air dried, and exposed to X-ray film (Blue Brand; Eastman Kodak Co., Rochester, N.Y.) overnight. Antigen-positive plaques were picked, plaque purified at least two times (using the screening procedure to pick positive plaques), and finally stored in SM buffer.

Preparation and analysis of recombinant lysogens. Lysogens of λgt11 recombinant phage were prepared as follows. Recombinant phage were used at multiplicities of infection of 10 to 100 to infect *E. coli* Y1089. Single colonies grown at 32°C on LB plates were tested for sensitivity at 42°C. Potential lysogens (cultures sensitive to 42°C) were prepared for Western blot analysis as follows. The cultures were grown in LB with ampicillin (100 μg/ml) at 32°C to an A_{600} of 0.5 and shifted to 42°C for 20 min. The cultures were then divided into two flasks and shifted to 37°C, and IPTG (final concentration, 10 mM) was added to one of the flasks. Incubation at 37°C was maintained for 1 to 3 h before harvesting by centrifugation at 3,000 × *g* at 4°C. The supernatant medium was drained, and the pellet was suspended in hot electrophoresis sample buffer (5) and immediately heated in a boiling water bath for 3 min prior to storage at −80°C or polyacrylamide gel electrophoresis.

Affinity purification of antibody against recombinant protein antigens. For the antibody selection procedure (10), recombinant phage were used to infect *E. coli* Y1090 on 139-mm petri dishes of LB-agarose so that there were approximately 10^5 plaques per dish. IPTG-saturated nitrocellulose (137 mm diameter) was used to overlay the plates for 3 h at 37°C. This piece of nitrocellulose, containing bound recombinant antigen, was washed with Tris-saline buffer and subsequently blocked with casein. The filter was then incubated for 3 h with absorbed rabbit antisera against *R. tsutsugamushi* Karp (diluted 1:100), and then washed as described above. After the last Tris-saline wash, the filter was washed with 0.15 M NaCl. The antibody bound specifically to recombinant antigens was eluted with 10 ml of glycine-saline buffer (0.2 M glycine [pH 2.8], 150 mM NaCl) and immediately neutralized to pH 7.0 with Tris. Antibodies selected in this manner were diluted threefold in casein for Western blot analysis.

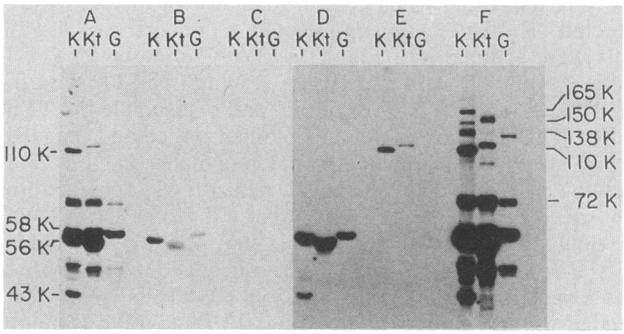

FIG. 1. Western blot analysis using rabbit anti-Karp strain sera and recombinant-antigen-selected antibodies against the Karp, Kato, and Gilliam strains of *R. tsutsugamushi*. Pooled hyperimmune rabbit sera against the Karp strain and antibodies affinity purified against recombinants or against λgt11 were reacted with nitrocellulose strips containing separated polypeptides of the Karp (K), Kato (Kt), and Gilliam (G) strains of *R. tsutsugamushi*. The sera used were pooled hyperimmune rabbit sera diluted 1:400 (A); affinity-purified antibody against λgt11Rts11 (B), λgt11 (C), λgt11Rts13 (D), and λgt11Rts14 (E); and pooled hyperimmune rabbit sera diluted 1:100 (F). The numbers on the left and right indicate the apparent molecular sizes (in kilodaltons) of the scrub typhus antigens. The 56K antigen was recognized by the antibody used in panels B and D, and the 110K antigen was recognized by the antibody used in panel E.

Polyacrylamide gel electrophoresis and Western blot analysis. A discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, using the buffers described by Laemmli (9), was used for the separation of both rickettsial and *E. coli* lysates. The gels consisted of 9% acrylamide cross-linked with *N,N'*-diallyltartardiamide. Electrophoresis was performed overnight at a constant 60 V.

Western blot analysis was performed by previously described procedures (12). Casein (2% in Tris-saline buffer) was used as a filler and as a diluent of antisera. Iodinated protein A was used for the detection of antibody bound to antigens recognized by rabbit anti-*R. tsutsugamushi* Karp or rabbit anti-β-galactosidase (Cooper Biomedical, Inc., Malvern, Pa.).

RESULTS

Antigens of *R. tsutsugamushi*. The hyperimmune rabbit sera used in this study reacted with several polypeptides of purified *R. tsutsugamushi* Karp, Kato, and Gilliam as determined by Western blot analysis (Fig. 1A and F). In the Karp strain, nine moderate to strong bands, with apparent molecular sizes of 165, 150, 138, 110, 72, 58, 56, 49, and 43 kilodaltons, were recognized. Polypeptides of similar molecular sizes were evident in all three strains. Similar analysis, using human convalescent sera from over 20 patients naturally infected with *R. tsutsugamushi*, has indicated that the 56K polypeptide is a dominant antigen, in that all human sera tested to date contain antibodies to this polypeptide antigen (unpublished data). Antibodies to several other scrub typhus polypeptide antigens (including the 58K and the 110K antigens) are also present in the majority of human convalescent

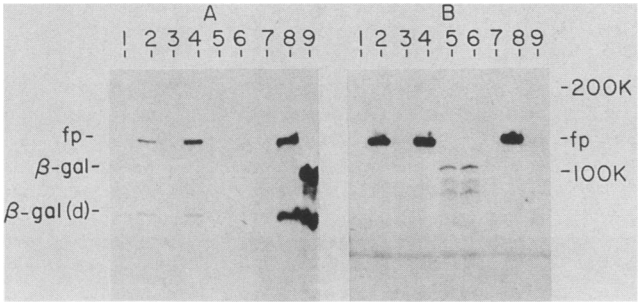

FIG. 2. Western blot analysis of lysogens with anti-β-galactosidase antibody and anti-Karp strain antisera. Lysates of lysogens grown either in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, 8, and 9) of IPTG were analyzed in a Western blot procedure by using either rabbit anti-β-galactosidase (A) or rabbit anti-Karp (B). (A) the fusion proteins (fp), β-galactosidase (β-gal), and a proteolytic degradation product of β-galactosidase [β-gal(d)] are indicated. (B) The molecular size markers (200K and 100K) and fusion proteins are indicated. The following lysogens were run on this gel: Y1089::λgt11Rts12 (lanes 1-2), Y1089::λgt11Rts13 (lanes 3-4), Y1089::λgt11Rts14 (lanes 5-6), Y1089::λgt11Rts10 (lanes 7-8), and Y1089::λgt11 (lane 9).

sera. The strong reactivity of the rabbit sera against several different rickettsial polypeptides permitted the use of this antisera for screening the recombinant libraries for a variety of rickettsial antigens.

Detection of recombinant phage synthesizing *R. tsutsugamushi* antigens. The genomic library contained approximately 15% recombinant phage as determined by the lack of β-galactosidase activity on X-Gal plates. By using the hyperimmune rabbit anti-Karp strain sera, which had been previously absorbed with *E. coli* BNN97, λgt11, and *E. coli* Y1090, to screen the recombinant library, five positive clones were detected after screening approximately 2,500 recombinant plaques. The positive plaques were plaque purified and subsequently amplified for further experimentation.

Analysis of recombinant lysogens. Western blot analysis of lysates from IPTG-induced and uninduced recombinant lysogens was used to determine whether (i) the cloned antigens were under the regulation of the *lac* promoter and (ii) the cloned antigen was fused to the *lacZ* gene product (β-galactosidase). With either rabbit anti-β-galactosidase or rabbit anti-*R. tsutsugamushi* Karp, recombinant lysogens λgt11Rts10, -11, -12, and -13 had high-molecular-weight protein antigens which were present only after the addition of IPTG (Fig. 2; data for λgt11Rts11 not shown). The β-galactosidase band in all of these lysogens had an elevated molecular weight as compared with the same antigen in the control IPTG-induced λgt11 lysogen. In addition, the antigenic activity, detected with the rabbit anti-Karp sera, comigrated with the β-galactosidase antigen, indicating that these cloned rickettsial antigens were fused to the β-galactosidase. One recombinant lysogen, λgt11Rts14, did not have large quantities of the β-galactosidase antigen in the IPTG-induced culture (Fig. 2A, lane 6). Expression of the cloned rickettsial antigen in λgt11Rts14 was *lac* promoter independent, as the antigen was present in both uninduced and IPTG-induced cultures (Fig. 2B, lanes 5 and 6). The antigenic polypeptide produced in the λgt11Rts14 lysogen ran as a triplet, with the slowest-migrating band having a molecular size of approximately 110K.

Determination of the relationship of the cloned antigen to native rickettsial antigens. Although rickettsial antigens were detected in the lysogens, it was not possible to identify precisely which of the eight antigens recognized by the rabbit anti-Karp sera were actually being synthesized by the lysogen, as the molecular weight of the cloned protein could not be accurately determined in the case of β-galactosidase fusion proteins or proteins that were truncated, degraded, or incorrectly processed. Therefore, to determine the actual rickettsial antigens synthesized by the recombinant phage, we used the antibody selection procedure to affinity purify antibodies reactive with the recombinants. These selected antibodies were subsequently used in Western blot analysis against purified rickettsiae. A representative Western blot demonstrating the rickettsial antigens recognized by the selected antibody is shown in Fig. 1. Antibody selected with recombinant λgt11Rts11 (Fig. 1B) reacted with a 56K polypeptide in the Karp strain and with polypeptides of similar molecular size in the Kato (54K) and Gilliam (57K) strains. Antibodies selected with recombinant phage λgt11Rts10, -11, -12, and -13 (Fig. 1D) all reacted in a similar manner. In addition, these selected antibodies also reacted with a lower-molecular-size antigen (43K) in the Karp strain, indicating that either two separate antigens (the 56K and 43K polypeptides) have been cloned or that the 43K and 56K polypeptides are related structurally and antigenically.

Antibody selected with clone λgt11Rts14 (Fig. 1E) reacted with the Karp 110K polypeptide antigen and the Kato 115K polypeptide, indicating that these two polypeptides shared cross-reactive determinants. In the Gilliam strain, antibody selected with clone λgt11Rts14 was weakly reactive with a 130K polypeptide, indicating that the cloned Karp 110K antigen and the Gilliam 130K polypeptide did not share strongly cross-reactive determinants. Weak reactivity with the Karp 56K polypeptide was also present with antibody affinity purified against recombinant λgt11Rts14. To determine whether antibody was nonspecifically selected by these procedures, λgt11 was also used to affinity purify antibody. This preparation of antibody did not react with any Karp, Kato, or Gilliam antigens in Western blots (Fig. 1C).

Reactivity of group-reactive and strain-specific MAb with the cloned 56K polypeptide. MAb which reacted with the 56K polypeptide in Western blots (Fig. 3) were used to probe the lysogens expressing the recombinant 56K polypeptide. MAb KCH47, previously reported to react with a scrub typhus group antigen (4), reacted with the fusion protein in lysogen Y1089::λg11Rts11 (Fig. 3C). MAb K13F88A, which recognizes a strain-specific determinant in the Karp strain (4), did not react with the fusion proteins synthesized by lysogen Y1089::λgt11Rts11 (Fig. 3B). Multiple antigen bands (56K, 43K, and other minor bands) were recognized by both of these MAb in lysates of rickettsial cells. This is most likely a result of various electrophoretic mobilities exhibited by the 56K polypeptide due to limited degradation and the heat-modifiable nature of this protein (6, 18).

Reactivity of human convalescent serum with recombinant proteins. The reactivity of the cloned scrub typhus antigens with human convalescent serum was determined by Western blot analysis of the constructed lysogens. Lysates of IPTG-induced lysogens expressing the 56K polypeptide antigen (λgt11Rts11, -12, and -13) as a fusion protein were reactive with the human serum (Fig. 4, lanes 2, 4, and 8). Uninduced cultures of these same lysogens were negative for specific rickettsial antigens. The 110K polypeptide expressed in lysogen λgt11Rts14 in both the uninduced and induced cultures was not recognized by the human serum used in the blots shown in Fig. 4 (lanes 5 and 6). This was not surprising,

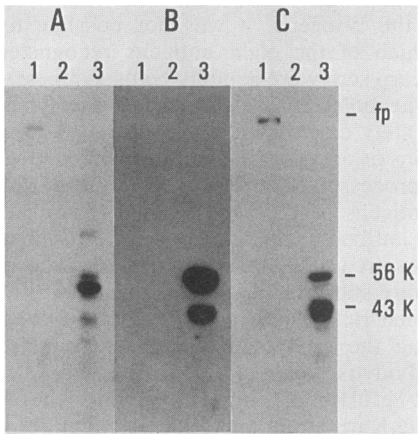

FIG. 3. Reactivity of group-reactive and strain-specific MAb with the cloned portion of the 56K polypeptide. Electrophoresed lysates of lysogens Y1089::λgt11Rts13 (lane 1), Y1089::λgt11 (lane 2), and *R. tsutsugamushi* Karp (lane 3) were reacted with rabbit anti-Karp sera (A), strain-specific MAb K13F88A (B), and group-reactive MAb KCH47 (C) in a Western blot procedure. Lysogen Y1089::λgt11Rts13 expressed a portion of the 56K polypeptide as a fusion protein (fp). The 56K and 43K polypeptides are noted.

as this human serum had a low level of antibody against the 110K polypeptide in Western blots with native Karp antigens (data not shown). The human serum (which had not been absorbed with *E. coli*) reacted with several other bands in the lysogens and the λgt11 control, and therefore these bands were considered to be *E. coli* antigens.

Southern blot analysis of the cloned rickettsial DNA. Purified, cloned rickettsial DNA inserts of the 56K and 110K polypeptide genes were used in Southern blot analysis against *R. tsutsugamushi* Karp DNA, L-cell DNA, and *R.*

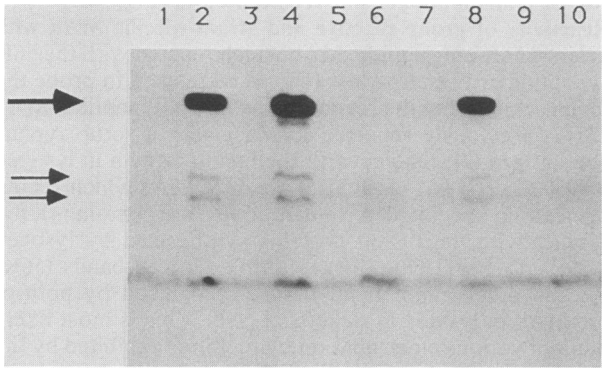

FIG. 4. Western blot analysis of lysogens expressing cloned rickettsial proteins with human convalescence serum. Electrophoresed whole-cell lysates of lysogens either uninduced (odd-numbered lanes) or induced (even-numbered lanes) with IPTG were incubated with an unabsorbed human serum obtained from an individual infected with *R. tsutsugamushi*. Paired lanes 1 and 2 (Y1089::λgt11Rts11), 3 and 4 (Y1089::λgt11Rts12), and 7 and 8 (Y1089::λgt11Rts13) are lysogens expressing a portion of the 56K polypeptide; a lysogen (Y1089::λgt11Rts14) expressing the 110K polypeptide is in lanes 5 and 6; and a lysogen of λgt11 is in lanes 9 and 10. The large arrow indicates antigenic fusion proteins containing a portion of the 56K polypeptide. The smaller arrows point to apparent degradation products of the fusion proteins. The other antigen bands on this blot are *E. coli* proteins recognized by this unabsorbed human convalescent serum.

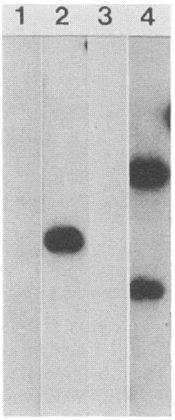

FIG. 5. Southern blot analysis of *R. tsutsugamushi* genomic digests with 56K and 110K gene probes. *Hin*dIII digests of L-cell DNA (lanes 1 and 3) or *R. tsutsugamushi* DNA (lanes 2 and 4) were hybridized with either a labeled 56K gene insert from clone λgt11Rts12 (lanes 1 and 2) or a labeled 110K gene insert from clone λgt11Rts14 (lanes 3 and 4). Lane 2 contains a 2.3-kb *Hin*dIII fragment which hybridized with the 56K gene probe, and lane 4 demonstrates that two *Hin*dIII fragments (3.6k and 1.6kb) hybridized with the 110K gene probe. Neither probe reacted with L-cell DNA (lanes 1 and 3).

conorii DNA. The 56K insert consisted of a single 0.7-kb *Eco*RI fragment, and the 110K polypeptide insert consisted of four *Eco*RI fragments with a total size of 5.4 kb. Against Karp DNA (prepared from rickettsiae grown in primary chicken embryo fibroblasts), the 56K probe reacted with a *Hin*dIII fragment of approximately 2.3 kb (Fig. 5, lane 2). The 110K probe, which contained all four *Eco*RI fragments, reacted with two genomic *Hin*dIII fragments of 3.6 and 1.6 kb (Fig. 5, lane 4). Similar fragments were found in rickettsial DNA prepared from L-cell-grown *R. tsutsugamushi* Karp (data not shown). The cloned 56K or 110K gene fragments did not react with restriction enzyme digests of uninfected L cells (Fig. 5, lanes 1 and 3) or with digests of *R. conorii* DNA (data not shown).

DISCUSSION

The lack of data concerning protective antigens of *R. tsutsugamushi* has made it difficult to select which antigens to clone for potential vaccine products. Past studies have indicated that the antigenic diversity of *R. tsutsugamushi* strains should be considered in vaccine construction because heterologous protection (defined by a variety of criteria) in humans or laboratory animals wanes after a short period of time (a few months to 1 year) (13, 16). Even though the protection against the heterologous strain is not as long-lasting as that against the homologous strain, there is some degree of resistance (i.e., lack of eschar formation, milder disease, and shorter duration of fever [16]) to the heterologous challenge, indicating that the cross-reactive antigens of scrub typhus strains may have some role in protective immunity. To perform detailed immunological analysis at both the cellular and humoral level on isolated rickettsial antigens, we are attempting to clone as many scrub typhus antigens as possible. Clones reported in this study were obtained from a λgt11 library constructed from rickettsial DNA digested with blunt-end-producing restriction enzymes. This library yielded clones expressing the 56K and 110K polypeptide antigens of *R. tsutsugamushi* Karp.

The 56K polypeptide (which corresponds to the 60K polypeptide of Hanson and the 56K polypeptide of Urakami et al.) appears to be a major outer membrane protein exposed on the surface of *R. tsutsugamushi* (7, 17). It is a dominant immunogen against which most animals and infected humans produce antibodies. Rabbit antibodies, affinity purified against the recombinant 56K polypeptide, recognized polypeptides of similar molecular sizes in the Kato (54K) and the Gilliam (57K) strains, indicating that the cloned portion of the 56K polypeptide contained group-reactive determinants. Furthermore, a group-reactive MAb (4) that recognized the 56K polypeptide and its modifiable form (43K) reacted with the 56K recombinant polypeptide. This MAb was previously characterized as a group-reactive antibody against a 50K polypeptide (7), as determined by radioimmunoprecipitation, and not against the 56K-43K pair that we have reported in this study. It is not clear why our results differ, but we used Western blot analysis (instead of radioimmunoprecipitation), which may have exposed determinants on the 56K that are not accessible in the radioimmunoprecipitation procedure. It is also possible that our 43K polypeptide and the 50K polypeptide of Hanson are the same antigen. The presence of a scrub typhus group-reactive region on the 56K polypeptide supports previous observations with polyclonal sera which recognized many different antigens (7, 17). In addition, other studies with MAb indicate that strain-specific determinants exist on this family of *R. tsutsugamushi* proteins (7). A strain-specific MAb (4) which reacted with the 56K polypeptide in Western blots did not react with the cloned portion of this antigen.

Expression of the recombinant 56K antigen was dependent on the presence of IPTG in the growth medium, indicating that the *E. coli lac* promoter was required for expression. In all four 56K antigen clones, fusion proteins consisting of a portion of the rickettsial antigen fused to β-galactosidase were present. The rickettsial DNA insert was approximately 0.7 kb in each clone. This sequence of DNA has a maximal coding capacity of about 25 kilodaltons of protein. The available data (DNA insert size, fusion protein size, and antigen cross-reactivity) suggest that all four 56K recombinants are identical. More precise data, such as DNA sequences, will be able to pinpoint differences if they exist. Cloning only a portion of a protein is not unexpected in the λgt11 system, and it may explain why the strain-specific MAb does not react with the cloned polypeptide. Molecular cloning of the strain-specific determinant(s) on the 56K polypeptide, which may be very important to the protective immune response, is now possible, since we have a portion of the gene to use as a probe.

Synthesis of the 110K polypeptide antigen was not dependent on the *lac* promoter, as expression occurred either in the presence or absence of IPTG. This suggests that a functional rickettsial promoter is included along with the structural gene in this particular clone (λgt11Rts14). Alternatively, another lambda promoter, such as *lom*, may be contributing to the synthesis of the 110K polypeptide (2). DNA sequence analysis of this clone will identify its genetic components more precisely. The polypeptide synthesized by clone λgt11Rts14 was approximately the same size as the native polypeptide (110K) in *R. tsutsugamushi* Karp, suggesting that the entire gene was cloned. This is supported by the size (5.4 kb) of the rickettsial DNA insert. By using the 110K polypeptide recombinant-antigen-selected antibodies, a cross-reactive 115K polypeptide antigen was found in the Kato strain, while in the Gilliam strain, a weakly reactive 130K polypeptide was present, indicating that group-reactive epitopes are on these proteins. The apparent size differences of these polypeptides also suggests that strain-specific sequences may also be located in these proteins.

The availability of recombinants expressing *R. tsutsugamushi* proteins will greatly assist in our understanding of these obligate intracellular bacteria. Aspects of pathogenicity, such as invasion of host cell membranes, elicitation of the immune response, and mechanisms of intracellular survival, might be more accessible to detailed study. As we are interested in the production of both prophylactic and diagnostic reagents for scrub typhus rickettsiae, our attention has been on cloning antigens. The 56K and 110K *R. tsutsugamushi* antigens are both recognized during a natural infection in humans (unpublished observations). The cloned portion of the 56K polypeptide was reactive with human convalescent serum, indicating that the group-reactive portion of the 56K polypeptide is included in the group of immunogens to which a naturally infected human responds. The recombinant 110K polypeptide was not reactive with the human convalescent serum. These recombinants (especially the 56K clones) can now be used as a source of rickettsial antigen for various immunological studies. As more *R. tsutsugamushi* proteins are cloned and characterized, we will be able, it is hoped, to identify those antigens required to stimulate a protective immune response.

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