JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1981, p. 333-341
0095-1137/81/090333-09$02.00/0

Vol. 14, No. 3

Isolation of Species-Specific Protein Antigens of *Rickettsia typhi* and *Rickettsia prowazekii* for Immunodiagnosis and Immunoprophylaxis

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Received 4 March 1981/Accepted 4 May 1981

A simple procedure for the selective isolation of the protective species-specific protein antigens (SPAs) of *Rickettsia typhi* and *Rickettsia prowazekii* was developed to permit use of the SPAs in the immunodiagnosis and immunoprophylaxis of typhus infections. Although the SPAs were readily extracted from lysozyme- or detergent-treated rickettsiae, as measured by rocket immunoelectrophoresis, other polypeptides were also present, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In contrast, both water and seven buffers, each at a 10 mM concentration and pH 7.6, were nearly equally effective in the selective release of the SPAs from whole cells by extraction for 30 min at 45°C. High-ionic-strength buffers and $MgCl_2$ abolished this SPA release, thus suggesting that divalent cations were important in the binding of the SPAs to the cell envelope. The efficacy of the dilute buffer extraction procedure for isolation of large amounts of SPAs was tested by further characterization of the supernatants obtained by centrifugation (200,000 × *g*) of two successive tris-(hydroxymethyl)aminomethane-hydrochloride buffer (Tris) extracts. With this procedure, between 10 and 15 mg of SPA was obtained from 100 mg of purified rickettsiae. Although low-molecular-weight ribonucleic acid fragments were released into the Tris extracts in significant amounts, only the SPAs were detected in significant quantities, as measured by polyacrylamide gel electrophoresis and rocket immunoelectrophoresis. The Tris extracts contained the same major and minor SPA polypeptides as those observed previously in SPA preparations obtained by extensive diethylaminoethyl-cellulose column chromatography, but the Tris SPAs were more satisfactory antigens in an enzyme-linked immunosorbent assay.

Although *Rickettsia typhi* and *Rickettsia prowazekii* have several common antigens (18), as might be expected from the 70% homology that exists between their genomes (12), these two species are readily distinguishable by several serological methods (reviewed in reference 8). In an earlier study, my co-workers and I demonstrated that species-specific antigens can be detected by rocket immunoelectrophoresis (RIE) of French pressure cell extracts of typhus group rickettsiae (4). These species-specific protein antigens (SPAs) were then further identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (SDS-PAGE) as the major band 1 protein described by Eisemann and Osterman (7). Other investigators have demonstrated that the protective immunogen(s) found in crude typhus vaccines is both species specific and labile to heating at 56 or 60°C for less than 45 min (2, 9, 10, 20). Because the SPAs have similar heat labilities, are species specific, and constitute 10 to 15% of the total rickettsial cellular protein (4), I believe that the SPAs are the protective immunogens found in the crude typhus vaccines. Furthermore, because the SPAs are associated with the rickettsial cell envelope (4, 13, 24), they may be exposed for direct interaction with components of the immune system of the host.

Silverman et al. (22) have recently summarized the considerable information that has accumulated regarding the ultrastructure of typhus group rickettsiae. A substantial mass of antigenic material appears to be exterior to the outer membrane and is easily removed or released from the rickettsiae. Much of this antigenic material is a group-reactive, electron-lucent slime layer (polysaccharide) capsule (21, 22). The protective vaccine antigen of Golinevitch et al. (9) and Golinevitch and Voronova (10)

was also readily released from the cell but contained a significant amount of protein. These Russian authors further suggested that the protective vaccine antigens were the regularly repeating globular structures demonstrated on rickettsial cells by them and others (14, 15) by electron microscopy of negatively stained preparations. These structures may be identical to the microcapsular layer observed in thin sections with the electron microscope, because both are associated intimately with the outer membrane of the cell envelope (16, 21). Indeed, Osterman and Eisemann (13) demonstrated that the SPA (band 1 protein) was present on the rickettsial surface because both it and band 4 protein could be preferentially iodinated by the chloramine T or lactoperoxidase methods. However, my coworkers and I (4) and Smith and Winkler (24) have observed that band 1 protein of *R. prowazekii* is distributed in both the membrane and supernatant fractions after French pressure cell disruption of the rickettsiae. This is also true for *R. typhi* (4). This ready solubilization of the SPAs would be unusual for tightly bound intrinsic membrane proteins. However, a large variety of gram-positive and gram-negative microorganisms have surface proteins which are exterior to the cell membranes and are readily extracted and purified by simple procedures (23, 26).

This paper shows that the SPAs of *R. typhi* and *R. prowazekii* can be selectively extracted from these rickettsiae without appreciable cellular lysis; consequently, the SPAs do appear to be surface proteins. In addition, conditions for the optimal selective release of the SPAs are defined. These SPA extracts were partially characterized both chemically and antigenically and were shown to be useful immunodiagnostic reagents in an enzyme-linked immunosorbent assay (ELISA). More extensive molecular characterization of the SPAs and examination of their usefulness in a variety of serological procedures are described in other papers (G. A. Dasch and S. Halle, manuscripts in preparation; G. A. Dasch and A. L. Bourgeois, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D8, p. 39). Finally, in other studies by G. A. Dasch and A. L. Bourgeois and by A. L. Bourgeois and G. A. Dasch (*in* W. Burgdorfer and R. L. Anacker, ed., *Rickettsiae and Rickettsial Diseases*, in press) and by A. L. Bourgeois et al. (A. L. Bourgeois et al., manuscripts in preparation) it has been shown that these SPA extracts are also highly immunogenic and immunoprophylactic in a guinea pig model for typhus infections.

MATERIALS AND METHODS

Materials. The origin, passage histories, and biological and biochemical characteristics of the rickettsial strains used in this investigation have been described elsewhere (3, 29). All rickettsiae were purified from frozen pools of infected yolk sacs of embryonated chicken eggs by two cycles of Renografin density gradient centrifugation in K36 buffer and filtration through a glass filter as described previously (5, 28). All buffers and enzymes were obtained from Calbiochem-Behring Corp., La Jolla, Calif.

General methods. SDS-PAGE was carried out with a neutral phosphate continuous buffer system with cylindrical 10% (wt/vol) acrylamide gels (6 by 75 mm) (27). RIE was done with hyperimmune serum from rabbits infected with the typhus rickettsiae, 1% Litex agarose (Accurate Chemical and Scientific Corp., Hicksville, N.Y.), and tris(hydroxymethyl)aminomethane (Tris)-Tricine buffer precisely as described previously (4). Malate dehydrogenase (EC 1.1.1.37) was assayed spectrophotometrically (17). The acid solubility of extracts containing nucleic acid material absorbing at 260 nm was determined by precipitating insoluble material with 0.5 N perchloric acid at 0°C for 10 min, centrifuging the extract at 3,000 rpm for 10 min, and washing the precipitate once in the perchloric acid; the optical density of the pooled perchloric acid extracts was then compared with that of the initial extract. The orcinol reactivity of the nucleic acid extracts was determined by the method of Schneider (19). The direct ELISA for antigen was the same as that used previously (4).

Preparative isolation of SPAs by Tris extraction. Well-washed (K36 buffer) Renografin density gradient-purified rickettsiae (100 to 200 mg of protein) were suspended in 10 ml of 10 mM Tris-hydrochloride (pH 7.6) buffer at 0°C. The suspension was then shaken at 200 rpm for 60 min in a water bath at 45°C. The extracted cells were pelleted from the suspension by centrifugation at 17,300 × *g* for 15 min, the cell pellet was resuspended in 10 ml of Tris, and the extraction was repeated. The twice-extracted cell pellet was sonicated in 10 ml of Tris as described previously (4). Intact cells were collected by centrifugation at 17,300 × *g*, and the cells were resuspended in 10 ml of buffer. After additional sonication, the remaining intact cells were removed by centrifugation at 7,700 × *g* for 5 min, and the two sonic extracts were combined. The consecutive Tris extracts 1 and 2 and the pooled sonic extracts were each separated by centrifugation into large membrane fragments (pellet centrifuged at 100,000 × *g* for 60 min), small membrane fragments (pellet centrifuged at 200,000 × *g* for 120 min), and the remaining 200,000 × *g* supernatant fluid. Each 200,000 × *g* supernatant fluid was sterilized by passage through a 0.22-μm-pore filter.

RESULTS

Selective extraction of the SPAs from typhus group rickettsiae. Outer membrane antigen complexes are often readily released from gram-negative microorganisms, including the trench fever rickettsia (11), by shaking heavy suspensions of cells in Tris-based buffers at 60°C. However, because the rickettsial SPAs are labile even at 56°C, small-scale analytical exper-

iments were initially conducted at 45°C. Because the typhus group rickettsiae were not readily lysed by osmotic shock, even after ethylenediaminetetraacetic acid (EDTA) or lysozyme treatment (not shown) or both, solutions of low ionic strength could be employed. Solubilization of the SPAs was monitored by RIE (Table 1), and the release of other proteins was detected by SDS-PAGE (Fig. 1). Surprisingly, SPA was released in significant amounts from *R. prowazekii* cells, whether pretreated with lysozyme or not, after most treatments (Table 1). The specificity of the RIE assay for SPA was also confirmed, because, as was expected (4), SDS interfered with RIE by denaturing the SPA, whereas pronase (and trypsin to a lesser extent) apparently degraded any SPA that was released. The lack of SPA release with the $MgCl_2$ extractant suggested that divalent cations are important in binding the SPA to the rickettsiae. Indeed, damage to the cell envelope by pretreatment of the rickettsiae with lysozyme or inclusion of Triton X-100 in the extractant prevented the $MgCl_2$ suppression of SPA release (Table 1). Furthermore, EDTA treatment of the ricksettiae had little effect on SPA extraction as compared with the Tris control, possibly because the Renografin used to purify the rickettsiae already contained both EDTA and sodium citrate; divalent cations essential for tight binding of SPA to the cell were probably already removed before the extraction procedure.

TABLE 1. *Release of SPAs from R. prowazekii after various treatments of purified whole cells*

Treatment[a]	Rocket peak height (mm) of:			
	Supernatant fluid (0.5 μl)[b]		Pellet (5 μl)[c]	
	No lysozyme treatment	Lysozyme pretreatment	No lysozyme treatment	Lysozyme pretreatment
Control	19	17	>45	34
5 mM EDTA	15	17	>45	>45
5 mM $MgCl_2$	0	14	>45	>45
1% Triton X-100	24	27	18	15
1% Triton X-100–5 mM EDTA	26	28	17	12
1% Triton X-100–5 mM $MgCl_2$	17	28	>45	19
1% DOC	21		33	
1% SDS	0		0	
100 μg of trypsin per ml	16		40	
100 μg of pronase per ml	0		35	

[a] Two hundred microliters of Renografin density gradient-purified cells (1 mg of protein per ml in 10 mM Tris-hydrochloride buffer [pH 7.6]) was held at 0°C or pretreated with 50 μg of lysozyme per ml at 37°C for 60 min and then added to 200 μl of various solutions in 10 mM Tris to give the final concentrations indicated. The cells were then shaken at 45°C for 1 h at 200 rpm. DOC, Deoxycholate.

[b] Supernatant fluid was obtained after centrifugation of each fraction (400 μl) for 10 min in an Eppendorf microfuge at 12,800 × *g*.

[c] The extracted cell pellet was resuspended in 200 μl of 2% Triton X-100–10 mM EDTA (pH 7.6) for the assay.

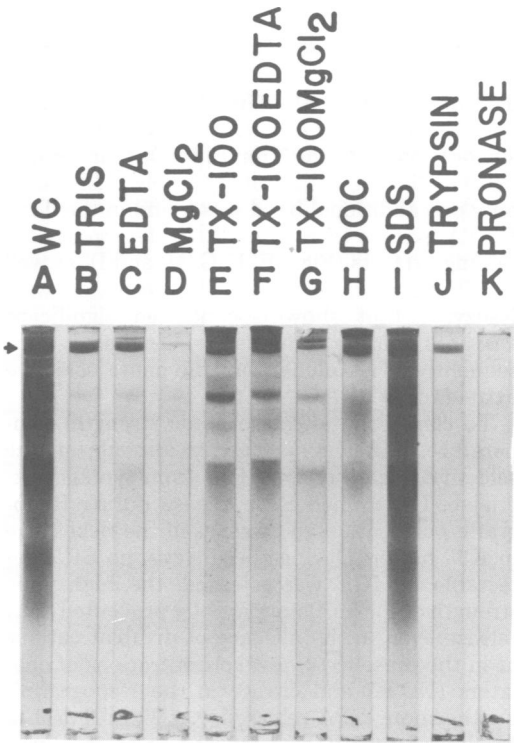

FIG. 1. *Analysis by continuous SDS-PAGE of supernatant fractions obtained by extraction of R. prowazekii Breinl strain. (A) Protein (50 μg) of Renografin-purified whole cells (WC). (B–K) Supernatants (100 μl) obtained from 50 μg of protein from purified cells (no lysozyme pretreatment) extracted with the indicated solutions in 10 mM Tris buffer as described in Table 1, footnote a. Band 1 (SPA) is indicated with the arrow. TX-100, Triton X-100; DOC, deoxycholate.*

With the exception of the SDS treatment, which lysed the cells completely, residual SPA activity was found in all extracted cell pellets. Cell pellets treated with extractants containing Triton X-100 had the least SPA activity. However, much of this Triton X-100-dependent extraction was due to cell lysis, particularly in the absence of $MgCl_2$ or after lysozyme pretreatment (Table 1).

The soluble extracts obtained (Table 1) varied considerably in their protein complexity as determined by SDS-PAGE (Fig. 1). As expected from previous identification of the SPA protein as band 1 (4), the band 1 staining intensity of

each extract paralleled the SPA activity of the extract detected by RIE (Table 1). Surprisingly, only band 1 was released in significant levels by Tris alone (Fig. 1B). Band 1 protein recovery was diminished by the addition of $MgCl_2$, trypsin, or pronase to the Tris (Fig. 1D, J, and K), as expected based on the data in Table 1. Although only one additional band was found in the Tris-EDTA extract (Fig. 1C), all the detergent-containing extracts (Fig. 1E, F, G, H, and I), as well as all the extracts from cells pretreated with lysozyme (not shown), contained significant amounts of other proteins besides band 1. Consequently, Tris alone was the most selective extractant of SPA.

To clarify the effects of ionic strength, temperature, buffer, and other treatments on the selective release of the SPAs from typhus rickettsiae, I examined SPA release during Renografin purification and washes of the rickettsiae in K36 buffer. During these steps, no SPA detectable by RIE was released; the high ionic strength of the buffer apparently prevented SPA release even in the absence of divalent cations or in the presence of low concentrations of chelators (EDTA and citrate in the Renografin). Furthermore, SPA release in 0.2 M LiCl or 0.15 M NaCl–5 mM EDTA for 60 min at 45°C was significantly reduced as compared with that in 10 mM Tris-treated cells; however, in every case, only band 1 protein was detected in the extracts by SDS-PAGE (not shown). The reduction in SPA release in the presence of $MgCl_2$ or high-ionic-strength buffers was not due to insolubility of the SPAs because Tris-released SPAs were soluble in these buffers.

For determining whether Tris buffer had a specific role in the extraction of SPA, the cells were suspended in water alone or in a series of buffers, all at 10 mM and pH 7.6 (Table 2). Differential effects were observed upon initial suspension of the rickettsial cells in the buffers at 0°C, but levels of SPA released after 30 min of extraction at 45°C were all very similar, except when bis-Tris propane was used. Similar buffer effects were seen upon a second resuspension and extraction of the cells. Because recoveries in bis-Tris propane of band 1 protein as detected by SDS-PAGE were also low, it is unlikely that this buffer had a direct effect on the antigenicity of the SPA as measured by RIE, which would account for the low activity observed. Most importantly, however, only band 1 protein was released in significant amounts with all of these buffers, as measured by SDS-PAGE (not shown). Lysis of cells was minimal, and the twice-extracted cell pellets did not contain appreciable amounts of SPA as detected by either RIE or SDS-PAGE (not shown).

TABLE 2. *Extraction of SPAs of R. typhi with different buffers*[a]

Buffer (10 mM, pH 7.6)	Rocket peak height (mm) at indicated times and temp of:			
	First extract (0.4 μl)		Second extract (4 μl)	
	0 min, 0°C	30 min, 45°C	0 min, 0°C	30 min, 45°C
H_2O	23	29	>50	48
Tris	14	26	18	48
Bicine	8	22	10	46
Tricine	15	29	8	44
TES	12	29	10	>50
Glycylglycine	17	27	15	>50
Bis-Tris	22	32	38	>50
Bis-Tris propane	4	16	1	32

[a] Samples (2.1 mg each) of Renografin-purified *R. typhi* cells were suspended in 700 μl of each of the buffers at 0°C, shaken at 45°C for 30 min, and centrifuged at 17,000 × *g* for 10 min to pellet the cells. A portion (100 μl) of the initial cell suspension was also centrifuged immediately at 12,800 × *g* for 10 min to give the 0 time extraction. The second extraction of the cell pellet in 600 μl of buffer was identical to the first. Rocket assays for antigen activity were conducted on the supernatant fractions. None of the final cell pellets retained significant rocket activity when suspended in 500 μl of 1% Triton X-100 (4 μl was assayed). TES, 2-{[tris-(hydroxymethyl)methyl]-amino} ethanesulfonic acid.

In other experiments (not shown), no differences in the release of SPA were detected between *R. typhi* and *R. prowazekii*. SPA recoveries after Tris extraction were nearly identical at 45 or 50°C but were significantly lower at 0°C for 60 min. Maximum SPA recovery in both the first and second Tris extractions was achieved after 20 to 30 min at 45°C. Extending the extraction to 60 min resulted in a slight decline in SPA recovery.

Characterization of fractions of typhus group rickettsiae obtained by a preparative selective Tris extraction procedure and by sonication. The results of the small analytical experiments summarized above suggested that the SPAs could be easily recovered in excellent yield by simple extraction in Tris buffer. However, when the procedure was applied to large amounts of the rickettsiae, it was found that small amounts of cell envelope material, in addition to the SPAs, were released during the Tris extractions. Consequently, the Tris-released supernatant and membrane materials were further compared with membrane and supernatant fractions obtained after sonication of the twice Tris-extracted rickettsial cell pellet.

The SDS-PAGE polypeptide compositions of *R. typhi* fractions are shown in Fig. 2, and the RIE analysis of *R. prowazekii* fractions is shown in Fig. 3. In all preparations, identically prepared fractions of each species were very similar in both assays (not shown). Both 200,000 × *g* Tris extract supernatant fractions consisted primarily of band 1 polypeptide, but small amounts of the minor polypeptides previously shown to have SPA activity by crossed immunoelectrophoresis and ELISA (4) were also present (Fig. 2A and B). These minor bands were not detected earlier by SDS-PAGE (Fig. 1) because the amounts of protein applied to the gel were considerably lower. The association of SPA with the outer membrane was indicated by the SDS-PAGE analysis of the membrane fragments released by the Tris extractions; polypeptides 3, 4, and 5 (and 6 in trace amounts), previously identified as outer membrane proteins in *R. prowazekii* (13, 24), and SPA polypeptide 1 were all present

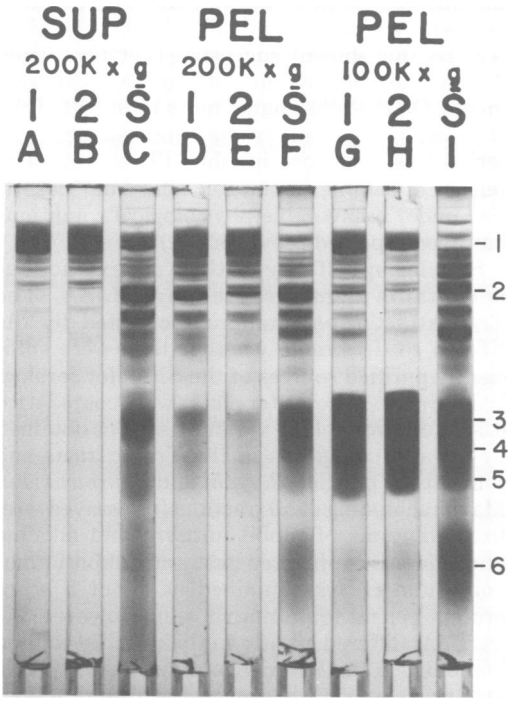

FIG. 2. *Analysis by SDS-PAGE of fractions of R. typhi Wilmington strain obtained by preparative Tris extraction and sonication. Consecutive Tris extracts 1 and 2 and the sonicate (S) of the Tris-extracted cells were each separated into pellets (PEL) by centrifugation at 100,000 × g (100K × g) for 60 min and 200,000 × g (200K × g) for 120 min and the remaining 200,000 × g (200K × g) supernatants (SUP). Protein (50 μg) was applied to each gel (anode below). Major proteins are numbered at right by the method of Eisemann and Osterman (7).*

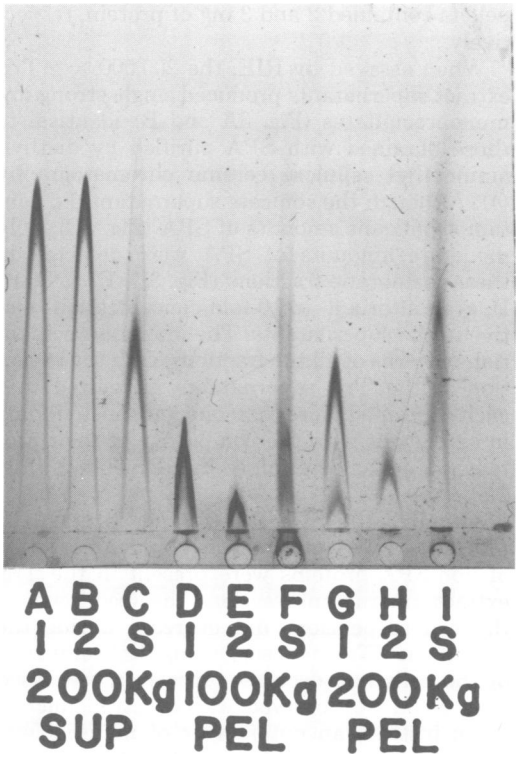

FIG. 3. *Analysis by RIE of fractions of R. prowazekii Breinl strain obtained by preparative Tris extraction and sonication. Abbreviations are as described in the legend to Fig. 2. Rickettsiae (100 mg) were extracted as described in the text. The Tris extract membrane pellets were suspended in 1 ml of Tris buffer, and the sonicate pellets were suspended in 5 ml (100,000 × g) and 3 ml (200,000 × g) of buffer. A 0.5-μl portion of each fraction was then assayed for antigen activity.*

(Fig. 2G and H). Interestingly, polypeptide 2 was apparently also associated with band 1 in the very small membrane fragments obtained at 200,000 × *g* (Fig. 2D and E). In addition, polypeptide 2 was present in large amounts in the sonicate 200,000 × *g* supernatant (Fig. 2C), suggesting that it could be readily solubilized like the SPAs. It was also found in the small membrane fragments obtained by sonication (Fig. 2F). Whether polypeptide 2 is important in the binding of SPA to the outer membrane is under investigation. Thus, the SDS-PAGE analysis (Fig. 2) suggested that only the 200,000 × *g* supernatants of the two Tris extracts were nearly free of unwanted polypeptides. In the two combined Tris extracts obtained from 100 mg of protein from purified rickettsiae, the 200,000 × *g* supernatant contained 10 to 15 mg of protein (SPA), and the 200,000 × *g* and 100,000 × *g*

pellets contained 2 and 3 mg of protein, respectively.

When assayed by RIE, the 200,000 × *g* Tris extract supernatants produced single strong immunoprecipitates (Fig. 3A and B) identical to those obtained with SPA purified by diethylaminoethyl cellulose column chromatography (4). Although the sonicate supernatant did contain significant amounts of SPA (Fig. 3C), only moderate amounts of SPA were detected in these membrane fractions (Fig. 3D, E, F, G, H, I), even after a 3- to 10-fold concentration relative to the supernatants. The immunoprecipitation patterns of all the fractions, with the exception of the Tris supernatants, suggested that each contained more than one antigen, a finding in agreement with the complexity of their protein profiles as determined by SDS-PAGE (Fig. 2).

Although the SDS-PAGE (Fig. 2) and RIE (Fig. 3) analyses indicated that only minor levels of non-SPA proteins were present in the Tris extract supernatant fractions, the absorbance of the cell suspensions did decrease during the extractions (Table 3), suggesting that some lysis of the cells had occurred. However, few lysed cells could be observed with electron microscopy; instead, an outer layer of material was clearly removed from the cell surfaces of the extracted cells (B. Merrell and G. A. Dasch, manuscript in preparation). The decreased absorbance of the cell suspension might have been due to a change in the optical properties of the cells. Consequently, malate dehydrogenase, a cytoplasmic marker enzyme, was measured in the soluble extracts as an alternative parameter of cell lysis (Table 3). Because only 88% of the rickettsiae were broken by sonication (based on turbidity; Table 3) and because some malate dehydrogenase may have been trapped in membrane fractions (24), the recovery of 15 to 20% of the total soluble malate dehydrogenase activity in the Tris extract supernatants constitutes a maximal measure of cell lysis during these extractions. The estimates of cell lysis based on absorbance and malate dehydrogenase activity were in fact quite comparable. However, a more striking measure of released cellular material was indicated by the disproportionately large (77 to 90%) amount of nucleic acid absorbance of the combined Tris extracts as compared with that of the sonic extracts (Table 3). Nearly all of the absorbance at 260 nm of the Tris extracts, however, was acid soluble (Table 3) and orcinol reactive (not shown), suggesting that it consisted of low-molecular-weight ribonucleic acid fragments. On the other hand, more than half of the retained sonicate supernatant nucleic acid material was acid precipitable (Table 3). The release of soluble ribonucleic acid from rickettsiae under unfavorable physiological conditions has been reported previously (1).

TABLE 3. *Characterization of the 200,000 × g supernatant fluids of the Tris extracts and of the sonicates of R. typhi and R. prowazekii*

Supernatant fraction	% Reduction in absorbance[a]	% Total malate dehydrogenase activity[b]	% Total absorbance at 260 nm[b]	% Acid-soluble absorbance at 260 nm in extract
R. typhi				
Tris extract 1	12, 12[c]	15, 6	51, 51	98
Tris extract 2	16, 7	6, 3	33, 26	94
Sonicate	88	79, 91	16, 23	47
R. prowazekii				
Tris extract 1	ND	16	85, 65	97
Tris extract 2	ND	3	5, 20	95
Sonicate	ND	81	10, 15	33

[a] Percent reduction in optical density at 420 nm of rickettsial suspension during extraction or sonication = 100 × (optical density of initial suspension − optical density of final suspension)/optical density of initial suspension. ND, Not done.

[b] Sum of activities or absorbances in Tris extracts 1 and 2 and sonicate = 100%.

[c] Two numbers represent results from two independent experiments.

Species specificity of the Tris extract supernatants demonstrated by ELISA. The Tris extract supernatants were tested by the ELISA to determine whether they were sufficiently purified sources of the SPAs for serological purposes. Microtiter plates were coated with 0.5-log dilutions of the various fractions obtained by ultracentrifugation of the Tris extracts and sonicates of purified *R. typhi* and *R. prowazekii*. Maximal binding of all fractions, as assayed with fixed dilutions of rabbit antisera and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, was obtained at about 5 μg of protein per ml (data not shown). However, the fractions differed greatly in their ability to bind homologous and heterologous antiserum (Table 4). As was previously observed with unfractionated total cell extracts (4), no species specificity was obtained with the three sonicate fractions or with the 100,000 × *g* pellets of the Tris extracts. Consequently, these fractions must consist primarily of typhus group antigens. In contrast, the 200,000 × *g* supernatants of the Tris extracts had the same degrees of species specificity observed previously with diethylaminoethyl cellulose column-purified SPAs (4). However, the Tris extract supernatants bound

TABLE 4. *Specificity of rabbit antiserum against R. typhi or R. prowazekii toward fractions of typhus group rickettsiae as determined by ELISA (4)*[a]

Antigen fraction	Optical density at 400 nm per 60 min[b]			
	R. typhi antibody		*R. prowazekii* antibody	
	RtAg	RpAg	RtAg	RpAg
Tris extract 1				
200,000 × *g* supernatant	1.14	0.45	0.16	0.80
200,000 × *g* pellet	0.77	0.40	0.32	0.70
100,000 × *g* pellet	1.04	0.85	0.73	0.80
Tris extract 2				
200,000 × *g* supernatant	1.29	0.49	0.20	0.87
200,000 × *g* pellet	0.76	0.42	0.39	0.70
100,000 × *g* pellet	1.08	0.92	0.84	0.85
Sonicate				
200,000 × *g* supernatant	1.58	1.30	1.24	1.07
200,000 × *g* pellet	1.33	1.27	1.18	1.17
100,000 × *g* pellet	1.20	1.15	1.21	1.18

[a] Plates were coated with 5 μg of antigen protein per ml, and the antigen was detected with rabbit anti-*R. typhi* antiserum no. 5 or anti-*R. prowazekii* antiserum no. 10 at a 1:1,000 dilution. Goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate was used at a 1:5,000 dilution.

[b] Controls with no antigen or with normal rabbit serum at a 1:1,000 dilution had similar low optical density readings, which were subtracted. RtAg, *R. typhi* antigen fraction; RpAg, *R. prowazekii* antigen fraction.

much more satisfactorily to the microtiter plates than did the diethylaminoethyl-purified SPAs (data not shown; cf. Fig. 8 of reference 4). The 200,000 × *g* membrane fractions of the Tris extracts were intermediate in species specificity, thus reflecting the admixture of SPAs and cell envelope antigens common to *R. typhi* and *R. prowazekii*, as had also been suggested by the SDS-PAGE (Fig. 2) and RIE (Fig. 3) results.

DISCUSSION

Purification of the SPAs of *R. typhi* and *R. prowazekii* by the selective Tris extraction procedure described here has several distinct advantages over the purification of SPAs by diethylaminoethyl cellulose chromatography from the soluble fraction of rickettsiae disrupted by the French pressure cell (4). First, recoveries by the latter procedure were low; SPA found in the membrane fraction after French pressure cell extraction was discarded, and SPA losses were considerable during the column separation and concentration steps. The present procedure is nearly quantitative for SPA recovery. Second, the Tris extraction is very rapid when reduced to the essentials: two 20-min Tris extractions, two 15-min centrifugations to remove the extracted cells, and a 2-h centrifugation at 200,000 × *g* to remove membrane fragments. Considerably more time is required just to assay fractions of one column separation for SPA activity. Third, the low ionic strength and heat used in the Tris extraction inactivate the rickettsiae, possibly by the activation of an endogenous ribonuclease which degrades the rickettsial ribonucleic acid to low-molecular-weight fragments. This inactivation thus obviates both the potential hazard posed by disruption of viable rickettsiae and the undesirable complications posed by the requirements of chemical or physical inactivation methods. Routinely, the two 200,000 × *g* Tris extract supernatants are pooled, filtered (0.22-μm-pore filter), and stored at 4°C. In 12 months no changes were observed in the physical, immunological, or chemical properties of the SPAs in such extracts. Finally, the ELISA plate binding properties of the Tris-extracted SPAs were superior to those obtained in earlier studies by diethylaminoethyl cellulose column chromatography (4). It is not known whether the difference in SPA binding is due to non-SPA materials also present in the Tris extracts, to a difference in the native properties of such SPAs (i.e., no proteolytic degradation), or to some other factor.

Many investigators have studied the release of cell wall-associated or surface proteins from bacteria (23, 26). However, although treatments used for the release of those proteins have often been relatively mild in contrast to the harsh treatments required for intrinsic membrane proteins, the simplicity of the buffer release reported here for the SPAs of typhus rickettsiae (Tables 1 and 2) was surprising. The best clue to the mechanism of SPA binding to the cell was the apparent suppression of its release by $MgCl_2$ and by the high ionic strength of the K36 (potassium phosphate-based) buffer used in the purification of the rickettsiae. Renografin density gradient centrifugation apparently effects an efficient ion exchange of cell wall divalent cations that are essential for SPA binding to the outer cell membrane. This cation exchange does not significantly diminish the viability of the typhus rickettsiae; in fact, damaged rickettsiae may be readily distinguished from viable rickettsiae by their greater density in Renografin density gradients (J. C. Williams and G. A. Dasch, unpublished observations). Furthermore, protective effects of Renografin on rickettsial viability have been observed for both the very labile scrub

typhus rickettsiae (6) and the typhus group rickettsiae (A. L. Bourgeois and G. A. Dasch, Abst. Annu. Meet. Am. Soc. Microbiol. 1980, D7, p. 39). Attempts to rebind the Tris-extracted SPAs to extracted cells either with added divalent cations or with elevations in buffer ionic strength have not been successful. It is not known whether this failure is due to alterations in the SPAs upon their release from the rickettsial cells, to alterations in the SPA surface attachment sites, or just to the failure to find the requisite conditions for the reassociation.

In intact *R. prowazekii* cells, the band 4 protein with an apparent molecular weight of 29,000 is the most readily iodinated (13, 25). However, because the SPAs can be released almost quantitatively without lysis of the cells by the Tris extraction, the SPA (band 1 protein, apparent molecular weight of 80,000 to 120,000) would appear to be the most superficial protein topologically—even though it was only the second most readily iodinated (13, 25). These observations may not be contradictory, because the iodination of band 1 protein may be relatively ineffective for a variety of reasons. First, band 1 protein incorporated relatively low amounts of labeled tyrosine relative to the other outer membrane proteins 3, 4, and 5 (13) and therefore may have been relatively deficient in tyrosine residues. Second, the centrifugation procedures may also have inadvertently removed iodinated SPA (13, 25). Third, the tyrosine residues of band 1 protein may be inaccessible or nonreactive during iodination (13, 25). Indeed, even in purified cell envelopes, band 1 was poorly iodinated, although band 3, 5, and 6 could be iodinated more readily than in the intact cell (25). Band 1 protein was more readily iodinated only when the ionic strength was greatly increased during the iodination procedure, a condition under which selective protein labeling was lost (13). The presence of four bands in the small amounts of highly purified outer membrane released during the Tris extractions (Fig. 2 G and H, bands 1, 3, 4, and 5) does attest to their intimate association in the intact cell. However, because bands 3, 4, 5, and 6 were only found in the membrane fractions, except possibly in very small amounts in the supernatant of the extensively sonicated cells (Fig. 2C), they may be classified as intrinsic membrane proteins. In contrast, the observation that band 1 protein is easily solubilized without appreciable cell lysis does support the view that it is more superficial on the rickettsial cell. Final conclusions as to the topological relationships of these major proteins, as well as of the major band 2 protein, will require more detailed correlations of biochemical studies with ultrastructural studies.

In conclusion, the SPAs of both *R. typhi* and *R. prowazekii* can be obtained in highly purified states and excellent yield by the simple selective Tris extraction procedure described here. The low degree of contamination of the Tris-extracted SPAs with extraneous proteins makes them most useful materials for the further molecular characterization of the SPAs and for the complete purification of the SPAs. Indeed, in other experiments by G. A. Dasch and A. L. Bourgeois (*in* W. Burgdorfer and R. L. Anacker, ed, *Rickettsiae and Rickettsial Diseases*, in press), the low contamination of the Tris-extracted SPAs with cell envelope proteins shown here was further confirmed by their relative freedom from endotoxin originating from the cell envelope; furthermore, although the SPA extracts were devoid of the nonspecific B-lymphocyte mitogenic activity found in other rickettsial fractions containing endotoxin, the SPAs elicited highly *R. typhi*- or *R. prowazekii*-specific transformation responses with lymphocytes from both guinea pigs and humans infected with these agents. In addition to this demonstration of their utility as specific humoral and cellular immunodiagnostic antigens for typhus infections, the purity and nontoxicity of the Tris extracts have permitted the direct demonstration that the SPAs are immunoprophylactic in guinea pigs against both local and systemic dissemination of the rickettsiae and that they elicit excellent titers of toxin-neutralizing antibodies, as reported by A. L. Bourgeois and G. A. Dasch (*in* W. Burgdorfer and R. L. Anacker, ed., *Rickettsiae and Rickettsial Diseases*, in press) and by A. L. Bourgeois et al. (A. L. Bourgeois et al., manuscripts in preparation).

ACKNOWLEDGMENTS

I gratefully acknowledge the devoted and expert technical assistance of Jonathan R. Samms and Walter G. Sewell in this investigation. I also thank Emilio Weiss for his constant encouragement and assistance with the manuscript.

This investigation was supported by the Research and Development Command, Department of the Navy, Research Work units MR0410501.0030 and M0095PN002.5053.

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