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Purification and Partial Characterization of a Type-Specific Antigen of *Rickettsia tsutsugamushi*

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A type-specific antigen (54- to 56-kilodalton polypeptide) in the envelope of *Rickettsia tsutsugamushi* was purified from each of three prototype strains (Gilliam, Karp, and Kato) by a combination of mild anionic detergent treatment, gel filtration, and reverse-phase high-performance liquid chromatography. The purified antigens from the three strains were shown to have similar amino acid compositions: primarily aspartic acid, glutamic acid, and glycine, with lesser amounts of cysteine, methionine, and tyrosine. The N-terminal amino acid sequences of the antigens were 74.3% homologous among the three strains.

Rickettsia tsutsugamushi, an obligate intracellular parasite, is the causative agent of scrub typhus, or tsutsugamushi disease. This organism is unique among *Rickettsia* spp. because several antigenic variants have been identified. Antigenic types of strains Gilliam, Karp, and Kato are commonly isolated, and these three strains are used as prototype antigens for serodiagnosis of scrub typhus. However, other antigenic types have been isolated in Thailand by Elisberg et al. (4) and in Japan by us (13, 17). Our previous studies (9, 12) demonstrated, by immunoblotting experiments with guinea pig hyperimmune sera and strain-specific monoclonal antibodies, that the strain-specific antigens of each strain are associated with the 54- to 56-kilodalton (kDa) polypeptides located on the rickettsial surface (the antigens in strains Gilliam and Karp are 56 kDa in molecular size, whereas the corresponding antigen in strain Kato is estimated as 54 kDa [12]). On the other hand, we found recently that strains with 54- to 56-kDa polypeptides that are antigenically different from those of the prototype strains Gilliam, Karp, and Kato are all avirulent in mice (11). This result suggests that the difference in molecular structure of the strain-specific 54- to 56-kDa antigens may be associated with rickettsial pathogenesis. Furthermore, treatment of intact rickettsiae with trypsin results in elimination of the 54- to 56-kDa polypeptides from the rickettsial surface (12), and the enzyme-treated rickettsiae lose the ability to adsorb to the host cell surface (16); these findings suggest that these polypeptides may be involved in the initial step of rickettsial infection.

These observations indicate the importance of investigating in more detail the 54- to 56-kDa polypeptides. As an initial step in this endeavor, the 54- to 56-kDa polypeptides were purified from each of the prototype strains (Gilliam, Karp, and Kato), and the amino acid compositions and N-terminal amino acid sequences of these strains were compared.

MATERIALS AND METHODS

Rickettsial strains and purification. *R. tsutsugamushi* Gilliam, Karp, and Kato were used throughout. The strains were triple plaque purified, propagated in suspension cultures of L929 cells, and purified by Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) density gradient centrifugation (14).

SDS-PAGE. Procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel) were as described previously (12). Rickettsial preparations were solubilized in sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 5 mM EDTA, 0.001% bromophenol blue, 0.065 M Tris hydrochloride buffer, pH 6.8) at 100°C for 5 to 10 min. After electrophoresis, the gels were stained with Coomassie brilliant blue or silver (8).

Protein determination. Protein content was determined by the method of Lowry et al. (7), with crystalline bovine serum albumin (Sigma) as the standard.

Sodium *N*-lauroyl sarcosine (Sarkosyl) extraction. Purified rickettsiae of strain Gilliam (3 mg of protein), suspended in 0.033 M Tris hydrochloride buffer, pH 7.4, containing 0.25 M

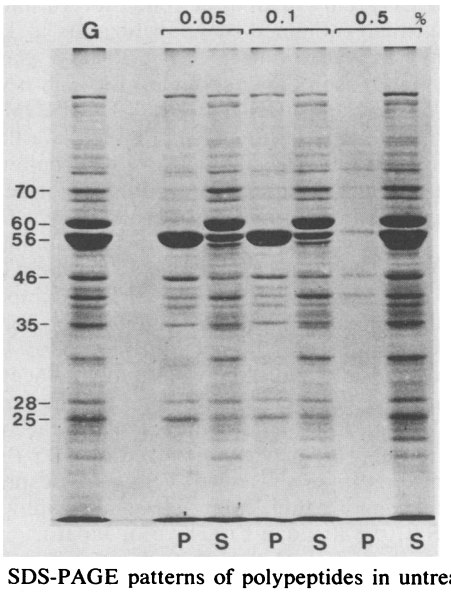

FIG. 1. SDS-PAGE patterns of polypeptides in untreated purified strain Gilliam (G) and in the soluble supernatant (S) and insoluble pellet (P) fractions obtained after Sarkosyl treatment (37°C for 30 min) at the concentrations indicated at the top. The columns were stained with Coomassie blue. Numbers at the left in Fig. 1, 2, 4, and 6 indicate molecular sizes (in kilodaltons) of the polypeptides.

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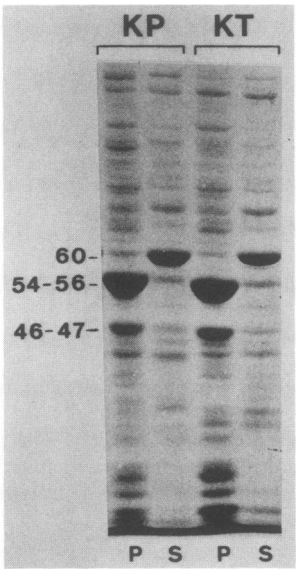

FIG. 2. SDS-PAGE patterns of polypeptides in the soluble supernatant (S) and insoluble pellet (P) fractions of strains Karp (KP) and Kato (KT) after treatment with 0.1% Sarkosyl.

sucrose, were divided into three equal portions, and the pellets (centrifuged at 2,800 × *g* for 20 min) were suspended in 10 mM sodium phosphate buffer, pH 7.4, containing 0.05, 0.1, or 0.5% Sarkosyl (Nakarai Chemicals, Ltd., Kyoto, Japan). After incubation at 37°C for 30 min, proteins in the soluble supernatant fluids and the insoluble pellets, separated by centrifugation at 10,000 × *g* for 1 h, were analyzed by SDS-PAGE.

Purification of the 54- to 56-kDa polypeptide. Purified rickettsiae (7 to 10 mg of protein) were treated with 0.1% Sarkosyl in phosphate buffer as described above. The insoluble pellet was solubilized with 10 mM sodium phosphate buffer, pH 7.4, containing 2% SDS, 5% 2-mercaptoethanol, and 5 mM EDTA by heating at 100°C for 10 min, and the supernatant fluid (obtained by centrifugation at 15,000 × *g* for 10 min) was loaded onto a TSK G3000SW glass column (8 by 300 mm; Tosoh, Tokyo, Japan) for high-performance liquid chromatography (HPLC; ULTROCHROM GTi system; Pharmacia). The proteins were eluted at a flow rate of 0.5 ml/min with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1 M sodium chloride by monitoring A_{280}, and the fractions were analyzed by SDS-PAGE.

The 54- to 56-kDa polypeptide fraction obtained as described above was applied to the TSK Phenyl-5PW RP column (4.6 by 75 mm; Tosoh) of an HPLC equipped with an automated gradient controller. Proteins were eluted with linear gradients of 0 to 20% acetonitrile in 0.05% trifluoroacetic acid for 5 min and then with 20 to 60% acetonitrile in 0.05% trifluoroacetic acid for 32 min, at a flow rate of 0.8 ml/min, by monitoring A_{215}. The fractions of each peak were pooled, and the proteins were analyzed by SDS-PAGE. The protein patterns in silver-stained gels were scanned with a double-wavelength thin-layer chromatography scanner (model CS-910; Shimadzu Co., Kyoto, Japan).

Analysis of amino acid composition and N-terminal amino acid sequence. Samples were hydrolyzed with 6 N HCl at 110°C for 24 h in evacuated sealed tubes, and amino acid compositions were determined with an amino acid analyzer (Hitachi model L-8500). Proteins were automatically sequenced by means of an Applied Biosystems sequencer (model 470 A) equipped with an on-line phenylthiohydantoin analyzer (model 120 A). Polybrene was used as a carrier (6).

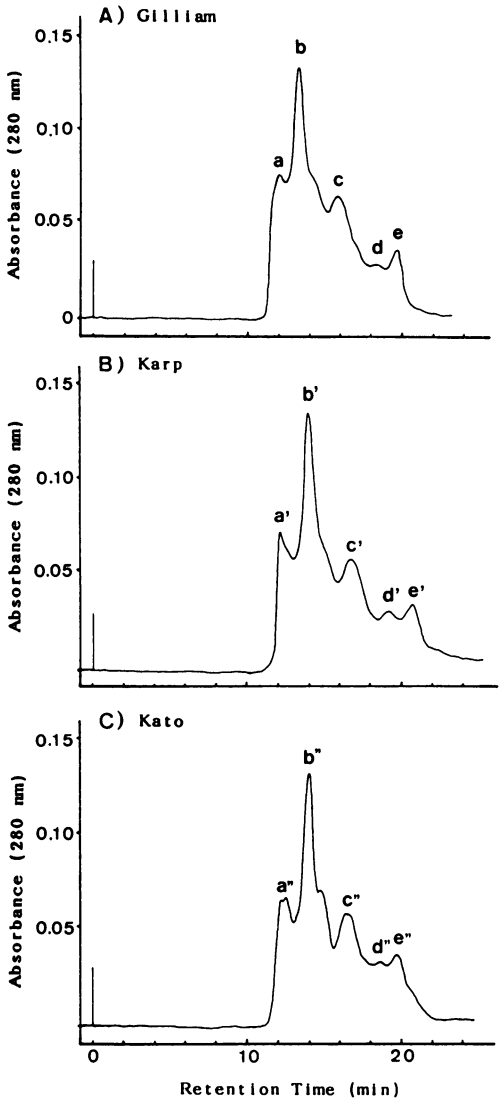

FIG. 3. Gel filtration chromatographic patterns of the insoluble fractions obtained by Sarkosyl treatment from strains Gilliam (A), Karp (B), and Kato (C). Effluents were monitored according to A_{280}.

RESULTS

Effect of Sarkosyl treatment of rickettsiae. To isolate and purify the 54- to 56-kDa polypeptide located on the rickettsial surface, outer membrane proteins were first separated from other proteins. In a preliminary test for this purpose, purified rickettsiae of strain Gilliam were treated with different concentrations (0.05, 0.1, or 0.5%) of Sarkosyl as described in Materials and Methods. SDS-PAGE analyses of the resultant soluble and insoluble fractions (Fig. 1) showed that in the 0.05 and 0.1% Sarkosyl treatments, the insoluble fraction contained a large quantity of 56-kDa polypeptide and some surface polypeptides of 46, 35, 28, and 25 kDa (12), whereas 60-kDa and several other polypeptides were in the soluble fraction. Treatment of rickettsiae with 0.5% Sarkosyl resulted in the solubilization of almost all polypeptides. These results indicated that Sarkosyl treatment of rickettsiae

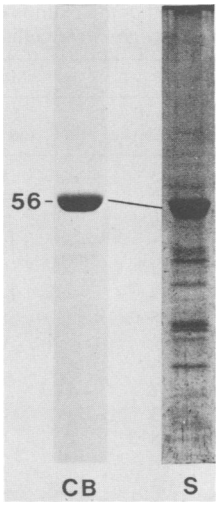

FIG. 4. SDS-PAGE patterns of the peak b fraction shown in Fig. 3A (strain Gilliam), stained with Coomassie blue (CB) and silver (S).

at a concentration of 0.05 or 0.1% effectively separated the rickettsial outer membrane proteins from other proteins. In addition, 0.1% Sarkosyl treatment of purified Karp and Kato by the same procedure produced SDS-PAGE profiles similar to that obtained for Gilliam (Fig. 2), which indicated that Sarkosyl treatment is useful for concentrating the 54- to 56-kDa polypeptides of all three strains.

Purification of 54- to 56-kDa polypeptides by HPLC. Each insoluble fraction of 0.1% Sarkosyl pellets obtained from strains Gilliam, Karp, and Kato was solubilized by treatment with SDS, and the proteins were separated by gel filtration chromatography with a TSK G3000SW glass column. The chromatographic patterns of the three strains were similar, revealing five main peaks (Fig. 3). The SDS-PAGE patterns of peak b in strain Gilliam showed a single band of 56-kDa polypeptide by Coomassie blue staining and this band as well as several others by silver staining (Fig. 4). The SDS-PAGE patterns of peaks b′ of strain Karp and b″ of strain Kato were similar to those shown in Fig. 4 (data not shown). Peaks a, a′, and a″ contained several proteins, mainly polypeptides larger than 60 kDa; peaks c, c′, and c″ consisted of 20- to 35-kDa proteins. In peaks d, d′, d″, e, e′, and e″, no proteins were detected by SDS-PAGE.

Each b, b′, and b″ fraction of Fig. 3 was further purified by reverse-phase HPLC (Fig. 5). A sharp peak was observed at a retention time of 27 to 28 min in each case, and the SDS-PAGE pattern showed a single band of 54- to 56-kDa polypeptide in the silver-stained gels (Fig. 6). The purity of the 54- to 56-kDa polypeptide obtained was estimated from the densitometric scanning profiles of the gels to be more than 90%. Yields of these proteins were approximately 0.12 to 0.18 mg from 2.2 to 3.0 mg of outer membrane protein (Sarkosyl-insoluble fraction).

Amino acid compositions and N-terminal amino acid sequences of 54- to 56-kDa polypeptides. The 54- to 56-kDa polypeptides purified from the three strains exhibited similar amino acid compositions (Table 1). Glutamic acid in strain Gilliam and glycine in strains Karp and Kato were the dominant amino acids. Cysteine, methionine, and tyrosine were present in only small or trace amounts. Tryptophan content was not determined.

Comparison of N-terminal amino acid sequences of the

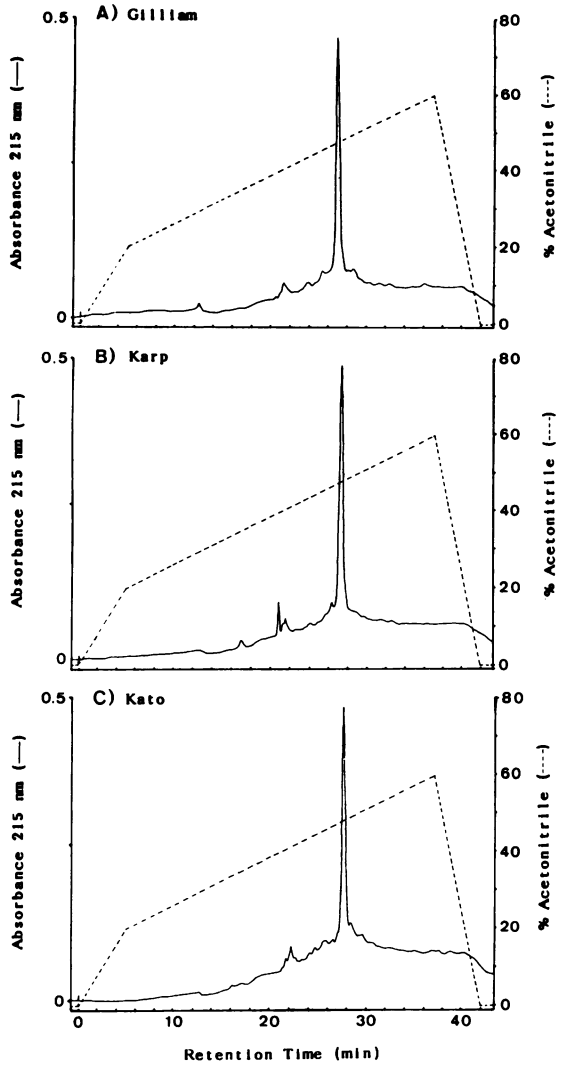

FIG. 5. Reverse-phase HPLC of the peak b, b′, and b″ fractions in Fig. 3. Elution was performed with acetonitrile (– – –) in 0.05% trifluoroacetic acid as described in Materials and Methods. Effluents were monitored according to A_{215} (——).

polypeptides from the three strains (Fig. 7) showed unique differences. Among the 34 to 35 residues, differences were seen at seven positions: at positions 15, 19, 27, 34, and 35 in strain Gilliam, at position 30 in strain Karp, and at position 5 in strain Kato. Deletion of an amino acid between positions 7 and 8 was observed in strain Karp. Analysis of the 54-kDa polypeptide of strain Kato revealed small amounts of lysine and alanine at positions 8 and 26, respectively, in addition to the main component, glycine. No phenylthiohydantoin amino acids were found at position 11 in any of the strains. We suspect that this position may be occupied by a cysteine residue.

Except for these differences, the N-terminal amino acid sequences were similar. Homologies among the 34 to 35 residues at the N-terminal sequences were 74.3% (identity of 26 residues) among all three strains and 77.1% (27 residues), 82.4% (28 residues), and 88.6% (31 residues) between Gilliam and Karp, Gilliam and Kato, and Karp and Kato, respectively.

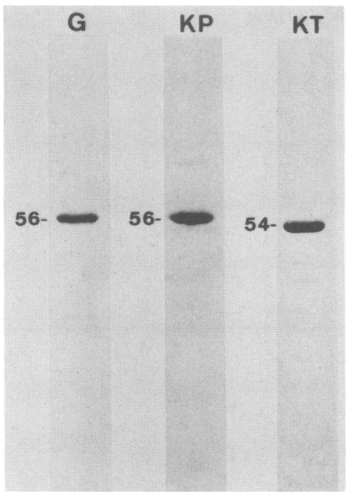

FIG. 6. SDS-PAGE profiles of polypeptides in the sharp-peak fractions shown in Fig. 5 at a retention time of 27 to 28 min. Gels were silver stained. G, Gilliam; KP, Karp; KT, Kato.

TABLE 1. Amino acid compositions of 54- to 56-kDa polypeptides from *R. tsutsugamushi* Gilliam, Karp, and Kato

Amino acid	Mol%[a]		
	Gilliam (56 kDa)	Karp (56 kDa)	Kato (54 kDa)
Aspartic acid	11.3	9.0	11.3
Threonine	4.9	3.6	4.1
Serine	5.5	7.7	7.2
Glutamic acid	14.2	13.4	11.8
Proline	4.4	7.0	6.8
Glycine	11.2	14.2	14.2
Alanine	10.5	8.9	9.2
Cysteine	tr	0.8	tr
Valine	6.3	5.2	5.2
Methionine	1.7	1.5	0.8
Isoleucine	5.3	4.9	5.7
Leucine	7.8	6.2	7.1
Tyrosine	1.8	1.2	tr
Phenylalanine	3.3	3.1	3.5
Lysine	6.5	6.6	7.3
Histidine	2.0	3.9	2.7
Arginine	3.3	2.8	3.1
Tryptophan	ND	ND	ND

[a] tr, Trace; ND, not determined.

DISCUSSION

The 54- to 56-kDa polypeptides in *R. tsutsugamushi* are located on the rickettsial surface and show strain-specific antigenicity (9, 12). This study was designed primarily to establish a procedure for purifying the polypeptides from three prototype strains (Gilliam, Karp, and Kato) and secondarily to compare the amino acid compositions and N-terminal amino acid sequences of the strains.

In the Sarkosyl treatment and chromatography steps of the purification procedure, polypeptides of the three prototype strains were distributed almost identically; also, the SDS-PAGE patterns of each fraction were similar, which indicated that the polypeptides of the three strains have similar chemical and physical properties. The samples for purification were not treated with nuclease, which indicated that the rickettsial nucleic acids do not interfere with this procedure. The purified 54- to 56-kDa polypeptides reacted with strain-specific monoclonal antibodies against these polypeptides in a dot blot immunoassay (data not shown), which suggested that the polypeptides retain antigenicity after the purification procedure.

Amino acid compositions of the 54- to 56-kDa polypeptides from the three prototype strains were similar to each other and also seemed to resemble those of the major outer membrane proteins of other gram-negative bacteria, including *Neisseria gonorrhoeae* (5), *Escherichia coli* (3), and *Chlamydia trachomatis* (2a); i.e., they contained large quantities of glycine, alanine, aspartic acid, and glutamic acid and lesser amounts of tyrosine, cysteine, and methionine.

Comparison of amino acid sequences at the N termini of the 54- to 56-kDa polypeptides of the three strains showed high levels of homology, although some unique differences were also observed. On the basis of the proposed triplet codons of amino acids, each amino acid conversion observed in these experiments seems to be possible by base substitution as follows: GAA or GAG to GAU or GAC for the Glu-to-Asp conversion at position 5; GGX to GCX (X indicates A, G, C, or U) for the Gly-to-Ala conversion at position 15; AUX to GUX for the Ile-to-Val conversion at position 19; GCX to GUX for the Ala-to-Val conversion at position 27; ACX to GCX for the Thr-to-Ala conversion at positions 30 and 35; and UCX to CCX for the Ser-to-Pro conversion at position 34. It is not known whether the N-terminal sequence relates to the strain-specific antigenicity of the polypeptide. The hydrophobic amino acid content of the polypeptide was in the range of 36.8 to 39.3% (hydrophobic amino acids used were proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine) and, in the 34 to 35 residues at the N-terminal sequence, was 28.5% in Gilliam, 44.1% in Karp, and 40.0% in Kato.

We reported recently the lack of peptidoglycan in *R. tsutsugamushi* (1). *C. trachomatis*, an intracellular parasite, is also well known to lack peptidoglycan (2), and several findings suggest that the chlamydial outer membrane may maintain structural integrity by the disulfide bonding of proteins (10). In contrast, the envelope of *R. tsutsugamushi* is very fragile and easily solubilized by treatment with SDS alone in the absence of a reducing agent. Furthermore, the 54- to 56-kDa polypeptide, which is a major outer membrane protein, contained a trace amount of cysteine. These results

	5	10	15	20	25	30	35
56K-Gilliam	Ile Glu Leu Gly **Glu** Glu Gly **Gly** Leu Glu	X	Gly Pro Tyr **Gly** Lys Val Gly **Ile** Val Gly Gly Met Ile Thr Gly **Ala** Glu Ser **Thr** Arg Leu Asp **Ser** **Thr**				
56K-Karp	Ile Glu Leu Gly **Glu** Glu Gly – Leu Glu	X	Gly Pro Tyr **Ala** Lys Val Gly **Val** Val Gly Gly Met Ile Thr Gly **Val** Glu Ser **Ala** Arg Leu Asp **Pro** **Ala**				
54K-Kato	Ile Glu Leu Gly **Asp** Glu Gly **Gly** Leu Glu (Lys)	X	Gly Pro Tyr **Ala** Lys Val Gly **Val** Val Gly Gly Met Ile Thr Gly **Val** Glu Ser **Thr** Arg Leu Asp **Pro** **Ala** (Ala)				

FIG. 7. N-terminal amino acid sequences of the 54- to 56-kDa polypeptides of strains Gilliam, Karp, and Kato. Substituted amino acids are shown in boldface. Amino acids in parentheses are minor components found by analysis of the polypeptide from strain Kato. X, Unidentified amino acid. Positions are numbered from the N termini. This sequence analysis was repeated twice with different purified batches of the polypeptides, and similar results were obtained.

indicate that the disulfide linkages may be weak, and this factor may help to explain the fragility and limpness of the rickettsial envelope, although dissociation of the oligomer of the 54- to 56-kDa polypeptide to the monomer was observed after 2-mercaptoethanol treatment of solubilized rickettsial samples (15).

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