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Characterization of the Madrid E Strain of *Rickettsia prowazekii* Purified by Renografin Density Gradient Centrifugation

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The avirulent Madrid E strain of *Rickettsia prowazekii* cultivated in chicken yolk sacs could be purified successfully with a Renografin density gradient method developed previously for *Rickettsia typhi*. Recovery during purification, viability, and lack of contamination with host cell components were similar for the two species, although yields of *R. prowazekii* per yolk sac were lower. Purified typhus rickettsiae provided satisfactory antigens in the complement fixation, Ouchterlony double-diffusion, and microagglutination tests. The retention of the typhus soluble group antigen during purification was readily demonstrated by complement fixation tests. However, removal of the soluble group antigen by ether treatment was not always adequate for the demonstration of type-specific particulate antigens. Heat-killed *R. prowazekii* cells gave higher serum microagglutination titers than untreated or formalized cells, a difference not noted for *R. typhi* cells. Although the protein profiles of whole cells and extracts of *R. typhi* and *R. prowazekii* on sodium dodecyl sulfate-polyacrylamide gels were relatively similar, a small but reproducible, difference in the electrophoretic mobilities of their malate dehydrogenases was detected. Purification of typhus rickettsiae on Renografin gradients has no apparent adverse effects on their metabolic or antigenic properties.

Isopycnic centrifugation in Renografin density gradients has proven to be an excellent final step in the purification of several obligate intracellular bacteria, including *Chlamydia psittaci* from L cells (10), *C. trachomatis* from yolk sacs (14) and HeLa cells (15), and *Rickettsia typhi* from both yolk sacs and mouse L cells (29). The microorganisms are recovered in high yield, contain negligible levels of host cell antigens, and exhibit excellent infectivity and metabolic activity. Cell-free extracts obtained from Renografin-purified *R. typhi* contained enzymes of the tricarboxylic acid cycle and several nucleotide kinases, but were devoid of enzymes of glycolysis and of the pyrimidine nucleotide salvage pathway (6, 31). Since some rickettsial enzymes have physicochemical properties that are quite distinct from those of the homologous enzymes of their host cells, the complete removal of these host cell enzymes from the Renografin-purified *R. typhi* was also readily demonstrated (6, 29, 31).

The extension of the Renografin density gradient centrifugation procedure to the purification of the Madrid E strain of *R. prowazekii* from yolk sacs is reported here. Rickettsial recovery, viability, and contamination with host cell components were evaluated. Some of the antigens of Renografin-purified *R. prowazekii* and *R. typhi* were examined for their suitability as serological diagnostic reagents. Finally, several protein fractions and the malate dehydrogenases of purified *R. typhi* and *R. prowazekii* were compared by polyacrylamide gel electrophoresis to determine whether these parameters might be useful in differentiating strains of typhus group rickettsiae.

MATERIALS AND METHODS

Preparation and biological assay of R. prowazekii from yolk sacs. The erythromycin-resistant Madrid E strain of *R. prowazekii* (30) was grown in the yolk sacs of embryonated chicken eggs and purified as described for *R. typhi* (29), except as noted in the text. Glutamate catabolism and hemolytic activity were also determined as described (29).

Preparation of soluble and particulate antigen fractions. Antigens were prepared by a modified ether extraction procedure (3, 25). Renografin-purified *R. typhi* or *R. prowazekii* were suspended in 0.04 M KPO_4 buffer, pH 7.0, at 0.5 to 2.0 mg of rickettsial protein per ml, mixed with an equal volume of 0.2% formalin in 0.85% NaCl, and incubated for at least 3 days at 25°C to inactivate the rickettsiae. The soluble antigen was then extracted by shaking the for-

malized cell suspension with 1.5 volumes of diethyl ether for 10 min at room temperature in a separatory funnel. The phases separated rapidly, with very little material at the interface. The turbid aqueous phase was then collected and centrifuged at 10,000 rpm for 15 min in a Spinco type 40 fixed-angle rotor to pellet the rickettsiae. Ether was then evaporated from the clear aqueous supernatant, which contained the soluble antigen, by passing filtered air through the solution. The particulate antigen was obtained by resuspending the rickettsial pellet in the formalized saline phosphate buffer, extracting it again with ether to remove residual soluble antigen, and then washing the rickettsial pellet again three times by centrifugation in the same buffer. It was then reconstituted to the initial volume of formalized cells. The antigens were stored at 4°C without loss of antigenicity.

Preparation of cell-free extracts and cell envelope fractions. Cell-free extracts were obtained by passing suspensions of Renografin-purified rickettsiae (0.4 to 2.0 mg of protein per ml) in 0.04 M KPO_4 buffer, pH 7.0, through a French pressure cell at 20,000 lb/in^2 twice, and centrifuging the crude extract at 6,000 rpm for 5 min in a Sorvall SS-34 fixed-angle rotor to remove intact cells. Extracts free of cell envelopes were then obtained by centrifuging the cell-free extracts again at 30,000 rpm for 3 h in a Spinco type 40 rotor. The pelleted cell envelopes were washed twice further by centrifugation in the same buffer.

Rickettsial extracts and cell envelopes were also prepared by digesting suspensions in lysozyme (100 to 200 μg/ml) for 15 min at 34°C, adding ribonuclease, deoxyribonuclease, and $MgCl_2$ to final concentrations of 100 μg/ml and 30 mM, respectively, followed by lysis with Triton X-100 at a final concentration of 2% until no further clarification was observed. Cell-free extracts and cell envelope fractions were then prepared by centrifugation as described above.

These fractions were used immediately or were frozen in aliquots at −70°C.

Preparation of antisera. Approximately 100 μg of protein of the Renografin-purified viable rickettsiae were inoculated intraperitoneally (*R. typhi*) or subcutaneously (*R. prowazekii*) into 3- to 4-kg white New Zealand rabbits. The rabbits were given a similar sample of rickettsiae after 4 to 6 weeks and bled 10 to 13 days later. Preparation of hyperimmune anti-yolk sac antiserum was described previously (29).

Polyacrylamide gels. Malate dehydrogenases were separated on Davis gels as described previously (6), except that the extracts were not dialyzed against the running gel buffer. Samples (50 to 100 μg of protein) for sodium dodecyl sulfate (SDS)-gel electrophoresis were solubilized in Laemmli (16) sample buffer by boiling for 3 min and were run on 10% neutral SDS gels (28).

Miscellaneous methods. Complement fixation tests were conducted on rickettsial fractions inactivated at 56°C for 30 min, unless they had been treated previously with formalin. Titers were determined by standard microtiter techniques (21, 27).

Ouchterlony double-diffusion tests were done with microtemplates after solubilizing the antigen with 0.5% SDS for 1 h at 37°C (8).

Rickettsial microagglutination titers were determined as described by Fiset et al. (9). Results obtained before or after staining with acridine orange were generally identical.

RESULTS

Purification of biologically active rickettsial preparations. The Madrid E strain of *R. prowazekii* did not differ significantly from *R. typhi* in its behavior during purification from infected yolk sacs (Table 1). Both species of rickettsiae banded at about the same density (1.20 g/cm^3) in the Renografin density gradients. Although total recovery of the rickettsiae, as measured by their capacity to catabolize glutamate or to hemolyze sheep erythrocytes, was nearly equally efficient for *R. typhi* and *R. prowazekii*, differences in initial harvests resulted in yields of fewer *R. prowazekii* cells (0.025 to 0.40 mg of protein per yolk sac) per yolk sac than of *R. typhi* (3 to 4 mg of protein per yolk sac) (29). To recover satisfactory amounts of *R. prowazekii* from yolk sacs, the equivalent of three to five egg harvests were generally used per gradient rather than the one to two used when infected with *R. typhi*. Fractionation of the more heavily laden gradients was facilitated by using an initial 20 to 40% linear Renografin gradient, which separated the rickettsial band from the very heavy band of yolk sac material at the top of the gradient more satisfactorily than the denser 30 to 45% gradient used previously with *R. typhi* (29). However, despite the large number of yolk sacs used, no host cell material was apparent in the final preparations of *R. prowazekii* when stained with the Gimenez method (12). Furthermore, host cell antigens, if detected at all, were present only at the highest concentration of purified rickettsial suspensions (1/5 of 0.1 to 1.0 mg of protein per ml) tested by the complement fixation method. On the other hand, rickettsial antigen was retained almost as well as hemolytic or metabolic activity.

Antigen characteristics of Renografin-purified typhus group rickettsiae. Rabbits readily developed antibodies against typhus rickettsiae when injected with viable Renografin-purified *R. typhi* and *R. prowazekii* (Table 2), as was anticipated from the infectivity for yolk sacs and hemolytic and metabolic activity of the preparations (Table 1). Species differences in the serum titers of these rabbits can be attributed either to the different routes of inoculation or to the lesser virulence of the Madrid E strain of *R. prowazekii*. Although the content of com-

TABLE 1. *Purification of typhus rickettsiae*

Rickettsia	Purification step	% Recovery				
		Normal yolk sac antigen (CF units)[a]	Hemolytic activity	Glutamate metabolism (CO_2 formed)	Egg infectivity	Rickettsial antigen (CF units)[a]
Rickettsia typhi[b]	1 (After high- and low-speed centrifugation)	(100)	(100)	(100)	(100)	
	2 (After BPA)[c]	3.2	96	37	78	
	3 (After gradient 1)					
	4 (After gradient 2)	<0.13	42	52	33	
R. prowazekii E strain[d]	1	(100)	(100)	(100)		(100)
	2	17	104	93		63
	3	0.045	43	42		36
	4	<0.014	39	46		22

[a] CF, Complement fixation.
[b] Calculated from data of Weiss et al. (29).
[c] BPA, Bovine plasma albumin.
[d] Mean of three experiments.

TABLE 2. *Complement fixation titers[a] of antisera against R. typhi and R. prowazekii using Renografin-purified rickettsial antigens*

Antiserum	Antigen				
	R. typhi		*R. prowazekii*		None (anticomplementary activity)
	Formalized whole cells[b]	Soluble Antigen[c]	Formalized whole cells[b]	Soluble antigen[c]	
Rickettsia typhi					
5	640	640	320	640	0
6	640	640	320	320	0
7	640	640	640	320	0
8	320	640	320	320	0
R. prowazekii					
9	40	80	160	160	40
10	320	320	320	320	20
11	160	160	160	160	20
12	160	160	160	160	20

[a] Expressed as reciprocal of serum dilution, mean of duplicate determinations.
[b] Twenty-five micrograms of protein per milliliter.
[c] Antigen diluted to the same final volume used with the formalized whole cells.

plement-fixing antigen was lost somewhat more rapidly during purification than biological activity of *R. prowazekii* (Table 1), the relative loss did not appear to be due to a significant stripping off of the "soluble" group antigen (19) from the rickettsiae. Both formalized intact cells of *R. typhi* and *R. prowazekii* and the aqueous supernatant obtained by ether extraction and centrifugation of a suspension of these cells contained antigens that reacted virtually identically in the complement fixation test to antisera against *R. typhi* or *R. prowazekii* (Table 2). Consequently, the typhus group soluble antigen is present in high titer in the purified rickettsiae of either species.

Although the soluble group antigen of typhus rickettsiae could be extracted from Renografin-purified rickettsiae, attempts to demonstrate type-specific antigens distinguishing *R. typhi* and *R. prowazekii* (19) have been less satisfactory. Some preparations of the washed particulate antigen fraction exhibited type specificity when compared with formalized intact cells or the extracted soluble antigen (Table 3), but this was not always the case. Similarly, precipitin lines obtained by using the Ouchterlony double-diffusion technique with various fractions of 0.5% SDS-solubilized *R. typhi* and *R. prowazekii* against homologous and heterologous antisera usually displayed several reactions of identity, as previously noted by Reiss-Gutfreund et al. (20). Alternative methods for preparing type-specific antigens, as well as the use of antisera with greater type specificity, are currently under investigation.

Rickettsial agglutination tests have generally employed yolk sac ether-extracted rickettsial antigens (9), but ether severely disrupts

the cell envelope of rickettsiae (18, 24, 26). Consequently, the use of intact rickettsiae obtained by Renografin purification as the starting material was regarded as particularly advantageous for the study of cell envelope antigens by the microagglutination test. Rickettsiae were inactivated by formalin treatment or by heating at 56°C. Some type specificity was exhibited by heat-inactivated antigens in the microagglutination test, since titers were comparatively lower with heterologous than with homologous antigens (Table 4). Maximum agglutination titers were similar to those obtained by complement fixation (Tables 3, 4). Heat-treated *R. typhi* cells gave serum titers comparable to those found using formalized cells. In contrast, heat-treated *R. prowazekii* cells gave higher microagglutination titers than untreated or formalized cells. This difference between heated and formalized *R. prowazekii* antigens was not seen in parallel complement fixation tests. In an experiment (not shown) in which the same preparation was heated for different periods of time, maximum microagglutination titers were obtained after heating formalized or untreated *R. prowazekii* at 56°C for 15 to 30 min, whereas longer periods of heating caused a decline in titer. Microagglutination titers determined with ether-extracted antigens were reported to be more type specific than those obtained by complement fixation (9). However, particulate antigens (= ether extracted) obtained from formalin-treated, Renografin-purified rickettsiae gave microagglutination titers similar to those of the formalized cells. Heat-inactivated rickettsiae were lysed by ether and did not yield satisfactory particulate antigens.

Polyacrylamide gel patterns of rickettsial proteins and malate dehydrogenases. The great similarity of the typhus group rickettsiae is reflected in their similar protein profiles obtained by SDS-polyacrylamide gel electrophore-

TABLE 3. *Type specificity of antigen fractions prepared from Renografin-purified typhus rickettsiae*

Antigen		Antiserum titer[a]	
Species	Fraction	*R. typhi* 7	*R. prowazekii* 11
Rickettsia typhi	Formalized whole cells	1,280	160
	Soluble antigen	>2,560	160
	Particulate antigen	1,280	40
R. prowazekii	Formalized whole cells	1,280	160
	Soluble antigen	1,280	160
	Particulate antigen	160	160

[a] Reciprocal of serum titer determined by complement fixation.

TABLE 4. *Microagglutination titers[a] of antisera against R. typhi and R. prowazekii using Renografin-purified rickettsial antigens*

Antiserum	*R. typhi* antigen				*R. prowazekii* antigen				
	Prep 1		Prep 2		Prep 1			Prep 2	
	Formalized	Heated[b]	Formalized	Heated[b]	Formalized	Heated[b]	Untreated	Formalized	Heated[c]
Rickettsia typhi									
5	1,280		1,280	960	160	640	80	80	640
6	640		960	640	80	480	80	80	240
7	320	320	640	640	120	640	80	80	320
8	640	320	640	320	60	480	40	40	320
R. prowazekii									
9	40		40	20	80	320	120	80	160
10	80	40	20	60	40	160	40	40	120
11	80	40	160	60	80	320	60	40	80
12	80		80	60	80	480	80	80	120
Yolk sac	0		5	10	0	10	10	0	0
Control serum	0		5	10	0	5	10	5	15

[a] Expressed as reciprocal of serum dilution, mean of duplicate determinations.
[b] At 56°C for 30 min.
[c] At 56°C for 60 min.

sis. Our whole-cell patterns were similar to those reported by Obijeski et al. (17), who used a glycerol tartrate viscosity-density gradient centrifugation method to purify killed rickettsiae. SDS-gel protein patterns of cell envelope-free extracts or cell envelope fractions obtained by French pressure cell passage or lysozyme-Triton X-100 lysis procedures from *R. typhi* and *R. prowazekii* also showed relatively few differences between the two species.

In contrast to the complex patterns obtained in the above experiments, the use of a specific enzyme stain permitted the demonstration of a clear electrophoretic difference among homologous proteins. The mobilities of the malate dehydrogenases in cell-free extracts of purified *R. typhi* and *R. prowazekii* were compared by polyacrylamide gel electrophoresis (Fig. 1). These gels demonstrated the absence of the upper, slower-migrating, disperse band of the host cell yolk sac malate dehydrogenases in these preparations. No host cell enzyme was detected in the rickettsial extracts, even when much higher loadings of protein were employed. Most importantly, however, a small reproducible difference in the mobilities of the malate dehydrogenases of *R. typhi* and *R. prowazekii* was detected.

DISCUSSION

Renografin density gradient centrifugation is a highly satisfactory method for preparing highly purified, viable, antigenic, and metabolically active *R. typhi* and *R. prowazekii*. It has been employed successfully in the purification of several of the strains of *R. prowazekii* (J. C. Coolbaugh, personal communication) recently isolated from flying squirrels (4). There is little doubt as to its value for the typhus group of rickettsiae. However, the general applicability of the method to other intracellular parasites is uncertain, since it is not satisfactory for obtaining completely purified scrub typhus rickettsiae, *Rickettsia tsutsugamushi* (G. A. Dasch and E. Weiss, *in Second International Symposium on Rickettsiae and Rickettsial Diseases, Smolenice near Bratislava, Czechoslovakia, June 21-25, 1976*, in press).

Although the antigenic composition of unpurified typhus group rickettsiae has been studied extensively (5, 19, 20), the possibility cannot be discounted that artifacts may have been introduced into this analysis by the presence of host cell materials. For example, Shepard and Topping (23) found that no soluble group antigen could be obtained from Celite-purified typhus rickettsiae unless additional normal yolk sac or serum was first added back to the rickettsiae before they were extracted with ether to release the antigen. Furthermore, some authors have associated the "soluble" antigen with a capsule present around the typhus group rickettsiae that is readily disrupted during the purification of the rickettsiae (1, 2, 24). Since the experiments described in this paper have shown that a soluble group antigen, readily detected by complement fixation or immunodiffusion, can still be obtained from Renografin-purified, viable rickettsiae with the usual ether treatment, the "soluble" antigen is neither an artifact arising from the presence of host cell materials nor is it completely removed from rickettsiae purified by suitable procedures. However, because the ether-extracted soluble antigen, which appears to originate as fragments of the rickettsial capsule and/or cell envelope (18, 24, 26), is a mixture of very large protein-carbohydrate complexes (13, 19, 20) and was thought to be slightly degraded by some authors (11), ether extraction may not be the most satisfactory method for obtaining homogeneous antigenic components. The added difficulty encountered here and by other investigators (20) in demonstrating type-specific antigens, using the available complex mixtures of antigens, suggests that other methods for preparing antigens should be investigated. Renografin-purified viable typhus rickettsiae would seem to be an optimal starting material for these studies.

Previous investigations have demonstrated that mild heat (60°C) alters the specificity of typhus antigens (7, 22). It has also been suggested that some antigens, possibly those responsible for type specificity, are heat labile (7, 13). The results reported here on the increased agglutinability of typhus rickettsiae after heating parallel those of Shepard (22) on the increased precipitation (with antibody against unheated antigens) of soluble typhus antigens that have been heated. In contrast, complement fixation titers were not affected by heating in either study. Since heated typhus rickettsiae (56°C, 30 min) were completely lysed by ether, whereas unheated preparations remained relatively intact, mild heat apparently alters the conformation of some cell envelope component(s). Precipitin formation and rickettsial agglutination might be expected to be more sensitive than complement fixation tests to such changes.

The great similarity of *R. typhi* and *R. prowazekii* has been amply exemplified here by their similarity in purification behavior, metabolic and hemolytic activity, common antigens, and similar SDS-gel protein patterns. Consequently, it is particularly satisfying that a single, simple biochemical parameter, the electro-

FIG. 1. *Discontinuous polyacrylamide gel electrophoresis of yolk sac and rickettsial extracts. Disc gels, 5 mm, were run for 3 h at 4 mA per gel. The gels were then stained for malate dehydrogenase activity. (A) R. typhi, (B) R. typhi and R. prowazekii, (C) R. prowazekii, and (D) uninfected yolk sac.*

phoretic mobility of malate dehydrogenase in a polyacrylamide gel, can now be used to distinguish at least one strain of each of these species. This parameter may be particularly useful because the *R. typhi* malate dehydrogenase can be detected even in extracts of unfractionated infected-cell cultures (6). Whether the malate dehydrogenases of other strains of *R. typhi* and *R. prowazekii*, particularly those of the latter isolated from flying squirrels, also differ is presently under investigation.

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