Rickettsial Indirect Hemagglutination Test: Isolation of Erythrocyte-Sensitizing Substance

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Received for publication 15 March 1978

The erythrocyte-sensitizing substances (ESS) of *Rickettsia prowazekii* and *R. conorii* were characterized by biological and chemical criteria. ESS could be derived from either soluble or particulate complement-fixing antigens obtained by ether extraction of rickettsiae. The soluble complement-fixing antigen exhibited two peaks of serological activity in potassium tartrate density gradients. The particulate complement-fixing antigen coincided with the more dense peak but was distinguishable by its sedimentation in rate-zonal sucrose gradients. ESS was obtained from each of the complement-fixing-reactive gradient peaks by extraction with hot alkali and was quantified by a modified indirect hemagglutination test. These ESS preparations sedimented similarly in potassium tartrate gradients and were shown to contain protein and carbohydrate, both by colorimetric tests and by incorporation of radioactive precursors. The serological activity of ESS was unaffected by trypsin, but both antigenicity and erythrocyte-binding capacity were reduced after exposure to sodium metaperiodate. Highly purified ESS was rapidly inactivated by potassium tartrate and required stabilization with bovine plasma albumin.

The indirect hemagglutination (IHA) test for human antibodies to typhus and spotted fever group rickettsiae has received renewed attention because of favorable results obtained in rickettsial diagnostic studies comparing this technique with other serological procedures such as the complement-fixation, microagglutination, immunofluorescence, and Weil-Felix tests (11, 16). The IHA procedure is technically uncomplicated and practicable for routine diagnostic use, particularly when glutaraldehyde-stabilized erythrocytes are used as carriers for rickettsial antigen (16). However, the nature of this antigen, termed erythrocyte-sensitizing substance (ESS), remains obscure. The preparative technique developed, and later modified by Chang et al. (2-4), employed a preliminary ether extraction of infected yolk sacs to partially purify the rickettsiae, followed by treatment with hot alkali to produce an antigenically reactive substance that would adhere to erythrocytes. More recently, ESS has been prepared from rickettsiae propagated in tissue culture and subsequently purified by sucrose density gradient centrifugation (1). The source of ESS has been localized in the cell envelope fraction of rickettsiae (7, 12, 20), but its relationship to other serological reagents believed to be derived from the rickettsial cell envelope is obscure.

The purposes of this investigation were to: (i)

identify and quantify ESS precursor and ESS in rickettsial extracts; (ii) determine the physical and antigenic relationship between ESS and rickettsial complement-fixation antigens; and (iii) determine the chemical nature of serologically reactive components of ESS.

MATERIALS AND METHODS

Rickettsiae. *Rickettsia prowazekii* (Breinl strain) and *R. conorii* (Casablanca strain) were grown in embryonated chicken eggs (Spafas, Inc., Norwich, Conn.) by standard methods (17), or in spinner cultures of gamma-irradiated L-929 cells, as previously reported (6).

Preparation of soluble and particulate antigen. Infected yolk sacs were diluted to 20% (wt/wt) in physiological saline, homogenized in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) and centrifuged at $6,500 \times g$ for 1 h at 4°C. The heavy lipid pellicle and the supernatant fluid were discarded, and the pelleted rickettsiae were resuspended to the original volume with physiological saline. An equal volume of diethyl ether (reagent grade; Mallinckrodt, Inc., St. Louis, Mo.) was added, and the suspension was shaken vigorously at 26°C for 5 min. In one experiment, sequential 5-min extractions were performed, as well as a 24-h extraction. Aqueous and ether phases were allowed to separate for 1 h at 26°C, and the aqueous phase was recovered. Soluble and particulate antigens were separated by centrifugation at $12,000 \times g$ for 1 h at 4°C. The pellet was resuspended in TEN buffer (6) to one-fifth the volume of the aqueous phase and was

designated particulate antigen (5 $\times$). The supernatant fluid was treated overnight at 4°C with an equal volume of 20% (wt/vol) trichloroacetic acid. The precipitate was sedimented by centrifugation at $240 \times g$ for 10 min at 4°C, adjusted to pH 7.2 with 0.1 N NaOH, resuspended in physiological saline to a final volume one-tenth the volume of the aqueous phase, and designated as the soluble antigen (10 $\times$).

Gradient centrifugation. Soluble (10 $\times$), particulate (5 $\times$), and ESS antigens were layered onto 10-ml potassium tartrate gradients, 0 to 35% (wt/wt) in TEN buffer. In some experiments the buffer contained 4 mg of bovine plasma albumin (BPA) per ml. Gradients were centrifuged at $148,000 \times g$ for 45 h at 4°C in a Beckman L2-65B ultracentrifuge, using an SW41Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Fractions (0.2 ml) were collected from the bottom of the tube. In a similar manner, soluble and particulate antigens were layered onto 4.4-ml sucrose gradients, 5 to 30% (wt/wt) in TEN buffer. Gradients were centrifuged at $2,900 \times g$ for 22 min at 4°C, using an SW50L rotor.

Analysis of gradient fractions for serological activity. Each fraction was assayed for complement-fixing (CF) activity by the CF-52 method (9) modified for the microtiter technique (15) and for ESS activity by the modified IHA test. Anti-complementary activity was titrated in every fifth fraction. Fractions from each peak of CF activity obtained in the potassium tartrate gradients were pooled, as were fractions from areas of the gradients which possessed no CF activity. These pools were then dialyzed 18 h at 4°C against phosphate-buffered saline (PBS), pH 7.2, and assayed for CF and ESS activities. A portion of each of these pools were adjusted to 0.2 N NaOH, heated at 100°C for 30 min according to the method of Chang et al. (3), dialyzed, and assayed for CF and ESS activities.

Modified IHA technique. The IHA technique, which normally measures serum antibodies using ESS-sensitized glutaraldehyde-treated sheep erythrocytes (SRBC), was modified to quantify ESS activity. Unfractionated ESS or density gradient fractions were serially diluted in PBS containing 4 mg of BPA (PBS-BPA) per ml, using U-bottom microtiter plates and 0.025-ml diluters. An equal volume of washed SRBC, 0.5% (vol/vol) in PBS-BPA, was added to each well. Plates were covered, incubated in a humidified atmosphere for 1 h at 34°C, and occasionally agitated to resuspend any settled SRBC. Reference antiserum (0.025 ml) was added to each well; the plates were sealed, shaken vigorously, and incubated at 26°C for approximately 18 h. The last well to show complete hemagglutination was considered the end point and defined as containing 1 U of ESS activity. The reciprocal of this dilution was taken as the titer of the antigen, and total ESS units of any sample were calculated by correcting the titer of the 0.025-ml portion for the total volume of the sample.

Enzymatic and chemical treatment of ESS. ESS prepared from the aqueous phase of infected yolk sacs was mixed with an equal volume of trypsin immobilized on agarose beads (Enzite-trypsin, Miles Laboratories, Inc., Elkhart, Ind.) to a concentration of 10 mg/ml in 0.07 M phosphate buffer, pH 8.0. At 0, 0.25, 1, 3, 6, and 24 h, samples were centrifuged at $240 \times g$

for 5 min to pellet the immobilized trypsin. The supernatant fluids were assayed for ESS activity by the modified IHA technique. The activity of the Enzite-trypsin was determined at each time point by adding 0.05 ml of the incubated Enzite-trypsin to 1.45 ml of benzoyl arginine ethyl ester, 0.25 mM in phosphate buffer, pH 8.0, for 5 min, centrifuging, and determining the optical density of the supernatant fluid at 253 nm (13).

ESS was tested for sensitivity to sodium metaperiodate (NaIO₄). Samples of ESS were adjusted to contain 0.05 M NaIO₄ and incubated in the dark at 26°C and pH 6.4. At 0, 0.25, and 3 h, an equal volume of ethylene glycol monoethyl ether (Fisher Scientific Co., Silver Spring, Md.) was added to the samples at a final dilution of 1:20, to destroy the NaIO₄. ESS activity was measured by the modified IHA technique. The activity of the incubated NaIO₄ was assessed at each time point by reacting a sample containing 0.04 mg of α -methyl mannoside with an equal volume of 0.016M NaIO₄ for 45 min at 26°C, then diluting the mixture and determining the optical density at 225 nm (8).

The binding of chemically modified ESS to erythrocytes was determined using [³H]galactose-labeled ESS treated with NaIO₄ for 1 h. The modified, radioactive ESS (0.2 ml) was added to 0.0125 ml of packed SRBC and incubated at 34°C for 1 h. A control preparation containing SRBC and untreated, radiolabeled ESS, diluted 1:2 in ethylene glycol monoethyl ether, was similarly treated. After incubation, the suspensions were centrifuged at $240 \times g$ for 8 min, and the supernatant fluids were recovered. Samples of the supernatant fluids were counted in a liquid scintillation spectrometer, and the radioactivity bound to erythrocytes was calculated from the difference between input counts and unbound counts remaining in the supernatant fluid. The serological activity of chemically altered ESS was assayed by the modified IHA procedure.

Inhibition of the IHA test. Soluble CF antigen, sodium metaperiodate-treated ESS, or untreated ESS were tested as inhibitors of the IHA reaction between serum antibody and ESS-sensitized SRBC. In experiments comparing the inhibition of untreated and sodium metaperiodate-treated ESS, the untreated ESS was diluted with water to compensate for the volume of oxidant; then an equal volume of ethylene glycol monoethyl ether was added to each sample to a final concentration of 1:20. Samples were added to microtiter plates containing dilutions of immune serum. The plates were covered and incubated for 1 h in a humidified atmosphere at 34°C. An equal volume, 0.025 ml, of ESS-sensitized SRBC was added to each well, and the plates were incubated at 26°C for approximately 18 h. The last well to show complete agglutination was considered the end point. Inhibitory activity was evidenced by a reduction of agglutination titers in the presence of the test material.

Radioisotope procedures. Rickettsiae grown in cell culture were radiolabeled with ¹⁴C-amino acids (3 μ Ci/ml of growth medium) or [³H]galactose (5 μ Ci/ml of growth medium) as described previously (6). Detection and quantification of intact organisms or subcellular fractions were accomplished by counting 0.1-ml

samples dried on glass-fiber filters (Whatman GF/C, Maidstone, England) with 949 liquid scintillator in a Packard Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Miscellaneous reagents and techniques. Reference antisera for the modified IHA test were pooled human sera of patients convalescent from epidemic typhus or Rocky Mountain spotted fever. Immune sera used in the CF test were obtained from infected guinea pigs. Protein was determined by the method of Lowry et al. (10), using BPA as the standard. Carbohydrate was determined by the anthrone method (14), with galactose as a standard.

RESULTS

Extraction of ESS precursor with diethyl ether. Rickettsial suspensions, prepared as aqueous dilutions of infected yolk sacs, can be partially clarified by treatment with diethyl ether, which solubilizes some yolk sac lipids in the organic phase (5). After phase separation, the aqueous layer is known to contain particulate rickettsiae, a soluble group-specific antigen reactive in the CF test (18), and a precursor substance which yields ESS after treatment with hot alkali (3). It was of interest to determine if ESS precursor remained bound to rickettsiae during ether extraction or was released in a soluble form. Rickettsiae were subjected to either short, multiple extractions or a lengthy single extraction with diethyl ether at 26°C. The aqueous phase was separated into particulate and soluble components by centrifugation at $12,000 \times g$ for 60 min at 4°C. Each component was treated with hot alkali, dialyzed, and assayed for ESS content by the modified IHA technique. Table 1 shows that approximately 50% of the ESS precursor was extracted from *R. prowazekii* after a 5-min exposure to ether. Neither single, long-term exposure to the solvent, nor multiple, short-term extractions achieved

complete solubilization. Therefore, a 5-min extraction at 26°C was used in all further studies for preparation of ESS precursor.

Physical relationship of soluble CF antigen and ESS precursor. The soluble, aqueous-phase material derived from diethyl ether extraction of infected yolk sacs was concentrated 10× by trichloroacetic acid precipitation, adjusted to pH 7.2, layered onto a potassium tartrate density gradient, and centrifuged at $148,000 \times g$ for 45 h at 4°C. Figure 1 shows that soluble CF antigen sedimented as two discrete components, one banding sharply at fractions 8 to 10, and the other more broadly at fractions 25 to 37. No ESS activity was demonstrable in any untreated fraction of the gradient. Fractions were pooled, as shown by the brackets in Fig. 1, dialyzed to remove the anti-complementary potassium tartrate, retested for CF activity, treated with hot alkali, dialyzed, and assayed for ESS by the modified IHA technique. Table 2 indicates the CF and modified IHA reactivity of these pools before and after treatment with NaOH. It is apparent that ESS was obtained only from those fractions of the gradient which exhibited CF activity. Particulate rickettsiae, pelleted from the aqueous phase after ether extraction, were also analyzed on a potassium tartrate gradient. A single band of CF antigen was observed at fractions 8 to 12 (Fig. 2). This band was the only portion of the gradient which evidenced ESS activity after treatment with hot alkali (Table 2). Similar results were obtained when *R. conorii* was extracted with diethyl ether and the soluble and particulate aqueous-phase antigens were centrifuged in potassium tartrate gradients. Although particulate rickettsiae and the more dense component of soluble antigen banded in a similar region of the potassium tartrate gradient after equilibrium centrifugation, there were clear differences in their physical properties. The particulate, ether-extracted rickettsiae sedimented in a sucrose velocity gradient, whereas the soluble antigen did not enter a similar sucrose gradient, and all detectable CF activity remained at the meniscus.

The particulate and two soluble CF antigens possessed physical properties that enabled them to be separated by either density or velocity gradient centrifugation. To determine if the ESS derived from these CF antigens was similarly distinct, samples were centrifuged to equilibrium in potassium tartrate gradients. Figure 3 demonstrates that ESS derived from particulate CF antigen, each soluble CF antigen, and crude aqueous-phase rickettsiae all sedimented chiefly as a broad, diffuse band in the center of the gradient. This sedimentation profile was observed with ESS derived from the CF antigens

TABLE 1. Comparison of ESS-precursor recovery after single or multiple extractions of typhus rickettsiae with diethyl ether

Fraction	Length of extraction	
	5 min	24 h
Aqueous supernatant from first extraction	4,096 ^a	4,352 ^a
Aqueous supernatant from second extraction	1,760	
Aqueous supernatant from third extraction	408	
Corpuscular material remaining after extraction	928	4,096

^a Fractions were treated with hot alkali, dialyzed, and assayed for ESS activity by the modified IHA technique. Total ESS units were calculated by correcting the titer for the total volume of the sample.

FIG. 1. Sedimentation of soluble, CF rickettsial antigen from *R. prowazekii* in a potassium tartrate density gradient. ○, CF titer; ▒, anti-complementary activity. Brackets and associated numbers identify fractions which were pooled for subsequent analysis.

TABLE 2. Recovery of CF and ESS activities after equilibrium centrifugation of soluble and particulate typhus CF antigen

Pool	Before alkali treatment		After alkali treatment	
	CF	ESS	CF	ESS
Soluble antigen				
1	<2	<2 ^a	<2	<2 ^a
2	640	<2	320	2,560
3	<2	<2	<2	<2
4	896	<2	<2	1,792
5	<2	<2	<2	<2
Particulate antigen				
1	<2	<2 ^a	<2	<2 ^a
2	640	<2	320	2,560
3	<2	<2	<2	<2
4	<2	<2	<2	<2
5	<2	<2	<2	<2

^a Pools were assayed for ESS activity by the modified IHA technique. ESS and CF values were corrected for the total volume of each pool.

of both *R. prowazekii* and *R. conorii*. A small peak of ESS activity at fractions 5 to 7 was routinely observed after alkali treatment of ether-extracted particulate organisms. Based on the sedimentation profile of ether-extracted rickettsiae in potassium tartrate gradients (Fig. 2), this small peak of activity may represent particulate material from which ESS was incompletely solubilized by hot alkali.

ESS purified by this gradient technique was susceptible to inactivation by potassium tar-

trate. A peak of ESS containing 352 U of serological activity, as assayed by the modified IHA test, became completely unreactive after 4 h at 26°C or 24 h at 4°C. The loss of reactivity was not observed when crude ESS was mixed with a similar concentration of potassium tartrate. Purified ESS could be protected from inactivation by the addition of BPA at a final concentration of 4 mg/ml.

Antigenic relationship of soluble CF antigen and ESS. We investigated the antigenic relationship between ESS and its precursor substances through use of an IHA inhibition test. A reference typhus antiserum with a titer of 4,096 in the IHA test was incubated with soluble typhus CF antigen for 1 h; then free antibody was quantified in the IHA test. The titer was reduced to 4, although a control preparation of spotted fever soluble CF antigen had no effect on the IHA titer. Five different lots of typhus soluble CF antigen were tested, with each showing similar results.

We also attempted to use typhus ESS to inhibit the interaction of typhus soluble CF antigen and human antibody. We reasoned that if ESS was able to interact with antibodies directed against CF antigens, but was unable to fix complement (4), then the addition of ESS to a reference antiserum before introduction of soluble antigen should result in a reduction of the CF titer of the serum. These experiments were difficult to interpret because we found that after incubation of ESS with human immune serum, and subsequent introduction of complement and

FIG. 2. Sedimentation of ether-extracted, particulate, CF rickettsial antigen from *R. prowazekii* in a potassium tartrate density gradient. ○, CF titer; ▨, anti-complementary activity. Brackets and associated numbers identify fractions which were pooled for subsequent analysis.

FIG. 3. Sedimentation in potassium tartrate density gradients of ESS derived from various CF antigens of *R. prowazekii*. Δ, Soluble CF antigen pool 2; ▲, soluble CF antigen pool 4; ○, ether-extracted particulate CF antigen; ●, crude aqueous-phase rickettsiae.

soluble CF antigen, the indicator system was unreliable. ESS attached to hemolysin-sensitized SRBC and caused partial agglutination which obscured the normal CF pattern. In addition, separate experiments indicated that normal erythrocytes with typhus ESS attached were lysed in the presence of typhus immune serum and complement.

Chemical characteristics. The chemical nature of ESS was determined by two different approaches: (i) examination of ESS obtained from rickettsiae grown in tissue culture and intrinsically radiolabeled with ^{14}C -amino acids or $[^3\text{H}]$ galactose; and (ii) quantification of carbohydrate and protein by colorimetric techniques after density gradient purification of ESS ob-

tained from yolk sac-propagated organisms.

The radioactive profile shown in Fig. 4 illustrates the results of centrifuging, in the same potassium tartrate gradient, ESS obtained from rickettsiae radiolabeled with amino acids or galactose. Three peaks of radioactivity were observed, but, as expected, only two peaks, banding at fractions 9 to 12 and 26 to 35, exhibited ESS activity. Rickettsial antigen sedimenting in these regions of the gradient incorporated both radiolabeled galactose and amino acids. It is also clear that the potassium tartrate gradient separated ESS from serologically unreactive material which banded at fractions 37 to 54. Similar results were achieved with ESS obtained from radiolabeled *R. conorii*.

A chemical analysis of ESS by colorimetric techniques also indicated the presence of both carbohydrate and protein. To find sufficient material for chemical analysis, soluble antigen obtained by ether extraction of typhus rickettsiae was trichloroacetic acid precipitated. This step resulted in a 20-fold concentration of CF antigen, which was treated with hot alkali and centrifuged on multiple potassium tartrate gradients. The major peak of ESS activity, located at fractions 23 to 38 (Fig. 3), was obtained from each gradient, pooled, dialyzed, and lyophilized. When resolubilized, the final preparation of ESS was concentrated approximately 100-fold from the original ESS. This preparation contained 2.2 mg of protein and 1.2 mg of carbohydrate per ml.

The contribution of each of these moieties to serological reactivity was determined by treating samples of typhus ESS with trypsin or sodium

metaperiodate. Table 3 shows that trypsin treatment did not affect either binding of ESS to erythrocytes, or the subsequent agglutination of sensitized cells by immune serum. Periodate, on the other hand, totally ablated serological reactivity of ESS within 15 min. Identical data were obtained with spotted fever ESS. To separately assess the role of carbohydrate in ESS binding, we used a preparation radiolabeled with [^3H]-galactose. Table 4 indicates that erythrocytes incubated with normal typhus ESS bound approximately 3.5 times more radioactivity than those exposed to periodate-treated ESS. The quantity of serologically reactive ESS bound to erythrocytes, as measured by the modified IHA test, indicated a more severe reduction in biological activity than could be accounted for solely by the reduction in binding.

The effect of periodate treatment on the capacity of ESS to interact with serum antibody was examined by use of an IHA inhibition test. A sample of typhus reference antiserum which exhibited a titer of 4,096 in the IHA test was exposed to equal quantities of normal and periodate-treated typhus ESS for 1 h; then free antibody was quantified by the IHA procedure. Absorption of the serum with normal ESS reduced the antibody titer to 256, whereas serum exposed to periodate-treated ESS showed a titer of 1,024.

DISCUSSION

Diethyl ether extraction of rickettsiae has been used routinely to partially remove contaminating host cell lipids from organisms propa-

FIG. 4. Sedimentation of ESS extracted from radiolabeled *R. prowazekii*. ○, ^{14}C -labeled amino acids; ●, [^3H]-galactose. The inset ordinate shows the ESS titer of radiolabeled fractions obtained from the potassium tartrate density gradient.

TABLE 3. *Effect of trypsin and sodium metaperiodate on typhus ESS*

Agent	Substrate	Time (h)					
		0	0.25	1	3	6	24
Enzite-trypsin (10 mg/ml)	ESS	64 ^a	64	32	64	64	64
	BAEE ^b	0.186 ^b	0.182	0.182	0.187	0.183	0.188
NaIO ₄ (0.05 M)	ESS	64 ^a	<2	<2	ND ^d	ND	ND
	α -Methyl mannoside	0.282 ^c	0.143	0.103	ND	ND	ND

^a ESS activity quantified by the modified IHA technique.^b Hydrolysis of benzoyl arginine ethyl ester (BAEE) measured as increase in optical density at 253 nm; figures indicate Δ optical density at each time point after a 5-min treatment with Enzite-trypsin.^c Oxidation by NaIO₄ measured as increase in optical density at 225 nm; figures indicate optical density at each time point as compared with a standard solution of NaIO₄.^d ND, Not done.TABLE 4. *Effect of periodate treatment on the binding of typhus ESS to erythrocytes and their subsequent agglutination by immune serum*

[³ H]galactose-labeled ESS	Radioactivity bound to SRBC (cpm)	ESS titer
Normal	424	128
Periodate treated	125	<2

gated in embryonated eggs (5). This technique was utilized by Chang et al. (3) in their initial studies with ESS, but it was not clear whether the solvent, which is known to extract CF antigen (18), also liberated ESS precursor from particulate rickettsiae. Our studies indicated that ESS precursor, as well as soluble CF antigen, was rapidly extracted from rickettsial organisms by diethyl ether and that soluble CF antigens from both typhus and spotted fever organisms could be resolved into two serologically reactive fractions by centrifugation in potassium tartrate gradients. The particulate rickettsiae remaining after brief ether extraction sedimented in the same region of the gradient as the denser form of soluble antigen. Other workers (19) have reported that particulate spotted fever rickettsiae were separable from soluble components during sedimentation in potassium tartrate-glycerol gradients, but only after lengthy extraction of Formalin-treated organisms with ether. In any case, our work indicates that the particulate CF antigen is distinct from the denser form of soluble CF antigen, since the two are separable on rate-zonal sucrose gradients, suggesting a substantial difference in physical conformation.

Our gradient centrifugation procedures were initiated with the goal of separating ESS precursor from CF antigen. However, the constant association of ESS-precursor activity with CF activity, and the failure to identify ESS precursor in any gradient fraction which did not also possess CF activity, soon suggested to us that the immediate precursor of ESS was CF antigen.

It appeared that alkali treatment modified the CF antigen in a manner which increased its capacity to bind to erythrocytes. Analysis of gradient-purified CF antigen before and after alkali treatment supported this hypothesis and further indicated a difference in the two forms of soluble CF antigen, as evidenced by differences in their residual CF activity. However, it is important to realize that although CF antigen is modified by the alkali treatment which produces ESS, distinct group-specific serological reactivity is retained (3, 4, 16). We do not yet understand the effect of alkali on rickettsial CF antigen, but studies with other bacterial antigens also indicate that exposure to heat and high pH increases binding efficiency to erythrocytes (8). Our studies indicated that such treatment of both species of soluble rickettsial CF antigen and particulate CF antigen resulted in similar products which met the criteria for ESS and were indistinguishable based on their sedimentation in potassium tartrate gradients. A small quantity of CF antigen associated with particulate rickettsiae was apparently converted to ESS by alkali without detachment from the organism. This finding was compatible with the electron microscopic observations of Tzianabos et al. (19), who reported that cell wall particles analogous to soluble CF antigen remained attached to spotted fever organisms even after extensive extraction and purification.

The antigenic relationship between soluble CF antigens and ESS was confirmed by the IHA inhibition test. Soluble CF antigen was capable of interacting with a reference antiserum and substantially reducing its reactivity in the IHA test. However, Chang et al. (4) suggested that antibodies reactive against particulate, washed, specific CF antigen were distinct from those reacting with ESS. These investigators also reported that ESS-antibody complexes were unable to fix complement in a CF test. Our results supported this observation but indicated that if ESS was bound to erythrocytes then reacted

with appropriate antibody, the complex would fix complement and result in lysis of the cells.

Chemical analysis of typhus ESS obtained from potassium tartrate gradients indicated a protein to carbohydrate ratio of 2:1. Previous analysis of crude preparations (16) indicated a ratio of 17:1 for typhus group ESS. These data suggested that our isolation procedure removed a substantial quantity of protein, although such purification was accompanied by ESS instability. Centrifugation of crude radioactive ESS on potassium tartrate gradients also indicated that ESS was a carbohydrate-protein complex which sedimented as one major peak of serological activity separated from other radiolabeled material. The role of the protein and carbohydrate components of ESS in the IHA test is undefined, but oxidation with periodate and enzymatic proteolysis both suggested an important function for the carbohydrate moiety. This finding was compatible with observations in other bacterial systems (8), but the role of carbohydrate seemed complex, since periodate oxidation affected binding of ESS to erythrocytes as well as interaction of ESS with serum antibody. Our studies do not preclude a requirement for protein in both of these activities, and more detailed investigations are required to fully define the role of both ESS components.

The usefulness of ESS may not be limited to its employment as a diagnostic reagent. ESS recovered from both typhus and spotted fever group rickettsiae possess common properties of erythrocyte binding and similar sedimentation in potassium tartrate gradients, yet retain discrete, group-specific antigenic determinants capable of interacting with humoral antibody. It is possible that mild chemical or enzymatic degradation of ESS could selectively remove components conferring common biological or physical properties and result in a relatively simple substance that could be examined for antigenic determinants conferring group specificity. Previous studies from this laboratory (6) have examined intact rickettsial organisms from the typhus and spotted fever groups in a comparative manner, using polyacrylamide gel electrophoresis. Although distinct differences were observed, protein patterns were complex, and a simple subcellular component such as ESS, which retains group specificity, could be valuable for more detailed analyses.

ACKNOWLEDGMENTS

We thank John E. DeWitt and Matthew J. Nypaver for excellent technical assistance.

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