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# Growth Characteristics and Proteins of Plaque-Purified Strains of *Rickettsia tsutsugamushi*

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**Six plaque-purified strains of *Rickettsia tsutsugamushi* (Karp, Gilliam, Kato, JC472B, TA716, and TA763) that fall into three categories of virulence for mice were compared by several parameters. Five of the six strains formed plaques of identical size in mouse cells, but each of three strains tested (representing three mouse virulence types) had a different doubling time in mouse cell cultures. Neither of these properties correlated strictly with virulence in mice, although the avirulent TA716 strain replicated much more slowly than the more virulent Karp and Gilliam strains. *R. tsutsugamushi* strain heterogeneity was also manifested at the polypeptide level by migration rates in sodium dodecyl sulfate-polyacrylamide gels of three of the major scrub typhus antigens (Sta110, Sta56, and Sta47), with those of Sta110 differing most widely. As expected, immunoblotting with polyclonal mouse sera showed substantial cross-reactivity among the major antigens of the six strains. Similar tests with Karp-induced monoclonal antibodies (MAb) demonstrated that some epitopes on Sta110 and Sta56 were shared by fewer than the six strains, but they identified no epitope unique to Karp. In contrast to the ready demonstration of antigenic heterogeneity in Sta110 and Sta56, four of the five Sta47-specific MAb reacted well with Sta47 from each of the six strains; the remaining MAb bound Sta47 from Karp and the Karp-like JC472B strain more strongly than Sta47 from the other four strains. The MAb also were useful in indicating the possible occurrence of Sta47 as dimers and trimers, the presence of Sta110 (as well as Sta56 and Sta47) in the rickettsial membrane, and the apparent interaction of the putative heat shock protein Sta58 with Sta47 or Sta47-Sta56 complexes.**

*Rickettsia tsutsugamushi* is a small, obligately intracellular bacterium and the etiologic agent of scrub typhus, which occurs naturally as a zoonosis involving chiggers of the genus *Leptotrombidium* and their small mammal hosts. Humans become incidentally infected when bitten by a rickettsia-carrying chigger. While several structural and biological properties distinguish *R. tsutsugamushi* from other members of the genus *Rickettsia*, a common feature among all rickettsiae is their possession of an outer membrane analogous to that of free-living gram-negative bacteria. A characteristic of *R. tsutsugamushi* unique among the *Rickettsia* species is its substantial variability in antigenic composition (including variability in the apparent outer membrane proteins) and in virulence, and a number of strains have been identified by classical serological methods.

The number of *R. tsutsugamushi* strains is not known, nor have strict criteria for classification been established. Many serological comparisons of *R. tsutsugamushi* isolates have been made, but few have been done with plaque-purified rickettsiae, largely because culturing these organisms is so cumbersome. Clonal purification of natural isolates is important, however, since the rickettsiae may exist as mixed populations both in chiggers and in mammals, including humans (5, 11, 48). Early serological studies of uncloned scrub typhus rickettsiae led to the concept of *R. tsutsugamushi* as an antigenic mosiac containing a wide mixture of determinants shared to varying degrees by different strains (25). More recent examinations of *R. tsutsugamushi* have confirmed this concept but also have ordered the complexity by looking at an array of antigenic polypeptides identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reaction with both polyclonal antibodies and monoclonal antibodies (MAb), all of which have helped in distinguishing rickettsial strains.

Several major protein immunogens have been identified, and genes encoding some of these have been cloned in *Escherichia coli* (30, 39–41, 43, 52, 53). Two quantitatively major antigenic proteins, Sta56 and Sta47 (nomenclature of Oaks, Stover, and colleagues [39, 52, 53]) (formerly referred to in our laboratory as 60K and 50K, respectively), are integral membrane proteins (20, 44, 52), and evidence points to their presence in the rickettsial outer membrane (21, 54). In addition to its abundance and immunodominance, Sta56 is notable for its heat modifiability (20, 22, 58) and its appearance as several bands in SDS-polyacrylamide gels (10, 58). Both strain-specific and cross-reactive epitopes have been identified in Sta56 and Sta47 (9, 10, 14, 20, 21, 38, 40, 41, 46, 52, 54, 55, 60). A third major immunogen, Sta58 (formerly called the 63K protein by us), is structurally related to the hsp60 family of stress proteins (6, 53). In limited studies, no strain-specific antigenic differences have been found, with the possible exception of one MAb which reacted with a 61-kDa protein, possibly Sta58, from only five of seven strains tested (10, 21, 40, 55). Restriction fragment length polymorphism analysis demonstrated some strain variation in Sta58 at the nucleotide level (51). Gene sequences encoding *R. tsutsugamushi* Karp and Gilliam Sta56 and Karp Sta58 have been published (43, 52, 53). Other *R. tsutsugamushi* genes which have been cloned encode 20- to 22-, 49-, 72-, 110-, and 150-kDa antigenic proteins (30, 40).

*R. tsutsugamushi* strains also vary in their virulence for mice and, possibly, for humans as well (1, 4, 8, 16, 31–33, 35, 47, 49). A single mouse gene (*Ric*) has been shown to affect virulence of a given scrub typhus rickettsial strain after intraperitoneal inoculation (16, 17), leading to the designa-

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TABLE 1. Characterization of *R. tsutsugamushi* strains

| Strain | Source | Virulence[a] in: | | Replication in cultured mouse cell lines | | Extraction (% cpm released[d] ± SE) |
|---|---|---|---|---|---|---|
| | | Susceptible mice | Resistant mice | Gt[b] (h ± SE) (*P*) | Plaque size[c] (mm ± SE) (*P*) | |
| Gilliam | Assam-Burma (human) | +++ | −−− | 10.6 ± 0.7 | 1.00 | 94.8 ± 1.3 |
| Karp | New Guinea (human) | +++ | +++ | 12.3 ± 0.7 (<0.01) | 0.75 ± 0.04 (<0.01) | 90.5 ± 3.2 |
| Kato | Japan (human) | +++ | +++ | ND | 1.02 ± 0.03 (NS) | 93.8 ± 2.0 |
| JC472B | Pakistan (gerbil) | +++ | +++ | ND | 1.09 ± 0.10 (NS) | 30.1 ± 7.9 |
| TA716 | Thailand (ground squirrels) | −−− | −−− | 18.2 ± 0.8 (<0.001) | 0.96 ± 0.09 (NS) | ND |
| TA763 | Thailand (rats) | −−− | −−− | ND | 0.98 ± 0.07 (NS) | ND |

[a] Virulence determined by resistance index: the number of infectious doses required to kill mice when injected intraperitoneally. +++, virulent, fewer than 10 infectious rickettsiae are lethal; −−−, not virulent, more than 10,000 infectious rickettsiae required for fatal outcome. Resistant and susceptible response is assigned to specific mouse strains based on the outcomes of infection with Gilliam (16). Virulence studies with JC472B were done on outbred mice. Results were taken from references 16, 34, and 49.

[b] Mean Gt, rickettsial doubling time, in mouse cell lines determined in seven individual experiments in which Karp and Gilliam strains were compared directly in C3H or BALB/3T3 cells and in three experiments directly comparing TA716 and Gilliam in C3H cells. In each experiment, the results were normalized to the mean Gt of Gilliam. Statistical evaluation was by the paired *t* test. ND, not done.

[c] Mean plaque diameter determined microscopically on day 14 in C3H mouse cell lines, relative to mean plaque diameter of Gilliam, which was determined as a control in each experiment and normalized to equal 1.00 mm. (The actual mean diameter of day 14 Gilliam plaques was somewhat less than 1.00 mm [24].) The numbers of experiments measuring 30 to 50 plaques each were 18 (Gilliam), 12 (Karp), 8 (JC472B), 6 (TA716), and 1 (Kato and TA763). Statistical evaluation was by Student's *t* test. NS, not statistically significant ($P > 0.05$).

[d] Rickettsial counts per minute in 13,000 × *g* supernatants after 30-min incubation at room temperature in 0.1% SDS in 10 mM phosphate buffer (pH 7.4). Rickettsial strains were compared in the same three experiments, and the reaction mixtures contained comparable quantities of protein. ND, not done.

tion of inbred mouse strains as relatively resistant or susceptible. *R. tsutsugamushi* strains can fall into three virulence types, based on their relative capacities to kill mice from innately resistant or susceptible strains: for highly virulent rickettsial strains (e.g., Karp and Kato), approximately one infectious organism is sufficient to cause death in any mouse strain; scrub typhus rickettsiae defined here as having intermediate virulence (e.g., Gilliam) readily kill mice of susceptible strains but not of resistant strains; and low-virulence strains (e.g., TA716 and TA763), even in relatively large doses, establish nonfatal infections regardless of the mouse strain. Rickettsial factors which lead to virulence have not been elucidated.

While increasing numbers of scrub typhus rickettsial isolates are being compared antigenically, little insight has been gained into the degree to which these organisms vary biologically. In this report, we compare six plaque-purified *R. tsutsugamushi* strains by a number of criteria: known virulence in mice, replication rates and plaque formation in cultured mouse cells, and polypeptide profiles and partial antigenic characterization using Karp strain-induced MAb reactive with Sta110, Sta58, Sta56, and Sta47. The *R. tsutsugamushi* strains studied include the three standard laboratory strains Karp (7), Gilliam (1), and Kato (50), all obtained from infected humans, two more recent animal isolates from Thailand, TA716 and TA763 (11, 12), and an animal isolate from Pakistan which originally was serotyped as Karp-like, JC472B (49). A better understanding of *R. tsutsugamushi* strain variation at both molecular and biological levels could lead to clues not only to the production of effective immunoprophylactic and immunodiagnostic tools, but also to the rickettsial properties which are important in the interactions of this highly pathogenic, intracellular bacterium with its host.

## MATERIALS AND METHODS

**Cell cultures.** The continuous mouse fibroblast cell lines C3H/10T1/2 clone 8 and BALB/3T3 clone A31 were derived from C3H and BALB/c mice, respectively. These and the Vero cells were purchased from the American Type Culture Collection (Rockville, Md.). The tissue culture medium for both maintenance of cell cultures and rickettsial growth in them was antibiotic-free RPMI 1640 medium supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.3) and 5% controlled process serum replacement-type 4 (C-4; Sigma Chemical Co., St. Louis, Mo.) (26). All cultures were incubated at 37°C in 5% $CO_2$ and 95% air (23).

**Rickettsiae.** Strains Karp, Kato, TA716, and TA763 were plaque purified by S. C. Oaks (42) and obtained as yolk sac suspensions from J. Osterman and T. Jerrells. Strains Gilliam and JC472B were donated by C. L. Wisseman, Jr., and were plaque purified in our laboratory. Working stocks of rickettsiae were collected in the late-log phase of their growth cycle in irradiated Vero cells (26). Traceable passage histories of the strains are as follows: Karp (embryonated chicken eggs [E] 58, tissue culture [TC] 13 to 38), Gilliam (animals [A] unknown, E 103, TC 11 to 31), Kato (E 172, TC 5 to 13), JC472B (A 3, E 10, TC 6 to 10), TA716 (A 8, E 10, TC 8 to 11), and TA763 (A 7, E 68, TC 4 to 7). The origins and virulence of each strain in mice are listed in Table 1.

Preparation of [$^3$H]leucine- or [$^{35}$S]methionine-radiolabeled scrub typhus rickettsiae in emetine-treated, irradiated Vero cells has been described previously (21). SDS-PAGE profiles of rickettsiae labeled with either amino acid and of unlabeled, Coomassie-stained, Renografin-purified rickettsiae were similar. Control experiments ensured that only rickettsial bands, and not host cell bands, were radiolabeled to a detectable extent (21, 28). Specifically radiolabeled rickettsiae were partially purified by differential centrifugation of disrupted, heavily infected Vero cells to separate them from the bulk of host cell material. For some purposes, partially purified rickettsiae were further separated from host material by centrifuging in 30-ml, discontinuous Renografin density gradients (45, 30, and 19% Renografin [E. R. Squibb & Sons, Princeton, N.J.]) in divalent cation-free, bovine serum albumin-containing attachment-penetration medium (10 mM potassium phosphate buffer [pH 7.3], 5 mM potassium glutamate, 45 mM NaCl, 62.5 mM KCl, 0.3% [wt/vol] bovine serum albumin, 0.1% [wt/vol] glucose) (23) at 27,000 × *g* in a Sorvall HB-4 swinging bucket rotor for 1 h. The purified rickettsiae were collected at the 30 to 45%

Renografin interface and washed free of Renografin by centrifugation and resuspension in the appropriate diluent.

**Plaquing and plaque purification of rickettsiae.** The microplaque assay in C3H or BALB/3T3 cells cultured in 24-well plates has been detailed previously (24) and modified only slightly since (26). Rickettsial stocks were clonally purified by plaquing rickettsiae which had been sufficiently diluted to result in a number of wells containing only one plaque. Such wells could be identified 2 weeks after plaquing by microscopic examination of unstained cultures, and the cells from wells having a single plaque were removed by scraping, sonically disrupted, diluted, and replaqued immediately for a total of three successive isolations. PFU recovered from a well having a single plaque averaged about $10^4$ after 2 weeks of incubation (mean of 39 independent observations) (18). This was more than enough to replaque without the intermediate amplification step required by earlier methods for plaque purification (42) and thus considerably reduced both the amount of handling and the time needed.

**Rickettsial growth curves.** Quantitative examination of *R. tsutsugamushi* replication in cell culture has been described in detail previously (23). Irradiation- or daunomycin-growth-inhibited cells (23) were infected in suspension and, after a 1-h absorption period, were distributed to slide chambers (Lab-Tek Division of Miles Laboratories, Inc., Naperville, Ill.) for further incubation at 37°C. Periodically, slides were fixed and stained for microscopic counting of cell-associated rickettsiae. For each time point, at least three replicates of 100 cells each were counted to determine the mean number of cell-associated rickettsiae per cell. Rickettsial generation times (Gt) were determined by regression analysis of the counts obtained during logarithmic growth, which occurred in the initially infected cells during the first 72 to 96 h (23). In obtaining comparative replication rates, Gilliam was included as an internal control in each experiment, because rickettsial Gt can vary somewhat from week to week, perhaps due to inconsistencies in the host cell cultures.

**Preparation of rickettsial membranes.** Crude membrane fractions were obtained from Renografin-purified rickettsiae which were disrupted by brief sonication in 10 mM HEPES buffer (pH 7.4) containing 5 mM EDTA (HE). The sonicated material was centrifuged at 13,000 × *g* for 2 min to remove whole rickettsiae and large debris, and the supernatant fluid was centrifuged at 200,000 × *g* for 2 h in the TLA 100.3 rotor of a Beckman TL-100 ultracentrifuge. The resulting 200,000 × *g* supernatant was held as the cytoplasmic fraction, and the pellet was resuspended in HE and recentrifuged at 200,000 × *g* to yield a washed membrane fraction (pellet) and membrane wash (supernatant).

**Antibody production.** Polyclonal rabbit and mouse antisera prepared by several injections of rickettsia-infected mouse livers and spleens were described in earlier studies (19). Control sera were taken from the same animals just before the first inoculation. MAb against the Karp strain of *R. tsutsugamushi* were developed by immunizing BALB/c mice intradermally with Renografin-purified viable rickettsiae ($10^5$ to $10^7$ PFU per mouse) and boosting 6 weeks later with an intraperitoneal injection of the same. Three days after the boost, the spleens were removed and homogenized. The washed, suspended spleen cells were fused with Sp2/0 myeloma cells (American Type Culture Collection) at a ratio of 5:1 with polyethylene glycol (1300 to 1600; American Type Culture Collection), and the mixture was distributed into 96-well plates, with $10^5$ myeloma cells added per well. Hybridomas were selected by growth in HEPES-buffered RPMI 1640 medium containing 20% fetal bovine serum, 0.1 mM hypoxanthine, 0.016 mM thymidine, and 0.0004 mM aminopterin (15). The hybridomas were screened for antibody production by enzyme-linked immunosorbent assay (ELISA), using Renografin-purified, sonically disrupted rickettsiae as the antigen and alkaline phosphatase-conjugated goat antiserum (Sigma) specific for mouse immunoglobulin G (IgG) heavy and light chains and IgM μ chains as the amplifying antibody. The cells from positive wells were cloned twice by limiting dilution (15), using irradiated Vero cells as feeder layers. Ascitic fluids were prepared by priming BALB/c mice intraperitoneally with 2,6,10,14-tetramethylpentadecane (Pristane; Sigma) and subsequently injecting $5 \times 10^6$ hybridoma cells into the peritoneal cavity. Normal ascitic fluid was prepared identically except that unfused Sp2/0 cells were used instead of hybridoma cells. Antibody subtype was determined by ELISA with a mouse isotyping kit (Bio-Rad Laboratories, Richmond, Calif.). Polypeptide specificity was determined by immunoblotting or, for the single MAb which did not immunoblot, by immunoprecipitation. The MAb were named according to the inducing scrub typhus rickettsial strain and their polypeptide targets, e.g., Kp56-a through Kp56-d were four clonally distinct anti-Karp Sta56-specific MAb.

**SDS-PAGE.** A standard Laemmli system employed 13% acrylamide–0.52% diallytartardiamide slab gels in Tris-glycine-SDS buffer (21, 28). For analysis of rickettsial proteins, specifically radiolabeled, partially purified rickettsiae were pelleted by centrifugation and resuspended in sample buffer (2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 5 mM phosphate buffer [pH 7], 100 μM phenylmethylsulfonyl fluoride). Samples were held either at room temperature or in a boiling water bath for 3 min before loading onto the gels. After electrophoresis at 55 mA, the gels were fixed in trichloroacetic acid and fluorographed by the method of Bonner and Laskey (3). $^{14}$C-labeled molecular weight markers were obtained from Amersham Corp. (Arlington Heights, Ill.). In some experiments, the relative amounts of protein in particular bands were determined by scanning densitometry of fluorographs or immunoblots.

**RIP and immunoblotting.** In standard radioimmunoprecipitation (RIP), rickettsial proteins were extracted with a mixture of 1% (vol/vol) Triton X-100 (TX), 0.5% (wt/vol) sodium deoxycholate (DOC), and 0.5 M NaCl in 10 mM phosphate buffer (pH 7.4) (TX-DOC-NaCl) (20, 21). The extracts were mixed with equal volumes of 40% serum or 10% ascitic fluids and held overnight at 4°C. The resulting immune complexes were collected with fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). After thorough washing in 1% TX plus 0.5% DOC in 10 mM phosphate buffer (pH 7.4) (TX-DOC), the antigen-antibody complexes were removed from the Pansorbin with sample buffer, either with or without holding in a boiling water bath, and stored at −70°C before SDS-PAGE.

To detect polypeptide epitopes accessible in unextracted rickettsiae, we performed a modified RIP (21). Washed, unextracted rickettsiae were incubated with antibodies or appropriate control fluids in sucrose-phosphate-glutamate-$MgCl_2$ buffer (SPG-Mg; 218 mM sucrose, 10 mM potassium phosphate buffer [pH 7.4], 5 mM potassium glutamate, 10 mM $MgCl_2$) for 1 h at 4°C and thoroughly washed again by centrifugation in SPG-Mg to remove unbound antibody or any nonparticulate antigen-antibody complexes. The immune complexes still associated with the 13,000 × *g*-pelletable fraction (P13, whole rickettsiae) were extracted with TX-DOC-NaCl and collected with Pansorbin as above.

For immunoblotting, proteins extracted in sample buffer

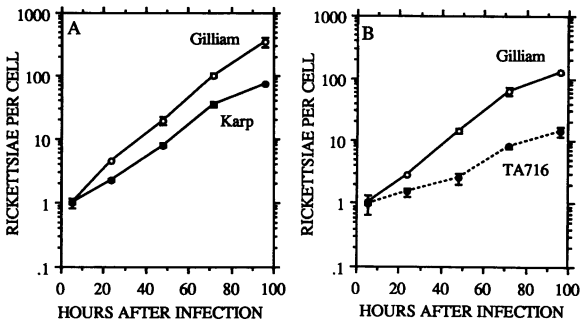


FIG. 1. Representative growth curves of *R. tsutsugamushi* Gilliam, Karp, and TA716 in daunomycin-inhibited, cultured C3H mouse cells. Each point is the mean of at least three replicate counts, and bars show the standard error. (A) Karp and Gilliam compared in the same experiment. (B) TA716 and Gilliam compared in the same experiment.

were electrophoresed in SDS-polyacrylamide gels as above, and the proteins were transferred from the gels to Immobilon polyvinylidene difluoride microporous membrane (Millipore Corp., Bedford, Mass.) (57). Polyvinylidene difluoride strips were equilibrated for 2 h (37°C) with phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% (wt/vol) Tween 20 (PBST) and 10% (vol/vol) C-4 (PBST/C-4). The strips were then incubated overnight (4°C) with ascitic fluid or mouse serum diluted 1:1,000 with PBST/C-4 which also contained 1 M D-glucose and 10% (vol/vol) glycerol. After incubation, the strips were washed three times for 5 min each (37°C) in PBST. Alkaline phosphatase-conjugated, goat anti-mouse IgG (heavy- and light-chain-specific) antibody diluted 1:1,000 in PBST/C-4 was added to the strips for 1 h (37°C). After three washes in PBST, the strips were incubated in a solution of *p*-Nitro Blue Tetrazolium (110 μg/ml), 5-bromo-4-chloro-3-indoyl phosphate (55 μg/ml), and 5 mM $MgCl_2$ in 100 mM diethanolamine buffer (pH 9.5) until color development was satisfactory (2).

## RESULTS

**Rickettsial replication in mouse cell cultures.** To determine whether rickettsial strain variability could be manifested by growth properties in cultured mouse cells, we determined (i) the sizes of plaques formed in mouse fibroblast cultures by all six strains and (ii) the Gt in these same cells of three strains of *R. tsutsugamushi*, representing each of the virulence types. Using Gilliam as an internal control in each of several experiments, four of the remaining five strains were found to form plaques the same size as Gilliam; only Karp, representing the most virulent phenotype in mice, formed smaller plaques (Table 1). Both Karp and TA716 had significantly longer Gt than Gilliam in growth-inhibited mouse cell lines, with that of TA716 being the longest (Table 1 and Fig. 1). This held true in a number of replicate experiments (Table 1) and, for Karp and Gilliam, in both C3H and BALB/3T3 cell cultures; the cell type did not influence the replication rates of these two rickettsial strains (27).

**Characterization of MAb.** Among the 12 stable hybridomas obtained from three fusions, antibodies to four polypeptide antigens were represented (Table 2). The three quantitatively major immunogens initially identified in our laboratory (Sta58, Sta56, and Sta47) all evoked MAb, and two MAb reacted with Sta110, described by Oaks et al. (40, 41). All but one of the MAb seemed to react as well with highly denatured antigen (immunoblotting) as with proteins extracted by milder detergents (RIP); Kp58-a was the single exception, being completely negative in immunoblots with whole-cell extracts. All the MAb were IgG, and IgG2a was predominant.

TABLE 2. Properties of Karp-induced MAb

| MAb | Subtype | Strain specificity[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Karp | Gilliam | Kato | JC472B | TA716 | TA763 |
| Kp110-a | IgG2a | +++ | −− | +++ | +++ | −− | ± |
| Kp110-b | IgG2a | +++ | −− | +++ | −− | −− | −− |
| Kp58-a[b] | IgG3 | +++ | +++ | +++ | +++ | +++ | +++ |
| Kp56-a | IgG2a | +++ | −− | 56 | +++ | −− | +++ |
| Kp56-b | IgG2a | +++ | −− | −− | +++ | −− | +++ |
| Kp56-c | IgG2a | +++ | −− | 56 | +++ | 56 | +++ |
| Kp56-d | IgG1 | +++ | 56 | −− | +++ | −− | −− |
| Kp47-a[c] | IgG3 | +++ | +++ | +++ | +++ | +++ | +++ |
| Kp47-b | IgG2a | +++ | +++ | +++ | +++ | +++ | +++ |
| Kp47-c | IgG3 | +++ | +++ | +++ | +++ | +++ | +++ |
| Kp47-d | IgG3 | +++ | +++ | +++ | +++ | +++ | +++ |
| Kp47-e | IgG2a | +++ | −− | ± | +++ | −− | ± |

[a] Determined in several experiments by immunoblotting, except for Kp58a, which was determined by RIP. +++, positive, and in the case of Sta56-specific MAb, several bands were blotted. 56, only the 56-kDa or 43- and 90-kDa bands were blotted; ±, weakly or inconsistently positive; −−, negative reaction. Assignment of strain specificity based on 5 to 10 individual determinations for each MAb.

[b] Immunoblots with Kp58-a were negative.

[c] Kp47-a reacted with apparent multimers of Sta47 as well as monomers and coprecipitated Sta58 as well as Sta56.

**SDS-PAGE profiles of extracted polypeptides.** The large number of *R. tsutsugamushi* polypeptides and the dependence of their SDS-PAGE migration patterns on the denaturing conditions complicates their identification, particularly among different laboratories. In our studies, individual polypeptides were identified by several criteria: (i) effect of denaturing conditions on migration in SDS-PAGE, (ii) immunoblotting and RIP with specific MAb, (iii) SDS-PAGE of Sta56 bands excised from polyacrylamide gels, and (iv) two-dimensional SDS-PAGE. The overall results of these tests were consistent for the six strains, and for simplicity only those for Karp are summarized in Table 3. In general, when whole-cell extracts, treated or not with heat or 2-mercaptoethanol, were electrophoresed, three patterns emerged: (i) some polypeptides (Sta110, Sta58) were unchanged, (ii) some (Sta56, Sta47, 30 kDa, 28 kDa) were modified to migrate at higher apparent molecular masses under more denaturing conditions, and (iii) some higher-molecular-mass forms (of Sta56, Sta47) apparently disaggregated to migrate more quickly when denaturing conditions were enhanced. In addition, some apparent proteolytic degradation of Sta56 was indicated (37).

SDS-PAGE profiles of proteins extracted from the six plaque-purified strains of *R. tsutsugamushi* were compared. As is true with the previously described strains (21, 28, 45, 54, 55, 60), TA716 and TA763 also have quantitatively predominant proteins migrating with apparent molecular masses of 58, 54 to 57, and 46 to 47 kDa when optimally denatured (Fig. 2 and Table 4). While the Sta58 proteins from each of the six strains migrated similarly, strain-related variations in the migration of other significant polypeptides did occur, even under the strongest denaturing conditions

TABLE 3. SDS-PAGE migration of *R. tsutsugamushi* Karp protein antigens extracted under different denaturing conditions[a]

| °C | 2-ME? | Approximate apparent molecular mass (kDa) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sta110 | Sta58 | Sta56 | Sta47 | 30 kDa | 28 kDa[b] |
| 100 | Yes | 110 | 58 | 56, (50, 53, 61)[c] | 47, (100, 150) | 30 | 28 |
| 24 | Yes | 110 | 58 | 43, 90, (>110) | 47, (100, 150) | 25 | 15, 21 |
| 100 | No | 110 | 58 | 56, (53), 61, (88) | 45, (100, 150) | 30 | 28 |
| 24 | No | 110 | 58 | 43, 88 | 45, (100, 150) | 25 | 15, 21 |

[a] Proteins were extracted in sample buffer (2% SDS) in the presence or absence of 5% 2-mercaptoethanol (2-ME); the extracts were held at either 100 or 24°C for 3 min before loading the gels, which were run under standard conditions. Results are from one- or two-dimensional SDS-PAGE and immunoblotting and RIP with MAb.

[b] This may consist of two distinct polypeptides (15 and 21 kDa).

[c] Numbers in parentheses refer to quantitatively minor polypeptide bands.

(Table 4). Fully denatured Sta56 from Karp and JC472B migrated with an apparent molecular mass of 56 kDa, but the migration rates of Sta56 from each of the other four strains were slightly different (see also Fig. 5). This pattern was consistent even for the less denatured 43-kDa and the less denatured, apparently dimeric 90-kDa forms. Sta47 was found in all strains, and that of the Karp and JC472B strains migrated slightly more slowly than the Sta47 from the other four strains (seen best in Fig. 5). The presence of 2-mercaptoethanol slightly retarded the electrophoretic migration of Sta47 (Table 3), suggesting that this reagent contributes to further unfolding of the molecule. Incomplete denaturation of Sta47 sometimes resulted in the appearance of both the 45- and the 47-kDa bands in immunoblots (see Fig. 5). The 28-kDa proteins from Gilliam and Kato migrated somewhat behind those from the other four strains. Because [$^{35}$S]methionine-labeled Sta110 was difficult to distinguish in SDS-PAGE, it was identified in immunoblots. This protein was most variable in terms of its SDS-PAGE migration (see Fig. 5). Of particular interest was the finding that Sta110 from Karp and the Karp-like JC472B differed so greatly in apparent size (110 kDa versus about 135 kDa), since the migration rates of all the other proteins examined in Table 4 were similar for both strains. With the exception of Karp and JC472B, none of the other strains could be strictly related to one another based on these SDS-PAGE data.

**Immunoblotting and RIP of Karp antigens.** Crude rickettsial membrane fractions, TX-DOC-NaCl extracts, and unextracted rickettsiae all were analyzed to clarify the probable location of the four Karp antigens recognized by our MAb. Nearly all the 28- and 30-kDa proteins, 85 to 95% of Sta110, and 75% of both Sta47 and Sta56 were found in the crude membrane fraction (200,000 × *g* pellet). Several other polypeptides reactive with polyclonal antibodies also were found in the crude membrane fraction, and some of these probably were related to Sta56. On the other hand, only 30% of Sta58 was associated with membranes under the conditions described. In the modified RIP assay, all the Sta56- and

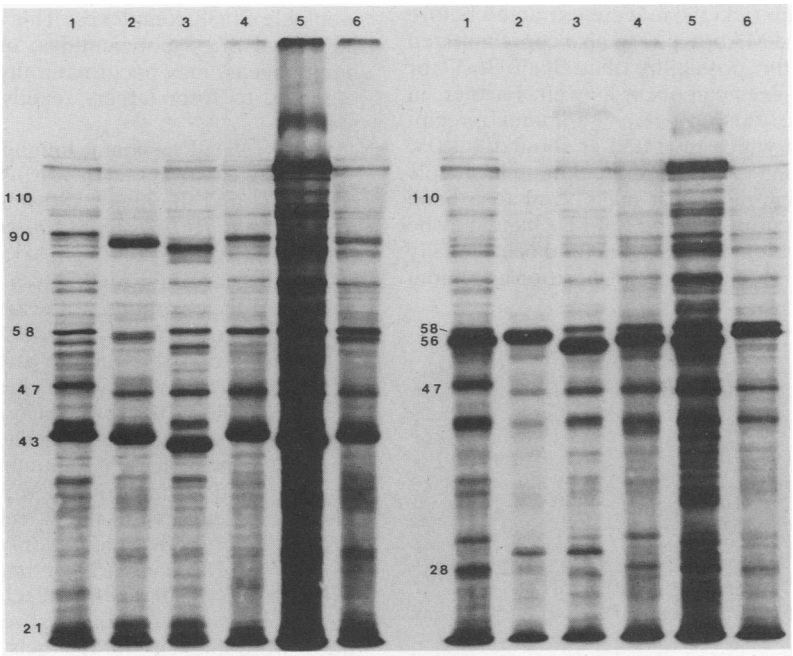


FIG. 2. SDS-PAGE of [$^{35}$S]methionine-labeled proteins of six strains of *R. tsutsugamushi*. Whole cells were extracted in sample buffer (containing 2-mercaptoethanol) and held at 100°C for 3 min (right panel) or not heated (left panel) before electrophoresis. Lanes: 1, Karp; 2, Gilliam; 3, Kato; 4, JC472B; 5, TA716; and 6, TA763. Side numbers represent the apparent molecular mass (in kilodaltons) of the major Karp bands.

TABLE 4. Electrophoretic migration[a] of identified protein antigens from *R. tsutsugamushi* strains

| Strain | Approximate apparent molecular mass (kDa)[b] | | | | |
|---|---|---|---|---|---|
| | Sta110 | Sta58 | Sta56 | Sta47 | 28-kDa |
| Karp | 110[c] | 58 | 56 | 47 | 28 |
| JC472B | 135 | 58 | 56 | 47 | 28 |
| Gilliam | 130[c] | 58 | 57 | 46 | 30 |
| Kato | 115 | 58 | 54 | 46 | 30 |
| TA716 | ND[d] | 58 | 55 | 46 | 28 |
| TA763 | 120 | 58 | 57 | 46 | 28 |

[a] Proteins were extracted under maximum denaturing conditions (sample buffer containing 2% SDS and 5% 2-mercaptoethanol, held in 100°C water bath for 3 min).

[b] As determined by slab SDS-PAGE in which the six strains were run side by side.

[c] Data from Oaks et al. (40, 41).

[d] ND, not determined; neither Sta110-specific MAb reacted with Sta110 from Ta716 so it could not be unequivocally identified.

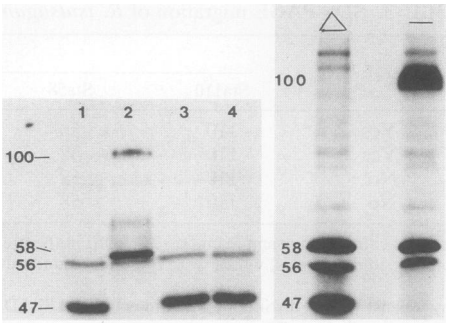


FIG. 4. Standard RIP of extracted *R. tsutsugamushi* Karp proteins with Sta47-specific MAb. Lanes: 1 to 4, immunoprecipitates with Kp47-b, Kp47-a, Kp47-c, and Kp47-d, respectively. These samples were not heated before SDS-PAGE. In the gel on the right, a portion of the same immunoprecipitate formed with Kp47-a was either heated to 100°C (△) or not (−) before SDS-PAGE. Side numbers represent the apparent molecular mass (in kilodaltons).

Sta47-specific MAb reacted with rickettsiae before their exposure to detergent (Fig. 3); this is consistent with a rickettsial surface location of the epitopes recognized by these MAb. In contrast, neither of the MAb specific for Sta110 was reactive in this modified RIP (not shown). Thus, these MAb which reacted well with extracted but not with unextracted Sta110 served as a partial control for the integrity of the rickettsiae during this procedure.

Figure 3 also shows that Sta47-specific MAb coprecipitated Sta56, and Sta56-specific MAb coprecipitated Sta47. This occurred whether the standard (TX-DOC-NaCl extracts) (Fig. 4) or modified (unextracted rickettsiae) (Fig. 3) RIP assay was performed. The coimmunoprecipitation of Sta47 and Sta56 suggested that these proteins were present in the same incompletely solubilized membrane fragments obtained by extraction with mild detergent. Additionally, in the standard RIP in which rickettsiae were extracted before exposure to antibody, one MAb, Kp47-a, also coprecipitated Sta58 (Fig. 4), raising the possibility that Sta58-Sta47 or Sta58-Sta47-Sta56 complexes can occur as well. Further, in the absence of heating to 100°C, the Kp47-a immunoprecipitate contained a protein which migrated at about 100 kDa, but none at 47 kDa; heating the immunoprecipitate in sample buffer converted this high-molecular-mass form to a band migrating at 47 kDa, indicating that the 100-kDa band may be an Sta47 dimer. Immunoblots with Kp47-a (and occasionally with Kp47-b) also showed faint bands at positions of about 100 kDa, in addition to the heavy band at 47 kDa (Fig. 5). In some blots, a 150-kDa band also could be seen.

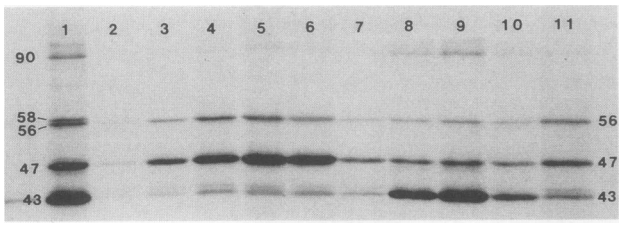


FIG. 3. Modified RIP of *R. tsutsugamushi* Karp proteins. Unextracted rickettsiae were treated with Karp-induced antibody or control fluids as follows. Lanes: 1, polyclonal mouse ascitic fluid; 2, normal mouse ascitic fluid; 3, Kp47-b; 4, Kp47-a; 5, Kp47-c; 6, Kp47-d; 7, Kp47-e; 8, Kp56-b; 9, Kp56-a; 10, Kp56-c; and 11, Kp56-d. Side numbers denote the apparent molecular mass (in kilodaltons). Samples were not heated before electrophoresis.

Among the panel of MAb described here, the one to Sta58 (Kp58-a) was unique in its reaction in the modified RIP. The nine Sta56- or Sta47-specific MAb which were reactive in this assay formed complexes with antigen which, after TX-DOC-NaCl extraction, remained in the 13,000 × *g* supernatant until Pansorbin was added. Kp58-a, however, consistently formed complexes with Sta58 which, after TX-DOC-NaCl extraction, mostly pelleted at 13,000 × *g* even before exposure to Pansorbin. Likewise, modified RIP with polyclonal antisera resulted in the sedimentation at 13,000 × *g* of most of the Sta58 (but not Sta56 or Sta47) before Pansorbin treatment. Controls which were treated identically except that antibody was omitted did not form sedimentable Sta58 complexes. This phenomenon suggests that individual Sta58 polypeptides, unlike the other two major immunogens, may occur naturally in such proximity to each other as to form larger, readily pelletable immune complexes.

**Strain-related variation.** Immunoblotting of proteins of the six rickettsial strains with Karp-specific polyclonal antisera confirmed that the major antigens (Sta58, Sta56, and Sta47) from various *R. tsutsugamushi* strains share some epitopes (Fig. 5) (9, 10, 14, 20, 21, 40, 41, 46, 54, 55, 60). To further assess the antigenic relatedness of these immunogens, we compared the reactions of the six strains with our panel of MAb (Fig. 5 and Table 2). All four of the Karp-induced, Sta56-specific MAb bound to Sta56 of at least three strains, but none reacted with all. Each of the MAb bound JC472B Sta56 in addition to Karp Sta56 (Fig. 5). MAb Kp56-d, the only one of the four which reacted with Gilliam Sta56, was the only one which did not immunoblot TA763 Sta56. TA716 Sta56 reacted with just one MAb. Immunoblotting with these MAb revealed several bands related to Sta56; in four instances of antibody binding, only the 43- and 90-kDa bands or the 56-kDa band (depending on whether or not the extracts were heated before electrophoresis) was seen in the immunoblots, even though several Sta56-related polypeptides were present in the gels. This unexpected finding suggests that the MAb bind with different affinities to epitopes in the various strains when Sta56 is in certain forms (aggregated, conformational, partially degraded [?]) of the protein. The unique reaction patterns of the four Sta56-

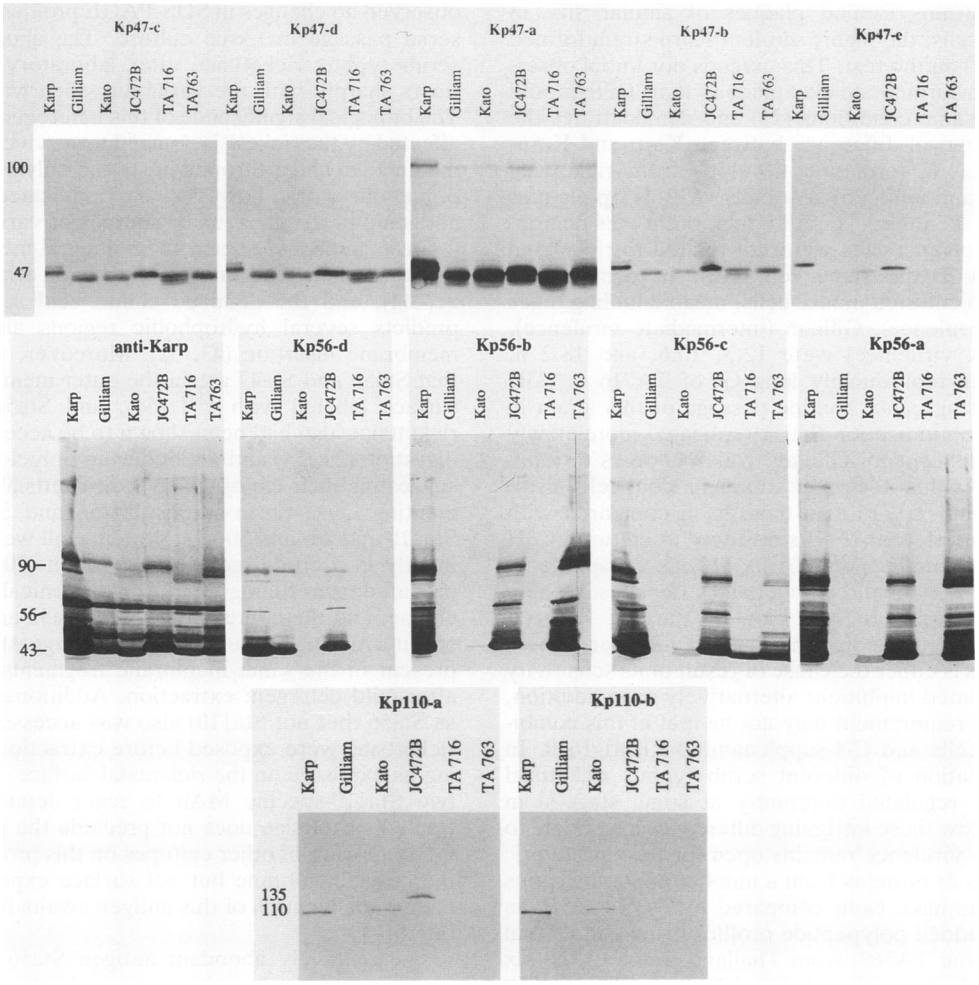


FIG. 5. Representative immunoblots of six strains of *R. tsutsugamushi* with Karp-induced Sta47-, Sta56-, and Sta110-specific MAb. Samples treated with Sta56-specific MAb were not heated before electrophoresis, while the others were. Side numbers represent apparent molecular mass (in kilodaltons).

specific MAb with protein extracts from the six strains suggest that each MAb recognizes a different epitope.

The epitopes defined by four of the five Sta47-specific MAb were conserved among all six of the strains tested (Fig. 5). Kp47-e failed to bind (or bound very weakly) to Sta47 of Gilliam, Kato, and the two Thai strains, but as strongly with JC472B as with Karp. This antigenic pairing of JC472B and Karp correlates with the slightly slower electrophoretic migration rate of Sta47 from these two strains compared with that of the other four Sta47. Note that Kp47-e did not appear to bind even homologous Sta47 as strongly as did the other Sta47-specific MAb. In addition to the apparent partial strain specificity of Kp47-e, the unique coprecipitation of Sta58 by Kp47-a suggests that at least three epitopes are recognized by the five MAb reactive with Sta47.

Both MAb specific for Sta110 displayed some antigenic strain specificity; one (Kp110-b) reacted with Sta110 only from Karp and Kato, while the other reacted with Sta110 from these two strains plus with JC472B and, very weakly, with TA763, but not with Gilliam or TA716 Sta110 (Fig. 5). The binding of Kp110-a antibodies to the Sta110 from Karp but not from JC472B is the first suggestion of an antigenic difference between these two strains. The one MAb to Sta58 immunoprecipitated Sta58 from all six strains (not shown).

An unexpected finding was the relative resistance of JC472B to extraction with SDS (Table 1). Scrub typhus rickettsiae in general tend to be quite susceptible to lysis by a number of detergents (18, 21), and all strains were readily solubilized by sample buffer. However, when Karp, Gilliam, Kato, and JC472B were treated in the same experiments with phosphate-buffered SDS, JC472B was refractory to SDS solubilization compared with the other three strains; while virtually all Karp, Gilliam, and Kato proteins were released to a 13,000 × *g* supernatant fraction by as little as 0.1% SDS; even 0.5% SDS failed to release more than 40% of JC472B proteins. This correlated with a resistance of JC472B Sta56 and Sta47 to SDS-induced release (18) and of Sta56 to denaturation (36).

## DISCUSSION

Six plaque-purified strains of *R. tsutsugamushi* from various animals and geographic areas and representing three virulence patterns in mice were compared by a number of criteria, including biological, structural, and antigenic aspects. In addition, some basic information on the subcellular fractionation of the major antigens and possible associations among the proteins or protein subunits was obtained.

Five of six strains formed plaques of similar size in cultured mouse cells; the highly virulent Karp strain formed smaller plaques than the rest. This extends our initial observation that Karp formed smaller plaques than Gilliam and Kato under the same conditions (24) and demonstrates the only known biological difference between Karp and Karp-like JC472B. Plaque formation develops from rickettsial growth, spread, and killing of host cells. Why Karp plaques are smaller than the others is not known, but it was demonstrated that the plaque sizes were not related to rickettsial replication rates in the same cell lines. In comparative growth studies in cultured mouse cells, mean doubling times of Karp (high virulence), Gilliam (intermediate virulence), and TA716 (low virulence) were 12.3, 10.6, and 18.2 h, respectively. The reproducibly long Gt of TA716 in C3H cells was surprising, since routine passage of this strain in Vero cells gave no indication that it replicated more slowly than the others (except for Gilliam, which appears to replicate slightly faster than the other strains in Vero cells also). Karp, Gilliam, and TA716 also have been compared with regard to their interferon (IFN) sensitivity in cultured C3H cells (27), which, unlike Vero cells (13), are capable of synthesizing IFN. Of the three rickettsial strains tested, only TA716 was susceptible to inhibition by purified IFN-$\alpha/\beta$ (27). It is possible, therefore, that TA716's slower growth rate in these cells is either the cause or result of its sensitivity to IFN-$\alpha/\beta$-mediated inhibition; alternatively or in addition, some nutritional requirement may not be met in this combination of C3H cells and C-4-supplemented RPMI 1640. In any case, replication of different scrub typhus rickettsial strains must be regulated differently at some stage(s) in cultured cells. How these intriguing differences may relate to the variations in virulence remains open for further study.

The properties of proteins from a number of scrub typhus rickettsial strains have been compared by SDS-PAGE; in this report, we added polypeptide profiles of two additional strains, TA716 and TA763, from Thailand. Each of the six strains examined was easily distinguished by SDS-PAGE, and the strain-related variable migration of some of the immunogenic polypeptides (Sta110, Sta56, and Sta47) during SDS-PAGE was confirmed and extended (21, 28, 40, 41, 45, 54, 55, 60). None of the strains except Karp and JC472B could be grouped by this method. Despite the demonstrated variation among scrub typhus rickettsial isolates, their SDS-PAGE profiles as a group (particularly the clustering of major proteins in the 46- to 58-kDa range) are readily distinguishable from those of other *Rickettsia* species. We also added to previous observations by us and by others (20, 22, 58) on the effects of denaturing conditions on the migration of individual polypeptides in SDS-PAGE. These are important not only in understanding the chemical natures of these proteins, but also in serving as identifying characteristics of the proteins. Better identification of proteins would be useful, for example, in serosurveys measuring the humoral responses to particular immunogens resolved by immunoblotting.

The SDS-PAGE profiles of plaque-purified Karp, Gilliam, Kato, and JC472B were similar to those we found earlier with these strains, before their plaque purification (21); thus, the plaque purification procedure did not select unique minor strains from the laboratory stocks. It is possible, however, that some selection occurred before this, when the isolates were originally adapted to laboratory passage. Some selection of faster-growing strains seems likely, since we demonstrated widely varying replication rates among different *R. tsutsugamushi* strains under fixed conditions. Moreover, we observed no changes in SDS-PAGE profiles during extensive serial passage in tissue culture. The apparent stability of scrub typhus rickettsiae after laboratory adaptation contrasts sharply with the variability seen within this species. The biological significance of this heterogeneity and how it is affected by the rickettsiae's need to survive and grow in both mammalian and arthropod hosts can only be surmised at this point; our work, however, has implicated two membrane antigenic proteins as major sources of variation.

Sta56 and Sta47 appear to be integral membrane proteins, based on their resistance to extraction with detergents (20, 21, 44), and the deduced amino acid sequence of Sta56 predicts several hydrophobic regions also suggestive of membrane insertion (43, 52). Moreover, there is evidence that Sta56 and Sta47 are in the outer membrane: both were surface labeled with $^{125}I$ (54), and Sta56 in unextracted rickettsiae also had been shown to be accessible to protease digestion (22, 54) and antibodies in polyclonal antisera (21), suggesting their exposure on the rickettsial surface. Supplementing these observations, Sta56 and Sta47, as well as Sta110 and 28- and 30-kDa proteins, all were found predominantly in a crude membrane fraction (200,000 $\times$ *g* pellet) prepared from Renografin-purified, sonically disrupted rickettsiae, and the coprecipitation of Sta56 and Sta47 by MAb specific for one or the other protein suggested that they were present in the same membrane fragments which remained after mild detergent extraction. Additionally, Sta47 as well as Sta56 (but not Sta110) also was accessible to MAb when rickettsiae were exposed before extraction, further indicating its exposure on the rickettsial surface. The failure of the two Sta110-specific MAb to react detectably with unextracted rickettsiae does not preclude the possibility of surface exposure of other epitopes on this protein or that it is in the outer membrane but not surface exposed. The precise membrane location of this antigen awaits further experimentation.

The relatively abundant antigen Sta56 has been widely studied and has long been recognized as a site of strain-specific as well as cross-reactive epitopes. In the present studies, none of the four Sta56-specific MAb bound to protein from all six strains, and the number of Sta56 bands reactive with the MAb varied by strain also, emphasizing the nuances involved in assigning strain specificity to antigenic determinants. Coupled with the strain-related structural variability demonstrated both by migration rates in SDS-PAGE and by protease (36)- or *N*-chlorosuccinimide (45)-generated cleavage products, these results support earlier conclusions that Sta56 is a highly variable protein. Multiple forms of Sta56 and the partial dependence of their electrophoretic migration on the denaturing conditions (10, 20, 58) were confirmed by two-dimensional SDS-PAGE, SDS-PAGE of excised Sta56, and immunoblotting with Sta56-specific MAb. Some of these bands apparently can occur as the result of reaggregation of the monomeric form of Sta56, and others may correspond to bands obtained by partial proteolytic digestion (37). The presence of Sta56-associated bands throughout the gels indicates the likelihood that other polypeptides excised from preparative SDS-polyacrylamide gels are contaminated with some form of this protein, so that experiments utilizing this technique must be interpreted with caution.

Our studies lead to the conclusion that Sta110 also is a major source of strain-related variation, as was indicated both by a relatively wide range of migration rates in SDS-PAGE (40, 41; this study) and by reaction with Karp-induced MAb. Neither of the two MAb specific for Sta110 reacted

with epitopes on all six strains. One reacted with only two strains and the other with four; no TA716 Sta110 was identified. The only antigenic difference detected so far between strains Karp and JC472B resides in this protein. Sta110 has been reported to be immunogenic in a large proportion of human infections as well as in hyperimmunized laboratory animals (41). The availability of Sta110-specific MAb has greatly enhanced our capacity to identify and study this protein, which does not appear as a major protein in [$^{35}$S]methionine-labeled rickettsiae.

In contrast to the specificity of the MAb directed to Sta56 and Sta110, the notable feature of the Sta47-specific MAb was their broad cross-reactivity. Only one of the five MAb displayed any strain specificity, by binding Karp and JC472B more strongly than Gilliam, Kato, or the two Thai strains. This binding pattern correlated with the electrophoretic migration of Sta47, since Karp and JC472B Sta47 migrated slightly behind Sta47 from the remaining four strains. In a similar screening of MAb induced by the Kawasaki strain, two of four MAb specific for Sta47 reacted with all six strains tested, while the other two reacted with two or three of the six strains (14). The relative conservation of Sta47 features among several strains suggests that this protein has a critical structural or functional role. RIP and immunoblotting results with MAb suggested, for the first time, that Sta47 occurs in dimeric and trimeric forms.

The putative heat shock protein Sta58, in contrast to Sta47, Sta56, and Sta110, was mostly in the subcellular fraction from which membrane had been removed. The intracellular location of other bacterial hsp60s appears to be variable. We earlier had noted the ease with which Sta58 was separated from the rickettsial pellets formed by centrifugation at 13,000 × *g* and which retained the bulk of the other major proteins (20, 22). In fractionation studies similar to that reported here, the hsp60 from a number of disrupted bacteria, including *Rickettsia typhi* and *Rickettsia prowazekii*, was found mainly in the supernatant which resulted from washing the membrane fraction by centrifugation at 200,000 × *g*; lesser amounts of hsp60 were found in the cytoplasmic and membrane fractions (6). The hsp60 (62.5-kDa protein) of *Coxiella burnetii*, another obligately intracellular gram-negative-like bacterium, was associated with a peptidoglycan-enriched subcellular fraction (59). More recently, *C. burnetii* hsp60 was determined to remain bacterium associated when these microorganisms were prepared from mechanically disrupted, infected host cells, but appeared to be secreted or translocated by the coxiellae after their natural release from infected fibroblasts (56).

Interestingly, one of the Sta47-specific MAb (Kp47-a) coprecipitated Sta58 in standard RIP with TX-DOC-NaCl-extracted proteins. Whether this represents a nonspecific or a chaperonin-type association (29) of Sta58 cannot be determined by our experiments. Also of interest was the finding that antibody-Sta58 complexes formed before detergent extraction of rickettsial proteins apparently were large enough after extraction to pellet during centrifugation at 13,000 × *g*. This was in contrast to the behavior of Sta56 and Sta47 and may be related to the natural occurrence of hsp60 proteins as large multimers (6). In the present work, the only MAb with specificity to Sta58 immunoprecipitated Sta58 from all six strains, and no strain-related variability in electrophoretic migration was noted either.

The importance of comparing strains at several levels is illustrated by the comparisons of Karp and JC472B. Despite their disparate sources (human in New Guinea and gerbil in Pakistan, respectively), the close relatedness of these two strains was apparent in antibody reactions with MAb to Sta56 (the only strain whose Sta56 seemed to completely overlap antigenically with Karp was JC472B) and with Kp47-e, which failed to strongly bind Sta47 from the other four strains. SDS-PAGE migration of Sta56 and Sta47 also was similar in Karp and JC472B, although some differences in SDS-PAGE profiles had been noted earlier (21, 28). On the other hand, structural variability in Sta56 from the two strains has been indicated by the different patterns of Sta56 peptides derived from partial trypsin digestion (36). The relative difficulty in solubilizing JC472B proteins (particularly Sta56 and Sta47) with SDS also suggests that some structural differences exist. It seems likely that, in the original serotyping of JC472B with polyclonal antisera (49), the strong cross-reactivity with dominant Karp immunogens Sta58, Sta56, and Sta47 could have masked the absence of Karp-specific epitopes on JC472B Sta110 and possibly other proteins. At the biological level, JC472B plaques in cultured mouse cells were larger than Karp plaques obtained in the same experiments; factors which might be responsible for this are unknown. Because JC472B has undergone far fewer laboratory passages than Karp, the possibility remains that at least some differences between them are due to this.

Aside from the similarities between Karp and JC472B, no pairing of any of the other strains could be made based on our data. The two animal isolates from Thailand were shown to have unique structural and antigenic features, and they appeared to be no more similar to each other than they were to the other strains examined. Gilliam and TA716 were the strains least reactive with the Karp-induced MAb to both Sta56 and Sta110.

In conclusion, some biological, structural, and antigenic characteristics of six plaque-purified strains of *R. tsutsugamushi* were compared in an attempt to begin to understand the factors underlying strain-related differences in antigenicity and virulence. We succeeded in showing different replication rates under standardized conditions, adding, along with plaque size variation, to our recent demonstration of differential susceptibility to IFN-mediated inhibition of rickettsial growth (27). The development and use of a panel of MAb has enhanced the identification of Karp strain polypeptides under different extraction and electrophoretic conditions, permitting the recognition of multiple forms of Sta47 and Sta56 in SDS-PAGE and, importantly, facilitating our identification of the counterparts of these and other antigens in additional scrub typhus rickettsial strains. The Karp-induced MAb also have provided a basis for assessing the degree of conservation of the target epitopes in five other clonally purified *R. tsutsugamushi* strains, leading to some general conclusions about the relative variability of the major immunogens. A future report will describe the modulation of rickettsial infection by some of the MAb, indicating for the first time the biological significance of some specific rickettsial components.

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