Analysis of Polypeptide Composition and Antigenic Components of *Rickettsia tsutsugamushi* by Polyacrylamide Gel Electrophoresis and Immunoblotting

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Polyacrylamide gel electrophoresis of lysates of purified *Rickettsia tsutsugamushi* revealed as many as 30 polypeptide bands, including major bands corresponding to molecular sizes of 70, 60, 54 to 56, and 46 to 47 kilodaltons. Compared with the polypeptide composition of the rickettsiae of Gilliam, Karp, and Kato strains and a newly isolated Shimokoshi strain, the major polypeptide in the Kato strain (54-56K) and in the Karp strain (46-47K) migrated a little faster and slower, respectively, than the corresponding polypeptides in the other strains. The largest major polypeptide (54-56K) was digestible by the treatment of intact rickettsiae with trypsin and variable in content in separate preparations, suggesting that the polypeptide exists on the rickettsial surface and is easily degraded during the handling of these microorganisms. Several surface polypeptides of rickettsiae, including the 54-56K and 46-47K polypeptides, were detected by radioiodination of intact rickettsiae followed by polyacrylamide gel electrophoresis of the lysate; however, the 70K and 60K polypeptides were not labeled. Immunoblotting experiments with hyperimmune sera prepared in guinea pigs against each strain demonstrated that the 70K, 54-56K, and 46-47K polypeptides showed antigenic activities. The 54-56K polypeptide appeared to be strain specific, whereas the 70K and 46-47K polypeptides cross-reacted with the heterologous antisera.

The incidence of scrub typhus fever (tsutsugamushi disease) decreased in Japan after the Second World War. Since 1975, however, the incidence of this disease began to increase, and in 1983 more than 700 cases were reported. The causative agent, *Rickettsia tsutsugamushi*, is unique among the *Rickettsia* spp. because of the presence of antigenic variants such as the Gilliam, Karp, and Kato strains (12). We recently isolated a strain (Shimokoshi) from a patient in the Niigata prefecture which was antigenically distinguished from those prototype strains (8). Other antigenic types were also discovered in Thailand by Elisberg et al. (3).

The study of these phenotypic variations at the molecular level is complicated by the difficulty in obtaining *R. tsutsugamushi* in pure form since the rickettsia organism is very fragile, sticks to host components, and is therefore not amenable to purification. However, several investigators have recently attempted the comparative analyses among rickettsial strains by modern techniques (2, 4, 5). We have successfully purified the microorganisms by Percoll density gradient centrifugation (9), making it easy to analyze their polypeptide composition and antigenic structure.

This study on *R. tsutsugamushi* describes a comparison of constitutional proteins among different strains by polyacrylamide gel electrophoresis (PAGE), the degradation of a polypeptide by treatment of the intact microorganism with trypsin, the detection of the rickettsial surface proteins by radioiodination, and immunoblotting to determine the antigenicity of the separated proteins and their strain specificities.

MATERIALS AND METHODS

Rickettsiae and their purification. *R. tsutsugamushi* Gilliam, Karp, and Kato strains were provided by A. Shishido, National Institute of Health of Japan, and were propagated in

L-cell suspension cultures as described previously (9). The Shimokoshi strain isolated recently in our laboratory was also grown in L cells by the same procedures. These rickettsiae were purified by Percoll density gradient centrifugation as described previously (9).

Besides these rickettsiae derived from L-cell cultures, rickettsiae passaged in chicken eggs were also used for a preparation of antisera as follows. Portions (0.2 to 0.5 ml) of rickettsial inoculum were injected into the yolk sacs of embryonated chicken eggs (5 to 7 days old), and when the embryos died 5 to 7 days postinjection, rickettsiae were collected from the homogenate of the yolk sacs by differential centrifugations.

Trypsin treatment of rickettsiae. A suspension of purified rickettsiae in 0.033 M Tris hydrochloride buffer (pH 7.4) containing 0.14 M NaCl and 0.001 M EDTA (NET buffer) was mixed with trypsin solution in 0.2 M Tris hydrochloride buffer (pH 7.4) at the final concentration of 100 µg/ml. The mixture was incubated at 37°C for 20, 40, or 60 min, diluted fivefold with NET buffer, and centrifuged at 2,600 × *g* for 20 min, and the pellet was washed twice with NET buffer by repeating the suspension and centrifugation procedure to remove the trypsin.

Radioiodination. Radiolabeling of the rickettsial surface proteins with ^{125}I was achieved by a modification of the method of Williams (13). Briefly, purified rickettsiae (about 1.6 mg of protein as analyzed by the method of Lowry et al. [7] with crystalline bovine serum albumin [BSA] as a standard) were suspended in 2 ml of phosphate-buffered saline (PBS)-EDTA (0.15 M NaCl, 0.015 M NaH_2PO_4, and K_2HPO_4 and 0.001 M EDTA [pH 7.4]) containing 200 µCi of carrier-free Na[^{125}I] (New England Nuclear Corp., Boston, Mass.) and 200 µg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.), and labeling was initiated by adding 10 µl of 0.006% H_2O_2. The mixture was kept shaking gently in a 37°C water bath, and two more 10-µl additions of H_2O_2 were

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made at 10-min intervals to accelerate the reaction. After incubation for 30 min, the reaction was terminated by the addition of 3 ml of ice cold PBS-EDTA, and the rickettsiae were washed three times with PBS-EDTA and centrifuged at 2,800 × g for 20 min. The final pellet was solubilized in a sample buffer for PAGE. Nonspecific labeling of rickettsiae was monitored in control reaction mixtures without lactoperoxidase.

Preparation of antisera. Hyperimmune sera against each strain of *R. tsutsugamushi* were prepared in guinea pigs by the intracerebral inoculation of purified intact rickettsiae propagated in L cells or chicken eggs as described previously (8). These sera were provided by M. Shibata, Public Health Laboratory in the Niigata prefecture, and by the Denka Seiken Co., Ltd., Tokyo, Japan. Anti-L-cell serum, also provided by the Denka Seiken Co., Ltd., was prepared in rabbits by the intramuscular inoculation of L-cell cytoplasmic fraction with complete adjuvant four times at 1-week intervals and was collected 1.5 months after the initial injection.

PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed by a modification of the Laemmli method (6). Samples for electrophoresis were dissolved in 0.0625 M Tris hydrochloride buffer (pH 6.8) containing 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue by heating at 100°C for 4 to 5 min. The stacking gel contained 4.5% acrylamide, 0.12% bisacrylamide, and 0.1% SDS in 0.125 M Tris hydrochloride buffer (pH 6.8), and the separation gel contained 10% acrylamide, 0.26% bisacrylamide, and 0.1% SDS in 0.375 M Tris hydrochloride buffer (pH 8.8). Each gel was polymerized by the addition of tetramethylenediamine and ammonium persulfate at final concentrations of 0.2 and 0.058%, respectively, in the stacking gel and 0.1 and 0.078%, respectively, in the separation gel. The total gel size was 14 cm wide, 1 mm thick, and 12.5 cm long (2.5 cm, stacking gel; 10 cm, separation gel). Electrophoresis was performed for about 6 h at constant electric current (20 mA) in 0.025 M Tris hydrochloride buffer (pH 8.3) containing 0.192 M glycine and 0.1% SDS. For the visible observation of separated polypeptide bands, the gel was fixed with a mixture of methanol, acetic acid, and deionized water (4:1:4 by volume) for 30 min at room temperature, stained with 0.25% Coomassie brilliant blue solution for 2 h, and then destained with 7% acetic acid. The molecular weight standards we used were rabbit muscle phosphorylase *b* (97,400), BSA (67,000), ovalbumin (45,000), bovine chymotrypsinogen A (25,000), whale myoglobin (17,800), and horse cytochrome *c* (12,400), purchased from Boehringer Mannheim Biochemicals, Federal Republic of Germany, and Schwarz/Mann, Orangeburg, N.Y.

For the detection of radiolabeled polypeptide bands after electrophoresis, the slab gel was dried and exposed to RX film (Fuji Photo Film Co., Ltd., Tokyo, Japan) by intensifying with a Quanta III screen (Du Pont Japan, Ltd., Tokyo).

Immunoblotting. Immunoblotting was accomplished by modifications of the techniques of Towbin et al. (10) and Burnette (1). Antigenic components were detected by using guinea pig hyperimmune sera against each strain of *R. tsutsugamushi* as the primary antibody and horseradish peroxidase-conjugated anti-guinea pig immunoglobulin G prepared in rabbits (Cappel Laboratories, Cochranville, Pa.) as the secondary antibody. Briefly, proteins separated by PAGE were transferred to a nitrocellulose sheet by electrophoresis in 0.025 M Tris hydrochloride buffer (pH 8.3) containing 0.192 M glycine and 20% methanol at 0.8 to 1.5 A

for 2.5 h. A portion of blotted sheet was cut out, stained with 0.2% Coomassie brilliant blue containing 40% methanol and 10% acetic acid for 5 min, and then destained with solution containing 90% methanol and 2% acetic acid for 5 min. The unstained part of the nitrocellulose sheet was soaked overnight in 0.5% Nonidet P-40 in PBS (0.15 M NaCl in 0.015 M phosphate buffer [pH 7.4]) to remove the SDS, washed four times with PBS containing 0.05% Tween 20 (Tween-PBS) for 30 min, immersed in 3% BSA (Fraction V; Sigma) in PBS at 37°C for 1 h to saturate protein-binding sites, and then reacted with a guinea pig antirickettsial serum diluted adequately (usually 1:30 or 1:100) in Tween-PBS containing 3% BSA for 1.5 h at room temperature. The sheet was washed again four times with Tween-PBS for 30 min, immersed in horseradish peroxidase-conjugated anti-guinea pig immunoglobulin G diluted 2,000-fold with PBS containing 3% BSA, and incubated for 2 h at room temperature. After the sheet was washed four more times with Tween-PBS, the peroxidase-associated bands were detected by immersing the sheet in a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 at room temperature for 5 min. The enzyme reaction was terminated by placing the sheet in running tap water.

In the immunoblotting procedure with anti-L-cell serum of rabbit, we used goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase, which was purchased from Miles Laboratories, Inc., Naperville, Ind.

Preparation of L-cell lysates. Whole L-cell lysates were prepared as follows. L cells washed twice with ST buffer (0.25 M sucrose in 0.033 M Tris hydrochloride buffer [pH 7.4]) were suspended in the sample buffer for electrophoresis, sonicated for 2 min, and then heated at 100°C for 10 min.

Lysate of the L-cell cytoplasmic fraction was then prepared as follows. L cells washed with ST buffer were homogenized in the same buffer with a Dounce homogenizer at 40 strokes (Kontes Glass Co., Vineland, N.J.), and the low-speed centrifugal supernatant was centrifuged at 10,000 × g for 20 min. The pellet was then solubilized in the sample buffer for electrophoresis by heating at 100°C for 10 min.

Both lysates were centrifuged at 15,000 × g for 20 min, and the supernatants were analyzed by PAGE and immunoblotting.

RESULTS

Polypeptide patterns of R. tsutsugamushi in PAGE. In PAGE analyses of the polypeptide compositions in two or three independently purified batches of the Gilliam, Karp, Kato, and Shimokoshi strains, about 30 visible bands appeared by Coomassie blue staining (Fig. 1). The overall patterns revealed the similarity among the batches of an individual strain and also among the different strains. However, some minor differences between the columns were also recognized, especially in some of the tiny bands; that is, some bands were different in content and some were lacking. These differences may be due to the contamination of host cell components in the preparations or to some other unknown factor(s), such as unstable properties of rickettsial proteins during the preparation process. The major bands corresponding to molecular sizes of 70, 60, 54 to 56, and 46 to 47 kilodaltons were commonly recognized in all columns. The distinguishable differences among the strains on these major bands were seen in the 54-56K (Kato) and 46-47K (Karp) polypeptide bands, which migrated a little faster (54K) and a little slower (47K), respectively, than the corresponding bands in the other strains (56K and 46K; see

also Fig. 4). Although the 60K bands were relatively invariant in the quantity of each column (Fig. 1), quantitative variations were recognized in the bands at the 54-56K region, with columns 5, 6, and 8 having less than the others. This seemed to be due to the instability of 54-56K polypeptides during the preparation of samples as discussed below. The 43K band was seen in some columns but not in the others (Fig. 1), also indicating a variable sensitivity to preparation.

Trypsin treatment of rickettsiae. As described previously (11), trypsin treatment of rickettsiae results in a decrease in infectivity due to the inability of the trypsin-treated rickettsia to attach itself to the host cell surface, suggesting the change of rickettsial surface structures by the enzyme treatment. PAGE analysis of the rickettsiae (Gilliam) before and after trypsin treatment (Fig. 2) demonstrated the disappearance of the 56K band as a result of this treatment, indicating that the 56K polypeptide exists on the rickettsial surface and is sensitive to the attack of proteolytic enzymes.

Radioiodination of rickettsial surface polypeptides. The surface proteins of Gilliam strain rickettsiae were detected by ^{125}I labeling with lactoperoxidase and PAGE analysis followed by an autoradiogram (Fig. 3). The 56K band was heavily labeled as expected, the 46K, 43K, 39K, 35K, 28K, and 25K bands were also labeled, and several minor bands were recognized above the 80K band.

Detection of rickettsial antigens by immunoblotting. Antigenic properties of the polypeptides derived from the Gilliam, Karp, Kato, and Shimokoshi strains and separated by PAGE were analyzed by immunoblotting (Fig. 4). Each rickettsial strain used was purified from infected L-cell cultures, and the antisera used were prepared in guinea pigs by injection of each strain of rickettsiae derived from L cells. The results obtained were summarized as follows. (i) The 54-56K polypeptide from each individual strain reacted strongly with the homologous antiserum but faintly or moderately with het-
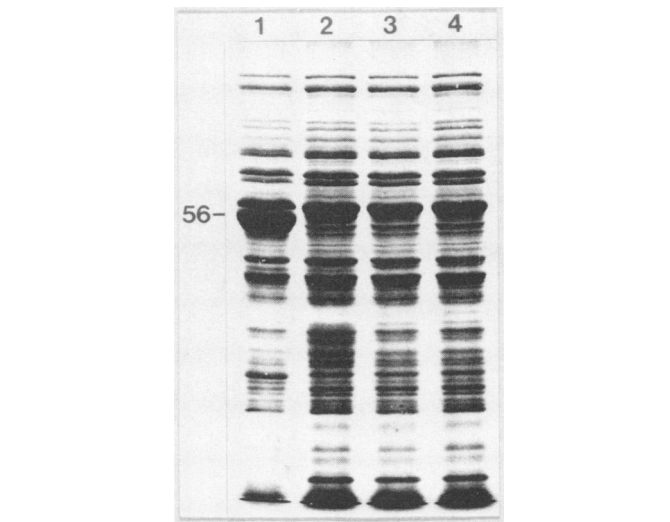

FIG. 2. Effect of trypsin treatment of *R. tsutsugamushi* (Gilliam) on the polypeptide composition. Columns: 1, rickettsiae before trypsin treatment; 2 through 4, rickettsiae treated with trypsin for 20, 40, and 60 min, respectively, at 37°C. The number on the left side indicates the molecular size (in kilodaltons) of a polypeptide which was diminished by treatment with trypsin.

erologous antisera, although anti-Gilliam and anti-Kato sera reacted to some extent with the 56K polypeptides of the Karp and Shimokoshi strains, respectively. These results show that 54-56K polypeptides have a strain- or type-specific antigenic nature. (ii) The 46-47K polypeptides reacted not only with the homologous antiserum but also with the heterologous antisera, although some strong reactivities with the homologous antiserum were also recognized. This suggests that the 46-47K polypeptides may have characteristics of a group- or species-specific antigenicity. (iii) Anti-Gilliam and anti-Karp sera reacted similarly with the 70K polypep-
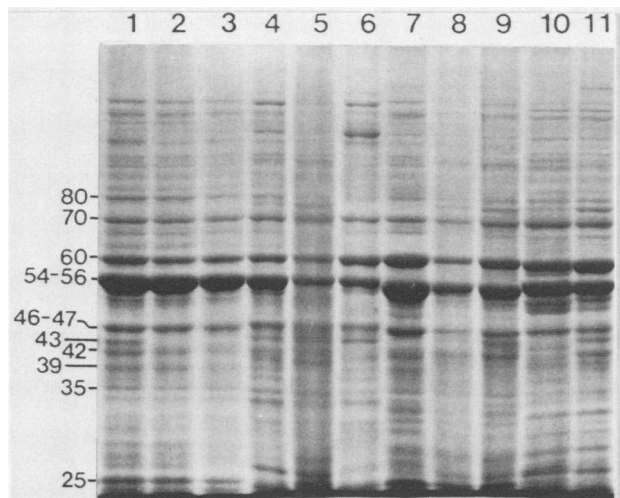

FIG. 1. Comparison of polypeptide patterns determined by PAGE among different strains of *R. tsutsugamushi*. Three independently purified batches of rickettsia strains (Gilliam, columns 1 through 3; Karp, columns 4 through 6; and Kato, columns 7 through 9) and two batches of Shimokoshi strain (columns 10 and 11) were solubilized in sample buffer, and the polypeptides were separated in a slab gel and detected by staining with Coomassie blue. Numbers on the left side indicate the molecular sizes (in kilodaltons) of the bands and were determined in comparison with the migrations of standard proteins.
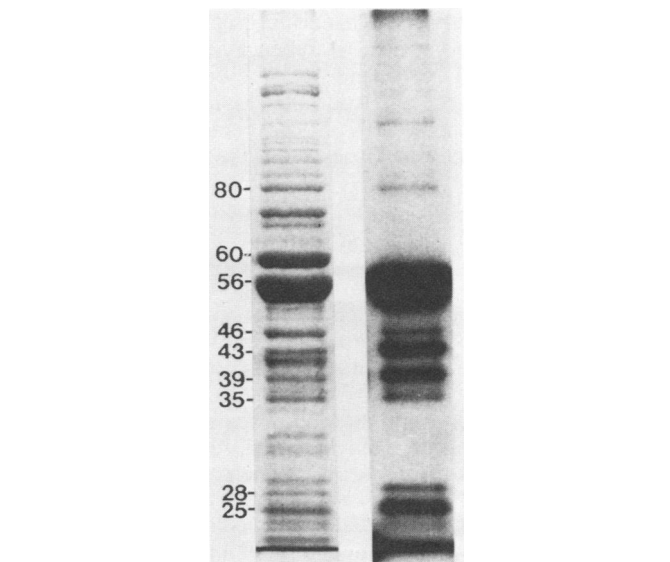

FIG. 3. PAGE pattern of radioiodinated polypeptides in rickettsiae (Gilliam). The left and right columns show the patterns of Coomassie blue staining and the autoradiogram, respectively. The numbers on the left side are the same as in Fig. 1.
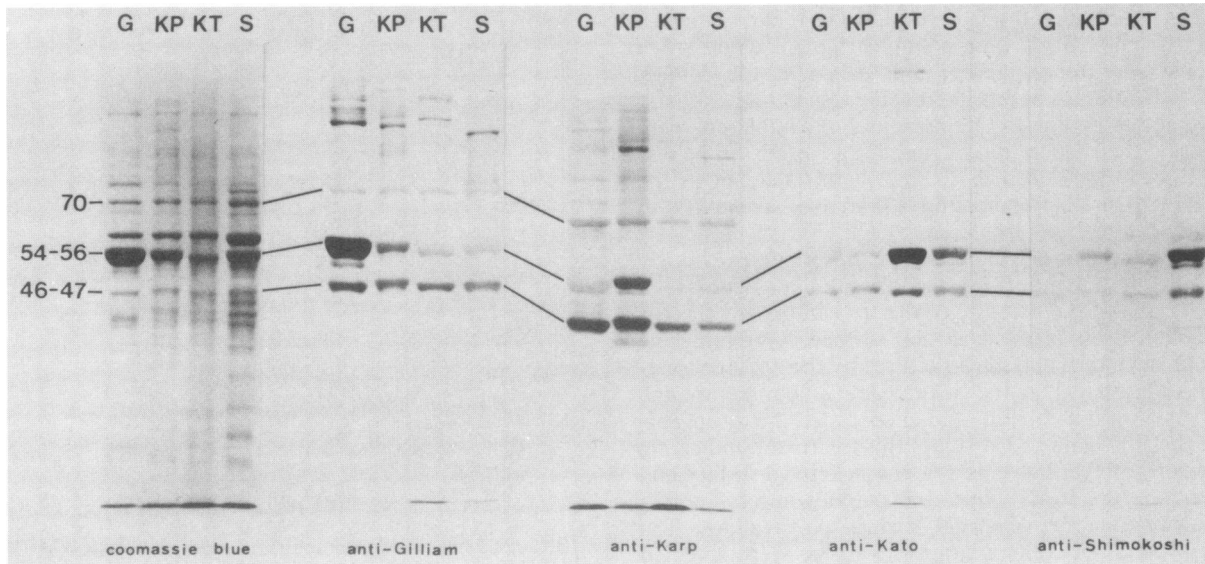
FIG. 4. Immunoblotting profiles of rickettsial polypeptides separated by PAGE. Rickettsial antigens were purified from L-cell cultures and antisera were prepared in guinea pigs immunized with rickettsiae also derived from L-cell cultures. Characters at the top indicated the rickettsial strain of each column: G, Gilliam; KP, Karp; KT, Kato; S, Shimokoshi. Each of the four columns in each group was stained with Coomassie blue or reacted with antisera as indicated at the bottom of the columns. The numbers on the left side are the same as in Fig. 1.

tides of all strains, suggesting that these polypeptides may also be group- or species-specific antigens. The 70K bands were not detected in the reactions with anti-Kato and anti-Shimokoshi sera, but this may be due to the low antibody titers observed in these sera.

Several other bands could be seen (Fig. 4), especially with anti-Gilliam and anti-Karp sera, but they have not yet been identified.

In these experiments (Fig. 4), rickettsial antigens used for both the immunoblotting and the preparation of antisera were derived from L-cell cultures. Therefore, it was necessary to distinguish the results described above from spurious reactions caused by host cell components which were contaminated in the antigens. This criticism can be excluded by the following observations. (i) When PAGE profiles of the lysates of whole L cells and L-cell cytoplasmic fractions were immunoblotted with the antisera used in Fig. 4, no bands were seen in the 70K, 54-56K, and 46-47K regions, and only tiny bands were observed at the lower positions of the columns (Fig. 5, panel B; only the results with anti-Gilliam serum are shown). (ii) In the immunoblotting with normal guinea pig serum before immunization, no visible bands were detected in the PAGE columns of rickettsiae and L-cell lysates (data not shown). (iii) Rabbit antiserum against the L-cell cytoplasmic fraction did not react with any rickettsial PAGE bands, although several bands were recognized in the column of L-cell lysates by this serum (data not shown). (iv) Immunoblots of rickettsiae derived from L cells with guinea pig hyperimmune sera against each strain of rickettsiae propagated in chicken eggs revealed essentially similar patterns to those of Fig. 4, demonstrating that the antisera apparently reacted with 54-56K polypeptides from the homologous strains, and also showed similar reactivities with 46-47K polypeptides from both homologous and heterologous strains (Fig. 5, panel C; only the results with anti-Gilliam serum are shown). The antigenicity of the 70K polypeptide was not clearly recognized in this case, probably because of the low antibody titer in the serum.

DISCUSSION

The PAGE analysis of the polypeptide composition in whole cell lysate of *R. tsutsugamushi* revealed as many as 30 protein bands by Coomassie blue staining. A comparison of the polypeptide patterns in Gilliam, Karp, Kato, and Shimokoshi strains revealed four main polypeptide bands (70K, 60K, 54-56K, and 46-47K) which were common in all
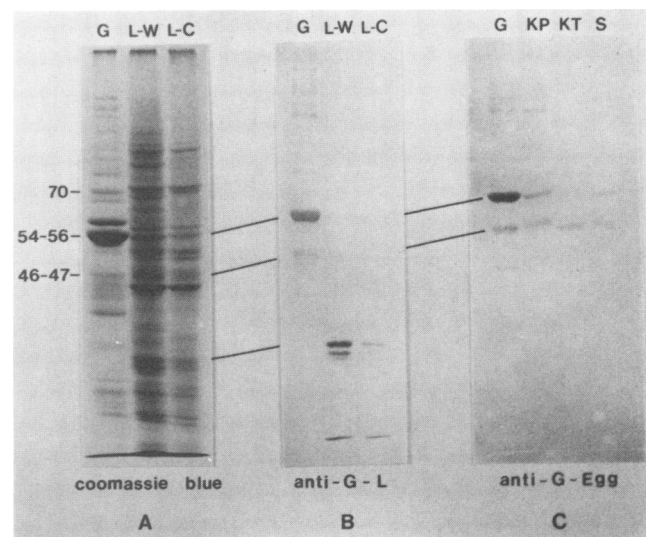

FIG. 5. Immunoblotting profiles of rickettsia and L-cell lysates. (Panel A) Coomassie blue staining of rickettsiae (G; Gilliam), whole L cells (L-W), and L-cell cytoplasmic fraction (L-C). (Panel B) Immunoblots of the same samples with the anti-Gilliam serum used in Fig. 4. (Panel C) Immunoblots of Gilliam, Karp (KP), Kato (KT), and Shimokoshi (S) strains derived from L-cell cultures with an antiserum against Gilliam strain propagated in embryonated chicken eggs. The numbers on the left side are the same as in Fig. 1.

strains. Among these, the 54-56K and 46-47K polypeptides were shown by radioiodination to be surface proteins of the microorganisms. Three of the four polypeptides (46-47K, 54-56K, and 70K) showed antigenicity in the immunoblotting experiments. Differences between strains were observed in the migration distance of corresponding polypeptides during PAGE, i.e., the largest main band at the 54-56K region in the Kato strain moved a little faster than those of the other strains, and the band at the 46-47K region in the Karp strain moved a little more slowly than those of the other strains. By PAGE analysis of immunoprecipitated preparations of *R. tsutsugamushi*, Hanson (4) found three major antigenic polypeptides with molecular sizes of 63, 60, and 50 kilodaltons in the Gilliam and Karp strains and four polypeptides of 63, 58, 57, and 50 kilodaltons in the Kato strain. When comparing these PAGE patterns with ours, the 63K, 57-60K, and 50K bands seem to correspond to our 60K, 54-56K, and 46-47K bands, respectively. Furthermore, both studies indicated the faster movement of the largest middle band in the Kato strain (57-58K [4] and 54K [this study]) than of those in the other strains (60K [4] and 56K [this study]), although we did not observe the separation into two bands in the Kato strain (57K and 58K [4]).

In our immunoblotting experiments, polypeptides in the 54-56K region showed strain-specific antigenicity reacting strongly with the homologous antiserum but not so much with the heterologous antisera. However, weak but clearly visible cross-reactions were observed between the antigens of Karp and Shimokoshi and the antisera against Gilliam and Kato, respectively. To define the meaning of these cross-reactivities, cloned rickettsiae must be used as a source of antigens, and experiments of this nature are in progress. The 56K polypeptide from the Shimokoshi strain, which was recently isolated in our laboratory from a patient, was found to be antigenically different from those of the prototype strains of Gilliam, Karp, and Kato. This supports the results of our previous study (8), although a little cross-reactivity was seen with the anti-Kato serum.

On the other hand, the 70K and 46-47K polypeptides showed apparent cross-reactivities with heterologous antisera, suggesting the possibility that these polypeptides are group-specific antigens. Hanson (4) mentioned the antigenic property in the 63K polypeptide and its existence on the surface of the rickettsia. Our study of the 60K polypeptide, which might correspond to the 63K polypeptide in the Hanson study, indicated neither antigenicity nor other evidence that it might be a surface polypeptide. Hanson did not mention the antigenicity of the 70K polypeptide. Eisemann and Osterman (2) detected six antigenic components in *R. tsutsugamushi* by PAGE followed by enzyme-linked immunosorbent assay, but the correspondence of their components to our antigenic polypeptides could not be defined.

Although several polypeptides on the rickettsial surface, including the 54-56K and 46-47K polypeptides, were detected by radioiodination, the 70K polypeptide was not labeled with ^{125}I despite our being able to detect its antibody production in the hyperimmune sera of infected guinea pig. Tyrosine residues in the 70K polypeptide, where ^{125}I binds predominantly, may be masked or sterically hidden in the rickettsia.

The 54-56K polypeptide was found to be sensitive to an attack of proteolytic enzyme such as trypsin. We sometimes observed differences in the contents of this polypeptide in different purified batches of rickettsiae and especially in batches prepared without rigid control, such as the samples handled at room temperature without cooling or prepared

from the infected cultures showing overgrowth of rickettsiae and vigorous cytopathogenic effects. These batches revealed decreased amounts of 54-56K polypeptide and under some unknown conditions, considerable amounts of 43K polypeptide were observed. This suggests that the 54-56K polypeptide is unstable.

We have mainly discussed here the four major polypeptides with molecular sizes of 70, 60, 54 to 56, and 46 to 47 kilodaltons. Polypeptides of larger and smaller molecular weights have shown some antigenicity, and they are now being examined in detail.

LITERATURE CITED

1. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
2. **Eisemann, C. S., and J. V. Osterman.** 1981. Antigens of scrub typhus rickettsiae: separation by polyacrylamide gel electrophoresis and identification by enzyme-linked immunosorbent assay. Infect. Immun. **32:**525–533.
3. **Elisberg, B. L., J. M. Campbell, and F. M. Bozeman.** 1968. Antigenic diversity of *Rickettsia tsutsugamushi*: epidemiologic and ecologic significance. J. Hyg. Epidemiol. Microbiol. Immunol. **12:**18–25.
4. **Hanson, B.** 1984. Characterization of *Rickettsia tsutsugamushi* outer membrane proteins, p. 240–243. *In* L. Leive and D. Schlessinger (ed.), Microbiology-1984. American Society for Microbiology, Washington, D.C.
5. **Hanson, B., and C. L. Wisseman, Jr.** 1981. Heterogeneity among *Rickettsia tsutsugamushi* isolates: a protein analysis, p. 503–514. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsia and rickettsial diseases. Academic Press, Inc., New York.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **192:**265–275.
8. **Tamura, A., K. Takahashi, T. Tsuruhara, H. Urakami, S. Miyamura, H. Sekikawa, M. Kenmotsu, M. Shibata, S. Abe, and H. Nezu.** 1984. Isolation of *Rickettsia tsutsugamushi* antigenically different from Kato, Karp, and Gilliam strains from patients. Microbiol. Immunol. **28:**873–882.
9. **Tamura, A., H. Urakami, and T. Tsuruhara.** 1982. Purification of *Rickettsia tsutsugamushi* by Percoll density gradient centrifugation. Microbiol. Immunol. **26:**321–328.
10. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76:**4350–4354.
11. **Urakami, H., T. Tsuruhara, and A. Tamura.** 1983. Penetration of *Rickettsia tsutsugamushi* into cultured mouse fibroblasts (L cells): an electron microscopic observation. Microbiol. Immunol. **27:**251–263.
12. **Weiss, E., and J. W. Moulder.** 1974. Genus *Rickettsia*, p. 883–890. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
13. **Williams, J. C.** 1981. Iodination of *Coxiella Burnetii* cells: optimal conditions for the iodination of exposed surface proteins, p. 473–482. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsia and rickettsial diseases. Academic Press, Inc., New York.