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Antigens of Scrub Typhus Rickettsiae: Separation by Polyacrylamide Gel Electrophoresis and Identification by Enzyme-Linked Immunosorbent Assay

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Antigens of plaque-purified *Rickettsia tsutsugamushi* strains Gilliam, Karp, and Kato were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were analyzed by an enzyme-linked immunosorbent assay. Six antigens were identified in each of the three prototype strains; in strain Gilliam, these antigens were located in the cell envelope fraction of the organisms. Reactivity of these isolated antigens with homologous or heterologous immune sera indicated that different macromolecules existed in all three strains, although they exhibited very similar mobilities during electrophoresis. Antigens of strain Gilliam reacted equally well with antibodies directed against Gilliam, Karp, or Kato rickettsiae. However, strains Karp and Kato each had two distinct antigens which did not react with heterologous antisera. *R. tsutsugamushi* antigens retained immunogenicity after electrophoresis, and antisera raised against them reacted with intact organisms and exhibited specificity in reactions with isolated antigens.

Numerous investigators have analyzed the proteins of typhus and spotted fever group rickettsiae by polyacrylamide gel electrophoresis (PAGE) techniques. Intact organisms (6, 14, 16), cell wall fractions (15, 22), and soluble antigens (15) have been examined to identify rickettsial species and establish the topographical relationship of peripheral proteins (15, 23). However, very little is known about the protein or antigenic composition of scrub typhus rickettsiae. Crude particulate and soluble antigens have been prepared from several strains of *Rickettsia tsutsugamushi* and used in complement fixation or other serological procedures (2, 11, 19, 20). Detailed antigen characterization has been inhibited by difficulties in establishing the homogeneity of scrub typhus isolates and in purifying the rickettsiae free from contaminating host cell debris, but a preliminary report by B. Hanson and C. L. Wisseman (ASM News **46**:608, 1980) of a study using non-plaque-purified strains of rickettsiae indicated successful radiolabeling of organisms and separation of antigens by PAGE.

Recent studies in this laboratory demonstrated reproducible plaque formation by scrub typhus rickettsiae and led to the isolation and propagation of plaque-purified isolates of the Gilliam, Karp, and Kato strains of *R. tsutsugamushi* (12). These antigenically homogeneous isolates were propagated in cell culture to reduce the contaminating host cell debris that is associated with organisms grown in the yolk sac of embryonated chicken eggs. However, complete purification of these organisms remains a problem, for the biophysical techniques which produced typhus and spotted fever group rickettsiae essentially devoid of host cell debris have proven less effective in purifying scrub typhus organisms (5).

We wished to examine the antigenic components of *R. tsutsugamushi* despite the unresolved technical problems in attaining complete purification of this organism. This paper describes (i) the separation of antigens of scrub typhus rickettsiae by sodium dodecyl sulfate (SDS)-PAGE; (ii) the identification of rickettsial antigens by reaction with scrub typhus immune sera in an enzyme-linked immunosorbent assay (ELISA); (iii) the detection of major antigens in the cell envelope fraction of rickettsiae; and (iv) a comparative antigenic analysis of the three major prototype strains of *R. tsutsugamushi*.

MATERIALS AND METHODS

Rickettsiae. *R. tsutsugamushi* strains Gilliam, Karp, and Kato were cloned by triple plaque purification, as described by Oaks et al. (12), and were propagated and intrinsically radiolabeled as previously described (6). At 2 to 3 days postinfection, cultures were homogenized for three 1-min cycles in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) and then centrifuged at 240 × *g* for 10 min at 4°C to pellet L929 cell debris. The rickettsiae in the supernatant were pelleted by centrifugation at 12,000 × *g* for 1 h at 4°C and were then washed once with 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, containing 2.0 M sodium chloride (Tris-NaCl).

The washed organisms were resuspended in 2.0 ml of Tris-NaCl, mixed with 2.0 ml of Sepharose 4B, layered over a 0.9-by-30.0-cm column (K9/30; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) containing 10.0 ml of Sepharose 4B, and eluted with Tris-NaCl. Fractions (1.0 ml) were collected and counted with Scint A liquid scintillation cocktail in a Prias PLD Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Peak radioactive fractions were diluted with 0.05 M Tris buffer, pH 7.2, centrifuged at 12,000 × *g* for 1 h at 4°C, and then resuspended in a small volume of the same buffer.

R. prowazekii strain Breinl was grown, radiolabeled, and purified as previously described (6).

Preparation of cell envelopes. Cell envelopes were prepared from *R. tsutsugamushi* strain Gilliam and from *R. prowazekii* as previously described (15). After the organisms were disrupted in a French pressure cell, centrifugation was increased to 12,000 × *g* for 1 h to ensure pelleting of any unbroken organisms. Cell envelopes were then treated with ribonuclease, deoxyribonuclease, and magnesium chloride, pelleted, and suspended in a small volume of TEN buffer (0.05 M Tris buffer, pH 7.5, 0.001 M ethylenediaminetetraacetic acid, and 0.1 M NaCl).

PAGE. SDS-PAGE was performed as described previously (6), using gels 5 mm in diameter by 75 mm in length. All samples contained approximately 100 μg of protein. Samples of cell envelopes were adjusted to contain 1.0% agarose before electrophoresis; however, all other samples were electrophoresed as aqueous preparations. After electrophoresis, the gels were quick-frozen on dry ice and sliced. Gel slices were either eluted and analyzed by ELISA or counted in a liquid scintillation counter. Radioactivity was expressed as disintegrations per minute. Migration is shown in all the graphs from left to right (cathode to anode).

Preparation of antisera against intact organisms. Female BALB/c mice (Flow Laboratories, Inc., Dublin, Va.), 8 to 10 weeks old, were inoculated subcutaneously with yolk sac-grown, plaque-purified *R. tsutsugamushi* strains Gilliam (10^5 plaque-forming units), Karp (10^5 plaque-forming units), and Kato (10^3 plaque-forming units) or intraperitoneally with yolk sac-grown *R. prowazekii* (10^6 plaque-forming units). Mice were anesthetized with carbon dioxide and bled from the right axillary artery 28 days after infection. Antibody titers, as determined by an indirect fluorescent-antibody test (13) using fluorescein-conjugated rabbit anti-mouse total globulins, were 320, 320, 640, and 80, respectively.

Preparation of the ELISA conjugate. The immunoglobulin G (IgG) fraction of rabbit antiserum against mouse IgG was conjugated to alkaline phosphatase as described by Avrameas (1) and modified by Yardley et al. (24). An ammonium sulfate suspension of alkaline phosphatase containing 1.0 mg of protein was centrifuged at 1,200 × *g* for 15 min at 4°C. The pelleted enzyme was mixed with 0.5 ml of rabbit anti-mouse IgG containing 2.5 mg of protein and then dialyzed for 2 days against 0.01 M phosphate-buffered saline, pH 7.2, with frequent changes of the buffer. The mixture was adjusted to contain 0.07% glutaraldehyde, incubated for 2 h at 26°C, and then dialyzed exhaustively against phosphate-buffered saline at 4°C. After dialysis, the conjugate was diluted to 5.0 ml with 0.05 M Tris buffer, pH 8.0, adjusted to contain 5.0% bovine serum albumin, sterilized by passage through a 0.45-μm Swinnex filter (Millipore Corp., Bedford, Mass.), and stored at 4°C.

The titer of the conjugate was determined by ELISA, using polystyrene tubes coated with 0.5 ml of purified mouse IgG, 1.0 mg/ml in 0.05 M carbonate buffer, pH 9.6 (coating buffer), and 0.5 ml of serially diluted conjugate. The dilution of conjugate which resulted in an absorbance value of 5.0 at 400 nm was chosen as the working dilution. A control tube containing mouse IgG was included in all experiments to ensure the stability of the conjugate.

Preparation of antisera against isolated antigens. Replicate samples of strain Karp organisms were digested with SDS, 2-mercaptoethanol, and heat and then electrophoresed on multiple gels. The gels were frozen, sliced, and divided into five antigen regions, based on the ELISA profile of a representative strain Karp gel. Region A consisted of slices 1 through 11 and included strain Karp antigen 1; region B, slices 12 through 25, antigens 2 and 3; region C, slices 26 through 31, antigen 4; region D, slices 32 through 46, antigens 5 and 6; and region E, slices 47 through 58. The corresponding regions from five gels were homogenized in a mortar and pestle and suspended in Snyder I diluent (10) to a total volume of 5.0 ml. On day zero, six groups of 8- to 10-week-old female BALB/c mice (Flow Laboratories), five mice per group, were inoculated subcutaneously with 1.0 ml of the homogenized regions of the strain Karp gels. Mice were boosted subcutaneously with similar inocula on days 5 and 10 and were bled from the retroorbital venous plexus 21 and 28 days after the last injection.

ELISA with isolated antigens. The ELISA was performed by using *R. tsutsugamushi* antigens directly bound to a polystyrene support (4), essentially according to the method originally described by Engvall and Perlmann (8). Gel slices were placed in polystyrene tubes (12 by 75 mm), eluted for 3 h at 37°C in 0.25 ml of coating buffer, and then stored at 4°C until use. For the ELISA, the tubes were washed four times with 5.0 ml of phosphate-buffered saline containing 0.05% Tween 20 and 0.2 mg of sodium azide per ml and then incubated with 0.25 ml of homologous or heterologous antiserum for 4 h at 26°C, with constant agitation. Anti-*R. tsutsugamushi* sera were used at a 1:80 dilution, and anti-*R. prowazekii* serum was used at a 1:40 dilution. After the primary antibody reaction, the tubes were washed four times with sodium azide and incubated with the appropriate dilution of the alkaline phosphatase–rabbit anti-mouse IgG conjugate on a mechanical shaker for 18 h at 26°C.

The tubes again were washed four times with sodium azide and reacted with 0.5 ml of *p*-nitrophenyl phosphate, 1.0 mg/ml in 0.05 M carbonate buffer, pH 9.8, containing 0.001 M magnesium chloride, at 26°C with constant agitation. Reactions were allowed to proceed until a bright yellow color appeared, or to a maximum time of 100 min, and were halted by the addition of 0.5 ml of 0.2 N sodium hydroxide. The absorbance value of each sample was determined at 400 nm with a Gilford model 240 spectrophotometer

(Gilford Instrument Laboratories, Inc., Oberlin, Ohio), and, if necessary, values were extrapolated to a maximum reaction time of 100 min.

ELISA reactions using antisera raised against the five regions of the strain Karp gels were performed only on the corresponding regions of homologous and heterologous gels. For example, anti-Karp region A was reacted only with region A of strain Gilliam, Karp, and Kato gels. Anti-region A serum was used at a 1:18 dilution; anti-region B serum, a 1:30 dilution; anti-region C serum, a 1:9 dilution; anti-region D serum, a 1:20 dilution; and anti-region E serum, a 1:14 dilution.

Reagents. Alkaline phosphatase, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate), and glutaraldehyde were purchased from Sigma Chemical Co., St. Louis, Mo. Rabbit anti-mouse IgG, IgG fraction, was obtained from Cappel Laboratories, Cochranville, Pa. Purified mouse IgG was obtained from Litton Bionetics, Kensington, Md., and fluorescein-conjugated rabbit anti-mouse total globulins were obtained from Microbiological Associates, Bethesda, Md. Falcon no. 2052 polystyrene tubes (12 by 75 mm) and Tween 20 were purchased from Fisher Scientific Co., Silver Spring, Md. Scint A liquid scintillation cocktail was obtained from Packard Instrument Co., Inc., Rockville, Md., and Sepharose 4B was obtained from Pharmacia.

RESULTS

Separation of *R. tsutsugamushi* antigens by PAGE. Scrub typhus rickettsiae were intrinsically labeled with ^{3}H-amino acids, partially purified, and electrophoresed in SDS-PAGE gels. The migration patterns for *R. tsutsugamushi* strains Gilliam and Karp were nearly identical (Fig. 1), with two high-molecular-weight proteins showing the greatest incorporation of radioisotope. The Kato strain differed from Karp and Gilliam because radioisotope incorporation occurred principally in a single protein corresponding to the higher-molecular-weight peak observed in the latter two strains. Smaller peaks of radioactivity were identifiable in each preparation, but the low radioisotope incorporation into these proteins precluded their use in distinguishing between strains.

Since our radiolabeling techniques had detected only two major structural proteins which were of minimal use in comparing scrub typhus strains, we utilized an ELISA technique to establish profiles of antigenic activity. After electrophoresis, antigens were eluted from individual gel slices and attached to polystyrene tubes. Antigen preparations from Gilliam, Karp, and Kato were reacted with homologous hyperimmune serum, washed, and treated with rabbit anti-mouse IgG conjugated to alkaline phosphatase. Enzymatic degradation of *p*-nitrophenyl phosphate was quantified by spectrophotometry and resulted in the antigen patterns seen in Fig. 1. The two peaks located between fractions 15 and 25 remained the most prominent antigens in Gilliam and Karp, but additional antigens were detected by the ELISA technique, and their mobility was reproducible when several preparations of a single strain of rickettsiae were examined.

The general antigen pattern in Kato was similar to that of Karp and Gilliam, but there was poor resolution of the antigen peak between fractions 20 and 25. Six antigen peaks were routinely and easily identified in each strain of scrub typhus rickettsiae. The antigens were numbered as shown in Fig. 1 for convenience of identification.

Antigenic composition of cell envelopes. The Gilliam strain of *R. tsutsugamushi* was intrinsically radiolabeled with ^{3}H-amino acids, and organisms were disrupted in a French pressure cell. Separation of cell envelope proteins by SDS-PAGE produced the pattern seen in Fig. 2A. The radioactive profile was similar to that seen with intact organisms and demonstrated the presence of proteins 2 and 3. When cell envelope preparations were examined by the ELISA procedure, more antigens could be identified. Antigens 1, 2, and 3 were present in the relative proportions observed in whole cells. Antigen 6 was also present, and cell envelopes appeared to be enriched in this antigen when the ELISA profile was compared with that obtained with intact rickettsiae. Small amounts of antigen with an electrophoretic mobility similar to that of Gilliam antigens 4 and 5 were also observed in this cell envelope preparation.

The localization of major protein antigens in the cell envelope of *R. tsutsugamushi* was not unexpected, since our previous studies with *R. prowazekii* (15) had demonstrated that the major proteins of this organism were also found in the cell envelope. We observed a significant difference, however, in the humoral immune response of mice to the cell envelope components of these rickettsiae. The antibody response to intact *R. prowazekii* was predominantly directed against a single cell envelope protein (Fig. 2B) and differed markedly from the broad response to multiple antigens after infection with *R. tsutsugamushi* (Fig. 2A). The antibody response to scrub typhus rickettsiae was not dependent upon rickettsial proliferation in the host. When organisms were inactivated by gamma radiation (7) and inoculated into mice, the ELISA pattern obtained with the mouse sera was identical to that observed after infection with viable organisms.

Strain and group specificity of individual antigens. Six major antigens were identified in three strains of *R. tsutsugamushi*, but it was not clear whether these antigens were unique to

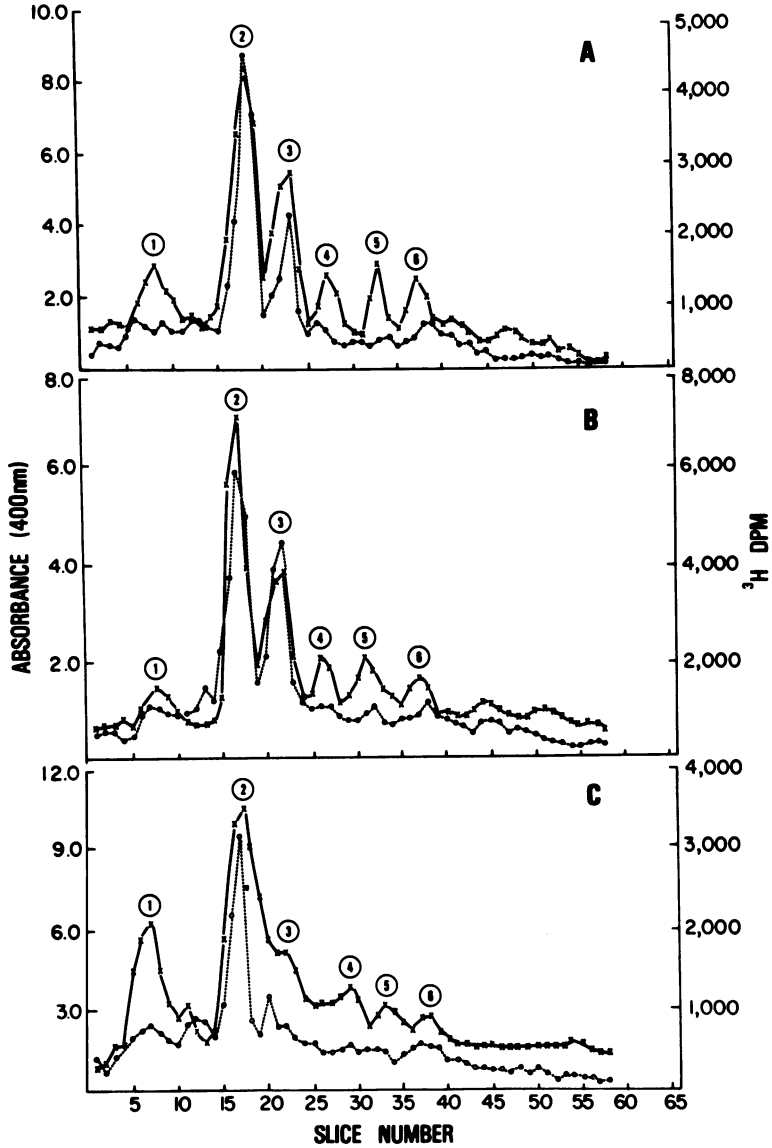

FIG. 1. *Separation of R. tsutsugamushi proteins and antigens by SDS-PAGE. (A) Strain Gilliam; (B) strain Karp; (C) strain Kato. Symbols:* ○, *Proteins intrinsically labeled with* ^{3}*H-amino acids;* ×, *antigens detected by ELISA of individual gel slices.*

each strain or were common among the major prototypes of scrub typhus rickettsiae. To distinguish between these possibilities, antigens from each strain of rickettsiae were separated by SDS-PAGE and then examined by the ELISA procedure, using both homologous and heterologous antisera. Antisera to Gilliam, Karp, and Kato each reacted with the major antigens of Gilliam (Fig. 3A), suggesting that the six Gilliam antigens or similar cross-reactive macromolecules were present in the antigenic mosaic of Karp and Kato organisms. Reaction of Karp antigen with each antiserum (Fig. 3B) produced a substantially different profile from that seen with Gilliam. Karp antigens 1 and 4 did not react with either Gilliam or Kato antiserum, and the reactions of Karp antigens 2 and 3 with Gilliam antiserum were also substantially reduced. The Kato profile (Fig. 3C) shared the major features seen with Karp antigens. Kato antigens 1 and 4 did not react with either Gilliam or Karp antiserum, and the reactions of Kato antigens 2 and 3 with both Gilliam and Karp antisera were dramatically reduced compared with the reac-

tions of these antigens with homologous antiserum.

The antigenic specificity exhibited by antigens 1 and 4 indicated that these antigens are not the same macromolecules in all three rickettsial strains, despite their nearly identical mobilities in SDS-PAGE gels. Antigens 2 and 3 from the three prototype strains showed cross-reactivity, but the disparity in the magnitudes of homologous and heterologous reactions seen with Karp and Kato suggested that variations also occur in these antigens.

Immunogenicity of individual antigens. It was of interest to determine whether antigens recovered from SDS-PAGE gels were immunogenic and whether the resulting antibodies had the same specificities as those obtained after infection with viable organisms. Karp strain rickettsiae were electrophoresed in multiple gels, and the gels were separated into regions corresponding to antigen 1, antigen 2 and 3, antigen 4, antigens 5 and 6, and the remainder of the gel (regions A through E, respectively). The designated regions from each gel were then combined, homogenized, and injected into mice. Antiserum for each region of the gel was obtained on days 21 and 28, pooled, and then assayed in the indirect fluorescent-antibody test against intact Karp organisms. The titers of the sera were as follows: anti-region A, 40; anti-region B, 160; anti-region C, 20; anti-region D, <20; and anti-region E, <20. The amount of antigen in each of these regions may have influenced the amount of antibody produced. If the same number of rickettsiae as used above were electrophoresed only a short distance into the gel, and all antigens were recovered in a single region for mouse inoculation, the resulting serum titer was 320. After assay in the indirect fluorescent-antibody test against intact organisms, the antisera were used in the ELISA procedure against *R. tsutsugamushi* antigens separated by SDS-PAGE. One profile in Fig. 4A illustrates the homologous reaction between Karp antigens and antisera against various regions of strain Karp gels. Despite the low titers of the antisera, Karp antigens 1, 2, 3, and 4 were easily identified. As anticipated, when these antisera were reacted with antigens from strain Gilliam rickettsiae (Fig. 4A), Gilliam antigens 1, 2, 3, and 4 were also

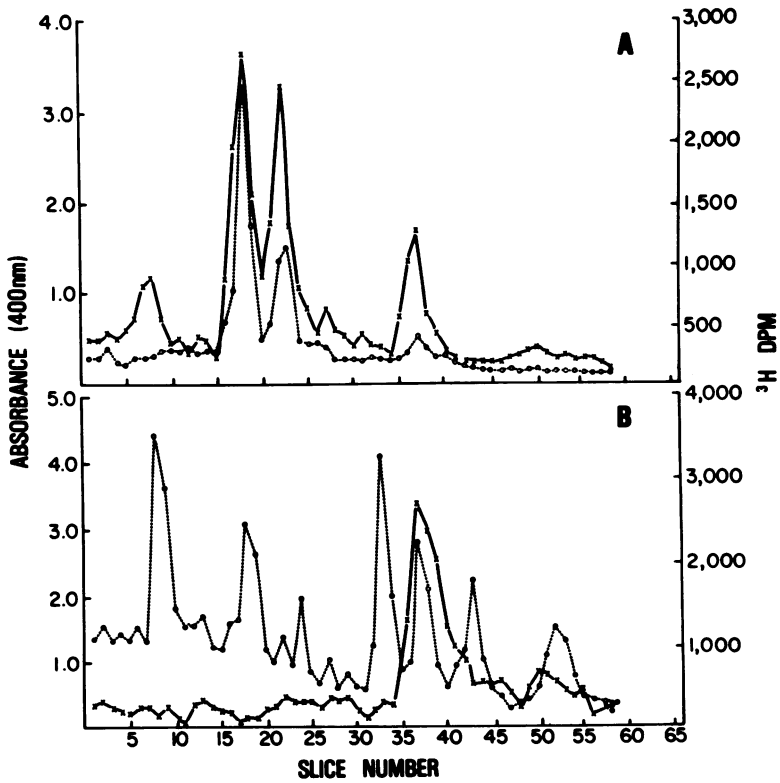

FIG. 2. *Separation of proteins and antigens from rickettsial cell envelopes. (A) R. tsutsugamushi strain Gilliam; (B) R. prowazekii. Symbols: ○, Proteins intrinsically labeled with ^{3}H-amino acids; ×, antigens detected by ELISA of individual gel slices.*

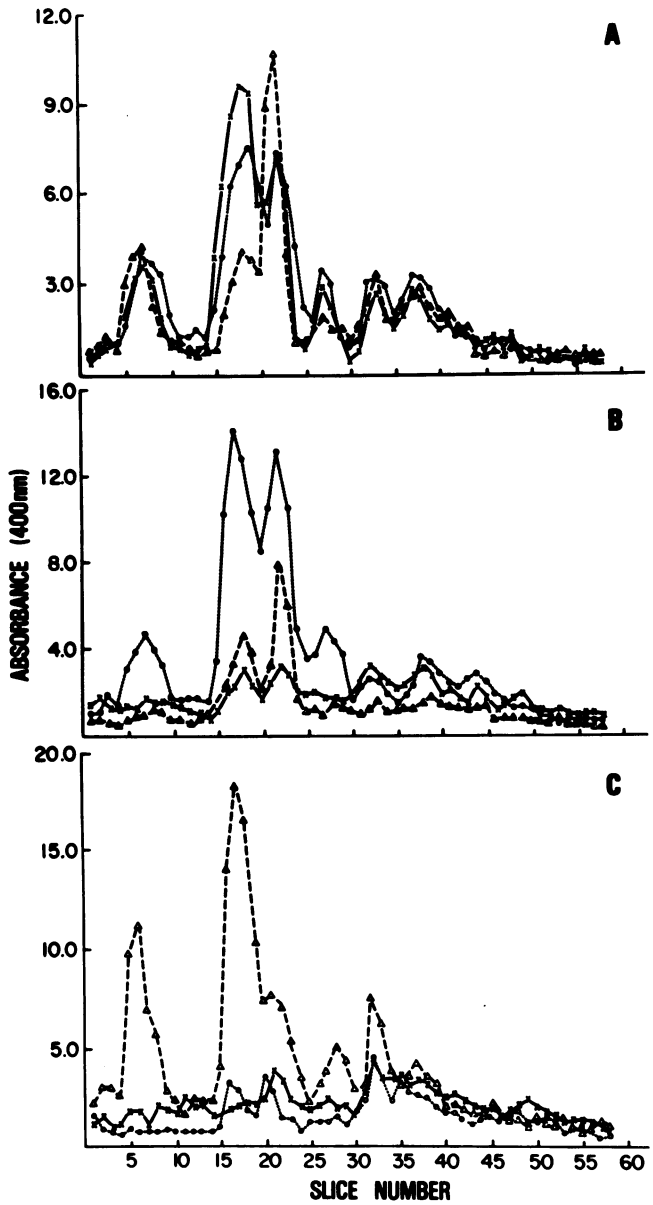

FIG. 3. *Reaction of homologous and heterologous antisera with separate antigens of R. tsutsugamushi in the ELISA. (A) Strain Gilliam; (B) strain Karp; (C) strain Kato. Symbols: ×, Antiserum to strain Gilliam; ○, antiserum to strain Karp; △, antiserum to strain Kato.*

detected. There was no reaction between Kato antigens and the anti-Karp gel sera (Fig. 4B). We recognized that this lack of reactivity could be due to an initial failure of Kato antigen attachment to the polystyrene tubes. To obviate this possibility, the initial reaction mixture was removed from each putative Kato antigen tube, and a high-titered homologous antiserum was added, followed by the usual ELISA reagent sequence. This technique clearly demonstrated the presence of antigen 2, the predominant antigen in this strain (Fig. 4B). A normal ELISA profile was not apparent throughout the gel after application of homologous antiserum, but we attribute this observation to the effect on Kato antigens of the harsh and uncontrolled alkaline conditions used for termination of the original colorimetric reaction.

Thus, it appears that antigens of scrub typhus rickettsiae recovered from SDS-PAGE gels re-

tained immunogenicity. Antisera obtained from mice injected with these antigens reacted both with intact organisms and with antigens recovered from gels. In addition, the antisera exhibit a specificity similar to that observed with antisera obtained after inoculation of mice with viable organisms.

DISCUSSION

This study has clearly demonstrated that scrub typhus antigens can be investigated without using highly purified organisms devoid of host cell debris. Use of the ELISA technique, in conjunction with antisera produced against plaque-purified strains of *R. tsutsugamushi*, provides positive identification of rickettsial antigens and with scrub typhus rickettsiae is more sensitive than the radioisotope procedures which we used.

Six major antigens were identified in each prototype strain of *R. tsutsugamushi*, and cell envelope preparations of the Gilliam strain contained each of the major antigens identified in similar intact organisms. Localization of major antigens in the cell envelope was anticipated, since our previous studies with *R. prowazekii* had demonstrated the presence of major proteins in cell envelope preparations (15). The topographical orientation of cell envelope antigens in scrub typhus rickettsiae is unknown, but it appears that their orientation is different from that established for epidemic typhus rickettsiae (15, 23). ELISA profiles obtained by reacting homologous mouse antiserum with *R. tsutsugamushi* and *R. prowazekii* indicated that multiple cell envelope antigens were recognized during experimental scrub typhus infection, whereas only a single protein from *R. prowazekii* elicited a detectable humoral response. It is possible that this difference in reactivity is caused by thermal lability or lack of renaturation of many antigens of epidemic typhus rickettsiae,

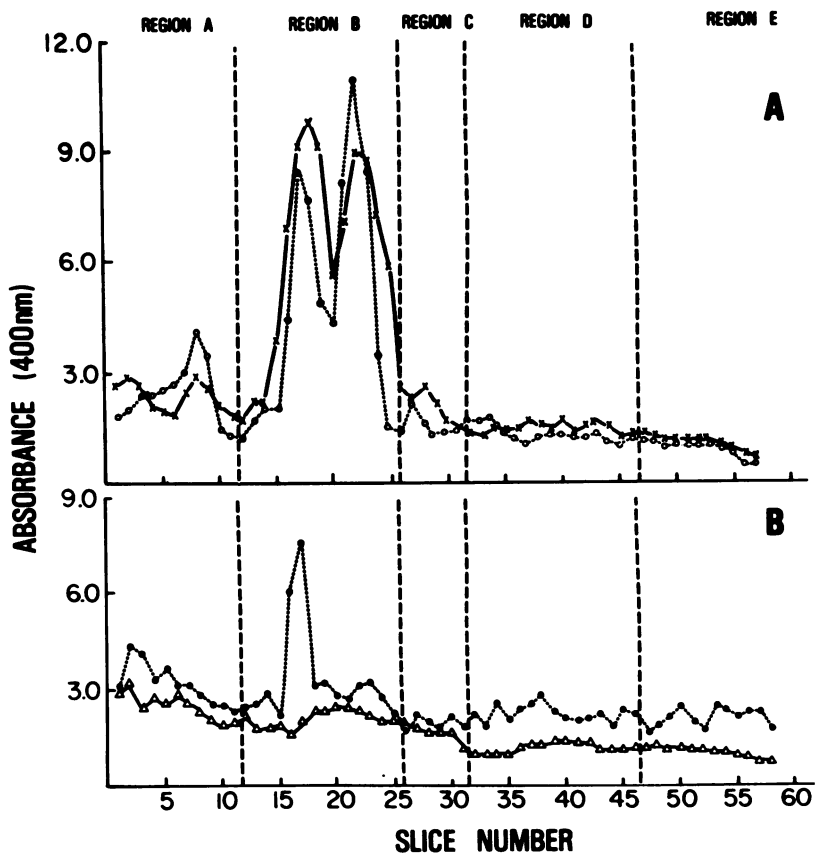

FIG. 4. *ELISA reaction of antisera against separate antigens of strain Karp rickettsiae with the three prototype strains of R. tsutsugamushi. (A) Strains Gilliam and Karp; (B) strain Kato. Symbols: ×, Gilliam antigens; ○, Karp antigens; △, Kato antigens; ●, Kato antigens detected after the initial reaction by using homologous antiserum obtained after immunization with intact organisms.*

but the observation is in agreement with our previous studies in which we demonstrated by extrinsic radioiodination that only two proteins were accessible for radiolabeling (15). Silverman and Wisseman (21) have demonstrated by electron microscopy that fundamental differences exist in the architecture of *R. tsutsugamushi* cell envelopes as compared with those of typhus group and spotted fever group rickettsiae. It is possible that differences in the reaction of mouse antiserum to *R. tsutsugamushi* and *R. prowazekii* antigens are an immunological manifestation of the morphological differences observed by electron microscopy. Since scrub typhus rickettsiae possess a reduced external slime layer relative to that seen in typhus group and spotted fever group organisms (17, 21), it is possible that a larger array of antigens is exposed and presented to the immunological apparatus of the host.

Before the introduction of plaque isolation techniques to the study of scrub typhus rickettsiae, there was concern that the broad antigenicity of these organisms could result from a mixed or heterogeneous population of rickettsiae isolated in nature and maintained through serial passage in embryonated chicken eggs. The prototype strains of rickettsiae used in this study were plaque purified three successive times before preparation of stocks for biochemical and immunological analysis. Thus, we were confident of the antigenic homogeneity of each prototype strain.

The overall patterns of reactivity between antigens of Gilliam, Karp, and Kato with homologous and heterologous antisera clearly indicate that antigens 1, 2, 3, and 4 are not identical in all three strains, whereas differences in antigens 5 and 6 are less apparent. We are uncertain of the physical conformation of these antigens, but two hypotheses seem attractive. It is possible that antigens from each strain exhibit similar electrophoretic mobilities because they consist of a similar backbone structure with an array of antigenic determinants unique to each rickettsial strain. The number and distribution of these determinants on the backbone could influence both the induction of antibody and the ability of determinants to bind antibody and react in the ELISA. Alternatively, it is possible that each antigen peak consists of several discrete antigens with similar electrophoretic mobilities under the PAGE conditions used in this study. In the latter case, use of different gel porosities might resolve chemically similar macromolecules.

Regardless of the physical nature of the separated antigens, our data do confirm that Gilliam antigens 1 and 4 react with antisera elicited by all three strains, whereas Karp and Kato antigens 1 and 4 react only with homologous antisera. Antigens 2 and 3 of all three strains share similarities; however, the pattern of reactivity of these antigens with homologous and heterologous antisera supports the assumption that they are not identical.

The presence of complex antigen mosaics with shared antigens between rickettsial strains is supported by in vivo data. The subcutaneous inoculation of mice with any of these prototype strains initiates a sublethal, immunizing infection which renders animals resistant to subsequent, potentially lethal intraperitoneal challenge with any other strain of *R. tsutsugamushi* (3; S. C. Oaks, Jr., G. A. Radlick, and J. V. Osterman, manuscript in preparation). It is tempting to speculate that antigens 1 and 4 of Karp and Kato may play a role in the virulence of these strains for mice, and the lack of such unique antigens may account for the limited virulence for mice of strain Gilliam (9). However, it must be kept in mind that although antibody studies of this nature are powerful tools in identifying specific antigens, the protective response of mice to scrub typhus infection is principally cell mediated (18), and we do not yet have knowledge of the antigens recognized by immunocompetent thymus-derived lymphocytes.

It was important to demonstrate that individual antigens recovered from gels retained immunogenicity and that antisera to these immunogens possessed the same specificity seen in antisera to intact organisms. Antigen stability must be demonstrated with each strain of *R. tsutsugamushi*, but the results of this investigation lend credibility to a continuing investigation of scrub typhus antigens, because they provide the technical basis for large-scale isolation and purification of individual antigens by immunosorbent column separation. Modulation of immunogenicity by use of appropriate adjuvants should elicit high-titered antisera useful for these immunological separation techniques. Unique antigens can be removed easily from complex mixtures, and cross-reacting antigens from each prototype strain can be examined to determine the extent of similarity.

We believe this investigation is important, because it provides an immunological examination of scrub typhus rickettsiae based on isolated antigens. Expansion of these studies to include other isolates of *R. tsutsugamushi* should provide sufficient data to describe the extent of antigenic heterogeneity in this species and to provide a rationale for development of an efficacious subunit vaccine.

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