JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1989, p. 377–384
0095-1137/89/030377-08$02.00/0

Vol. 27, No. 3

Production of Antibody to and Cellular Localization of Erythrocyte-Sensitizing Substance from *Rickettsia rickettsii*

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Received 26 September 1988/Accepted 28 November 1988

Antibodies to *Rickettsia rickettsii* erythrocyte-sensitizing substance (ESS) were raised in rabbits by using a derivatized ESS. The resulting antibodies reacted with *R. rickettsii* and cross-reacted with *Rickettsia conorii*, a member of the spotted fever group rickettsiae, but did not react with *Rickettsia typhi*, a member of the typhus group rickettsiae, *Legionella bozemanii*, or *Proteus vulgaris* OX19 or OX2. Immunoblot analysis indicated that ESS was present in more than one fraction and that the major haptenic fraction was proteinase resistant. Immunoelectron microscopy indicated that the antibodies to *R. rickettsii* were specific to components located on the cell surface and intracellularly to components between the cell wall and cytoplasmic membrane.

The term erythrocyte-sensitizing substance (ESS) was coined by Chang (6) and Chang et al. (5, 7) to describe the product obtained when *Rickettsia typhi* and *Rickettsia rickettsii* are boiled in 0.1 N sodium hydroxide. Antibodies to ESS, as well as to other components of the rickettsial cell, are produced naturally in infection or in the laboratory by immunization with whole rickettsiae of the spotted fever or typhus group.

The ESSs from *R. rickettsii* and *R. typhi* antigens have recently gained importance because of their increased use as antigens in indirect hemagglutination (IHA) (1, 4, 20, 31) and latex agglutination (16, 17, 20) tests for the serodiagnosis of Rocky Mountain spotted fever and endemic typhus, respectively. ESS from *R. typhi* was detected in biologic fluid from experimentally infected guinea pigs and was suggested (Y. A. El Batawi, Ph.D. thesis, University of Maryland, Baltimore, 1964, cited in reference 23) as a possible diagnostic marker for typhus infection.

Antibodies specific to *R. rickettsii* ESS were required for a study (in progress) to determine the levels of sensitivity of various diagnostic techniques in detecting ESS in a model system of cell culture supernatant fluid free of *R. rickettsii* organisms. Free ESS is, however, haptenic. Haptens have been made antigenic by coupling to proteins (11). The present report describes the preparation of antibodies to *R. rickettsii* ESS by using ESS coupled to tetanus toxoid (TT), the reactivity of these antibodies to *R. rickettsii* antigens, and the localization of ESS in whole cells. In this report, unless stated, ESS refers to ESS obtained from *R. rickettsii*.

MATERIALS AND METHODS

Antigens. To prepare ESS, the R strain of *R. rickettsii* was grown in egg yolk sacs (EYS) of chicken embryos (34) and was purified as described by Weiss et al. (40). ESS was prepared by boiling the organism in 0.1 N NaOH (1 mg of lyophilized rickettsiae per ml) (5). For immunoelectron microscopy, *R. rickettsii* was grown in monolayer cultures of L cells (1). The infected L cells were gently shaken with glass beads in tissue culture flasks for 10 s. The organisms and tissue culture fluid were then removed and centrifuged at 16,300 × *g* for 30 min at 4°C. Both the sedimented rickettsiae and host cells were used for microscopy.

To study the specificity of the antisera to the derivatized ESS, the following antigens were used (Table 1) *R. typhi* Wilmington and *R. conorii* Malish were grown as described for *R. rickettsii* (34), and *R. typhi* ESS was prepared as described previously (7). Also included were *Proteus vulgaris* OX19 (obtained from the Rocky Mountain Laboratories and maintained at the Wadsworth Center since 1960) and OX2 (ATCC 7829), *Legionella bozemanii* ATCC 33817, and *Legionella micdadei* ATCC 33204. Rickettsial antigens for the complement fixation (CF) tests were obtained from the Centers for Disease Control.

Preparation of ESS-MBS-TT. ESS (0.5 ml) was dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mM sodium chloride. After dialysis, 15 μl of dioxane containing 0.32 mg of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (19, 22, 26) (MBS; Pierce Chemical Co., Rockford, Ill.) was added, and the mixture was incubated with shaking at 30°C for 1 h. As a control, 0.5 ml of ESS solution was mixed with dioxane without MBS.

Each mixture was cooled to 5°C and applied to a Sephadex G-25 column (30 by 1.6 cm), which was equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM magnesium chloride and 50 mM sodium chloride. The activated ESS (ESS-MBS) and excess MBS were eluted with the equilibrating buffer at a flow rate of 0.5 ml/min. The fractions containing ESS-MBS were combined, and 0.2 ml of TT (a gift from F. McCarthy, Wyeth Laboratories Inc., Marietta, Pa.; protein nitrogen, 0.36 mg/ml; purity, 1,111 Lf (amount of toxin corresponding to 1 U of antitoxin)/mg of protein nitrogen) was immediately added. The mixture was incubated with shaking at 30°C for 1 h, and the reaction was terminated by adding cysteine to a final concentration of 1%. The mixture (ESS-MBS-TT) was then dialyzed against saline. As a control, we blocked the ESS-MBS with cysteine

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and then added TT to form a mixture of ESS-MBS-cysteine and free TT.

Determination of free amino groups. To determine the extent of coupling of ESS to MBS, tests for unsubstituted and/or monosubstituted amino groups in ESS and ESS-MBS were performed by the fluorescamine technique (38).

Production of antisera. Antibodies to ESS were prepared by injecting 0.5 ml of ESS-MBS-TT, mixed with 0.5 ml of Freund complete adjuvant, intramuscularly (i.m.) into NYS:(FG) rabbits. The injections were repeated without adjuvant at weekly intervals for 2 weeks. Rabbits that produced antibodies to ESS with titers ≤32 were euthanized 4 weeks after the first injection. Rabbits that produced relatively high antibody titers (>32) were bled periodically for up to 70 weeks and were then injected i.m. with Ketaset (Veterinary Products, Bristol Laboratories, Syracuse, N.Y.) and bled out by cardiac puncture.

As controls, we also attempted to prepare anti-ESS by injecting 0.1 ml of ESS or 0.5 ml of ESS-MBS-cysteine plus TT mixed with 0.1 ml of Freund complete adjuvant i.m. into NYS:(FG) rabbits. The protocol of injections and blood collections were as described for ESS-MBS-TT.

Antisera to purified, Formalin-killed *R. rickettsii*, *R. typhi*, and *R. conorii* were raised in New Zealand White rabbits as previously described (3). Antisera to the nonrickettsial bacterial antigens were raised in NYS:(FG) rabbits by injecting 1 mg (lyophilized antigens) suspended in saline and 1 ml of complete Freund adjuvant. The injections were repeated without the adjuvant at weekly intervals for 2 weeks, and the rabbits were bled out 1 week later.

Antisera to EYS was prepared by injecting 1 ml of 10% EYS in glycine buffer, mixed with 1 ml of complete Freund adjuvant, i.m. into NYS:(FG) rabbits. During the third week the animals were injected i.m. with Ketaset and bled out by cardiac puncture.

Antiserum to TT was prepared by injecting 0.1 ml of TT, mixed with 0.1 ml of Freund complete adjuvant, i.m. into NYS:(FG) rabbits. The injections were repeated as for the production of antibody to ESS-MBS-TT.

Serology. The microimmunofluorescence (micro-IF) test (27, 28) with *R. rickettsii* was performed with two conjugates: for micro-IF/immunoglobulin G (IgG), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (γ-chain specific); and for micro-IF/IgM, anti-rabbit IgM (μ-chain specific) (Organon Teknika, Malvern, Pa.). CF was performed as described previously (36). Rickettsial antibodies were detected by IHA with ESS or *R. typhi* ESS (4). Antibodies to TT were detected by IHA as described previously (8).

Immunoabsorption. Immunoabsorption was performed by a one-step protocol (15). For absorption with *R. rickettsii*, each diluted serum sample was mixed with *R. rickettsii* (0.477 mg of lyophilized organisms per ml) in equal volumes (final dilutions, 1:500 for anti-*R. rickettsii* and 1:100 for anti-ESS collected at week 20) and incubated at 37°C for 72 h with shaking. The suspension was centrifuged at 5°C at 4,000 × *g* for 30 min. The supernatant was saved for assay. For absorption of sera with ESS, latex-ESS was prepared by force adsorbing (14) 1 ml of ESS to 20 ml of latex suspension (Difco Laboratories, Detroit, Mich.). A 200-ml volume of 95% ethanol containing 0.5% sodium acetate was added with continuous stirring, and the mixture was left overnight at 5°C. The following day the suspension was centrifuged and the supernatant was discarded. The following day the suspension was centrifuged and the supernatant was discarded. The latex sediment was suspended in 20 ml of 0.1 M glycine-buffered saline (pH 8.2), and 2 ml of the same buffer containing 0.1% bovine serum albumin (BSA) was added. Latex-ESS was mixed with each diluted serum sample and processed as described above for absorption with *R. rickettsii*. Control sera were incubated with glycine-buffered saline (1:1) and processed as described above.

Electrophoresis and electroblotting. *R. rickettsii* in solubilizing buffer (21) was incubated in a water bath at 37°C for 5 min. The mixture was then divided into two portions. One portion was digested with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (18) and then used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The remaining portion was used in SDS-PAGE directly, without proteinase K treatment.

The antigen preparations and the molecular weight standards (Bethesda Research Laboratories-Life Technologies, Inc., Gaithersburg, Md.) were subjected to SDS-PAGE with the buffer system of Laemmli (21). The 5% stacking gel and 10% separating gel did not contain SDS (18). Electrophoresis was carried out at a constant current of 30 mA with 0.025 M Tris–0.192 M glycine in 0.1% SDS for 3 to 4 h.

Electroblotting was done on nitrocellulose paper with 25 mM phosphate buffer (pH 7.4). The transfer conditions were 250 mA for 18 h at 5°C. One lane for each hapten was stained with amido black (35); the remaining lanes were blocked in 50 mM Tris buffer (pH 7.4) containing 10% BSA for 5 h at 45°C. All lanes were then incubated overnight at 37°C with the sera and washed in the blocking buffer repeatedly for 30 min. Horseradish peroxidase-labeled, goat anti-rabbit IgG antiserum (heavy and light chain specific; Organon Teknika) was used to identify antibody. The lanes were incubated for 3 h with the conjugate and washed with 50 mM Tris buffer (pH 7.4). The enzyme substrate for color development was 4-chloro-1-naphthol (13).

Immunoelectron microscopy. Antibody-reacting sites of *R. rickettsii* were localized with secondary antibody-coated immunogold probes. Immunolabeling was attempted before embedding to localize external sites and after sectioning to localize internal sites.

R. rickettsii cells were fixed with 1% formaldehyde–0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 20 min at 25°C. After being washed with phosphate-buffered saline (PBS; pH 7.4), the cells were suspended in 0.05% sodium borohydride for 25 min at 25°C to enhance preservation of cellular antigenicity (10, 37). They were then washed with PBS until the release of hydrogen ceased.

External sites were localized by incubating the fixed cells with four rabbit antisera: anti-*R. rickettsii*, anti-ESS (collected at week 20), anti-TT, and anti-EYS (the last two were controls), each diluted 1:50 in PBS containing 1% BSA. Incubation was for 2 h at 25°C with gentle shaking followed by centrifugation and suspension in PBS containing 1% BSA. The cells were then treated for 30 min at 25°C with a 1:20 dilution (in PBS containing 1% BSA) of goat anti-rabbit IgG secondary antibody conjugated to 5-nm colloidal gold (Janssen Life Sciences Products, Piscataway, N.J.). Excess complex was removed by centrifugation, and the cells were postfixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h. After being washed in cacodylate buffer, the samples were encapsulated in 2% agar, dehydrated in ethanol, and embedded in Epon-Araldite. Thin sections were retrieved on copper grids and stained with uranyl and lead salts prior to viewing.

Internal sites were localized on sections of the above fixed cells essentially by the procedure described in the instructions provided for immunogold probes by Janssen Life Sciences. Briefly, sections of the material on nickel grids

TABLE 1. Immune responses to ESS in rabbits injected with lot 1 ESS-MBS-TT

Test and antigen	Titer at 20 wk after first inoculation		
	ESS[a]	ESS-MBS-cysteine plus TT[a]	ESS-MBS-TT
CF			
R. rickettsii	<4	<4	64
R. typhi[a]	<4	<4	<4
Micro-IF/IgG			
R. rickettsii	<16	<16	2,048
R. conorii[a]	ND[b]	ND	512
R. typhi[a]	<16	<16	<16
L. bozemanii[a]	ND	ND	<16
L. micdadei[a]	ND	ND	<16
P. vulgaris OX19 and OX2[a]	ND	ND	<16
IHA[c]			
R. rickettsii	<16	<16	1,024
R. typhi[a]	<16	<16	<16

[a] Control.
[b] ND, Not done.
[c] Antisera to EYS and TT did not react to either antigen in the IHA test.

were floated on BSA-Tris buffer (9) supplemented with 5% normal goat serum. The grids were divided into four sets. Each set was incubated with one of the four antisera diluted 1:50 in BSA-Tris buffer containing 1% normal goat serum, washed in buffer, and then incubated for 30 min with a 1:20 dilution of secondary antibody conjugated to 15-nm colloidal gold (Janssen Life Sciences), followed by staining with uranyl and lead salts prior to viewing. This resulted in double labeling, with the external sites labeled with 5-nm gold and the internal sites labeled with 15-nm gold. In some instances, in an effort to precisely identify the localization of a specific antigenic site, we applied a 5-nm gold probe to sections of previously unlabeled rickettsial cells.

RESULTS

Preparation of ESS-MBS-TT. ESS coupled to MBS, as indicated by an increase in A_{260} and a shift of the elution pattern. The fluorescamine test revealed a 44% decrease in fluorescence after coupling of ESS with MBS, indicating that some of the free amino groups (in denatured protein or amino sugar or both) in ESS had reacted with the maleimido group of MBS. Attempts to prove attachment of TT to ESS-MBS failed because the final preparation contained far more TT than ESS-MBS. We could not ascertain its attachment to ESS-MBS by SDS-PAGE and immunoblotting. However, the control results (Table 1) indirectly indicate that TT was attached to ESS-MBS.

Antibody to ESS. Two lots of ESS-MBS-TT were prepared, each with a different batch of ESS. Significant levels of antibody to ESS, determined by the micro-IF/IgG and IHA tests (titer, >32 for both tests) and CF test (titer, >4), were produced in one of four rabbits injected with lot 1 ESS-MBS-TT conjugate (Table 1) and in one of five rabbits injected with lot 2. Of the remaining rabbits, five were weakly reactive (titer, ≤32) and two were nonresponders (titer, <8). No antirickettsial IgM was detected by the micro-IF/IgM test.

Controls. No antibodies to ESS were produced with five rabbits injected with ESS or with five rabbits injected with ESS-MBS-cysteine plus TT, as shown by the CF, micro-IF, and IHA test results (Table 1). All three tests were also negative when the sera from rabbits injected with ESS-MBS-TT were tested with *R. typhi* antigens (Table 1). This lack of cross-reactivity of anti-ESS determined by the three serologic techniques was confirmed by SDS-PAGE and immunoblotting (results not shown), indicating that the antibodies were specific to ESS. In addition, antibodies to TT did not react with the unconjugated rickettsial antigens and erythrocytes (RBC) coated with EYS did not agglutinate when mixed with antisera to ESS.

TABLE 2. Titers of rabbit antisera to *R. rickettsii* and ESS after immunoabsorption with *R. rickettsii* and latex coated with ESS antigens

Antibody to:	Absorbed with:	Titer	
		IHA (ESS)	Micro-IF/IgG (*R. rickettsii*)
R. rickettsii	Buffer (control)	2,000	128,000
	R. rickettsii	<500	<500
	Latex-ESS	<500	128,000
ESS	Buffer (control)	400	400
	R. rickettsii	<100	<100
	Latex-ESS	<100	<100

The antisera to ESS did cross-react in the micro-IF test with *R. conorii*, a member of the spotted fever group, but did not cross-react with legionella or proteus antigens (Table 1).

Immunoabsorption profile. The immunoabsorption profiles of anti-*R. rickettsii* and anti-ESS are shown in Table 2. Absorption of anti-*R. rickettsii* with *R. rickettsii* reduced the titers to <500 (initial dilution) in both the micro-IF/IgG and IHA tests. Absorption with latex-ESS reduced the titer to <500 in the IHA test but had no effect on the micro-IF/IgG titer (15). Absorption of anti-ESS with *R. rickettsii* reduced both titers to <100 (initial dilution). These results indicate that anti-ESS did not contain a profile of antibodies to the whole array of *R. rickettsii* antigens. If present, the levels of antibodies to the whole array of antigens were below the level of sensitivity of the serologic tests.

Differences in the profile of antibodies to *R. rickettsii* antigens were also shown when the sera were incubated with electroblotted *R. rickettsii* (Fig. 1) and proteinase K-treated *R. rickettsii* (Fig. 2). Lane 1 in both figures was treated with amido black as a control. No protein bands were detected after the enzymatic digestion within the limit of sensitivity of the staining technique. The profiles of the antibodies to the antigen bands were as follows.

(i) Unabsorbed sera. With *R. rickettsii* (Fig. 1), anti-*R. rickettsii* (lane 2) and anti-ESS (lane 5) had different reactivity profiles. The major bands detected by anti-ESS, as indicated by the intensity of the bands, were mainly in the region below 43 kilodaltons (kDa). Anti-*R. rickettsii* yielded more bands and with greater intensity from 150 kDa down. With proteinase K-treated *R. rickettsii* (Fig. 2), some dissimilarities were seen (lanes 2 and 5) between 37.4 and 68 kDa, where the bands were more pronounced with anti-*R. rickettsii*, and the lower two bands (6.2 and 14.3 kDa) were detected by anti-*R. rickettsii* but apparently not by anti-ESS.

(ii) Absorption with *R. rickettsii*. With or without proteinase K treatment of *R. rickettsii*, the profiles of anti-*R. rickettsii* and anti-ESS absorbed with *R. rickettsii* were similar (Fig. 1 and 2, lanes 3 and 6). As expected, most bands were not detected, and those that were detected with anti-*R. rickettsii* were faint, probably due to remaining antibodies (the titer of the absorbed serum was <500) in this originally high-titered serum (titer, 128,000; Table 2).

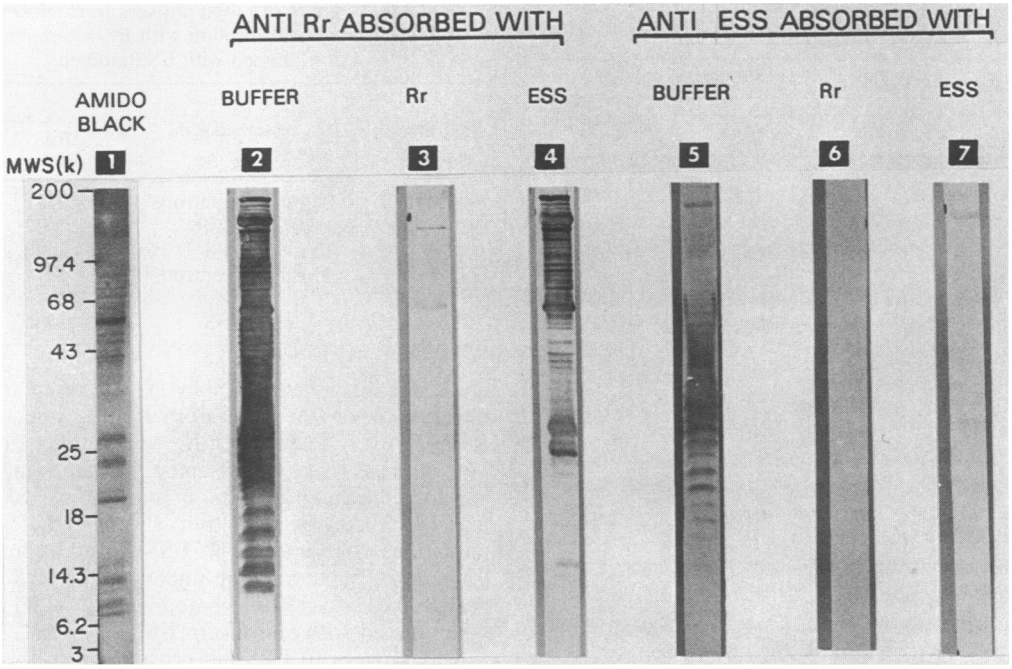

FIG. 1. Immunoblots of anti-*R. rickettsii* (anti Rr) and anti-ESS immunoabsorbed with *R. rickettsii* (Rr). Lane 1 was stained with amido black. MWS(k), Molecular weight standards in thousands.

(iii) Absorption with ESS. Striking differences were, however, observed between the antisera absorbed with ESS. The absorbed anti-*R. rickettsii* serum reacted with untreated *R. rickettsii* (Fig. 1, lane 4) but did not react with proteinase K-treated *R. rickettsii* (Fig. 2, lane 4). These results show that the reactivity of the antibodies remaining in anti-*R. rickettsii* serum after absorption with ESS appeared to be directed toward the proteinase K-sensitive bands of *R. rickettsii*. In contrast, the absorbed anti-ESS did not react with antigens in lane 7 of either Fig. 1 or 2, indicating that ESSs were mostly directed against fractions that were proteinase K resistant.

Immunoelectron microscopy. No immunogold labeling occurred when cells were treated with buffer or negative control serum before embedding (Fig. 3A) or after sectioning (results not shown). When the anti-whole *R. rickettsii* cell

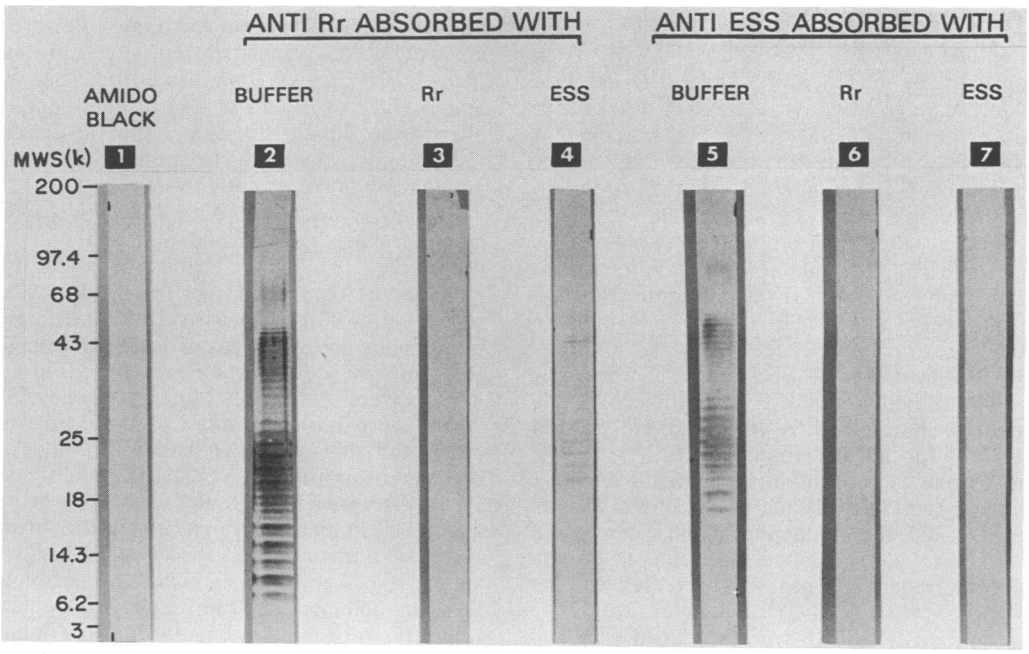

FIG. 2. Immunoblots of anti-*R. rickettsii* (anti Rr) and anti-ESS immunoabsorbed with proteinase K-treated *R. rickettsii* (Rr). MWS(k), Molecular weight standards in thousands.

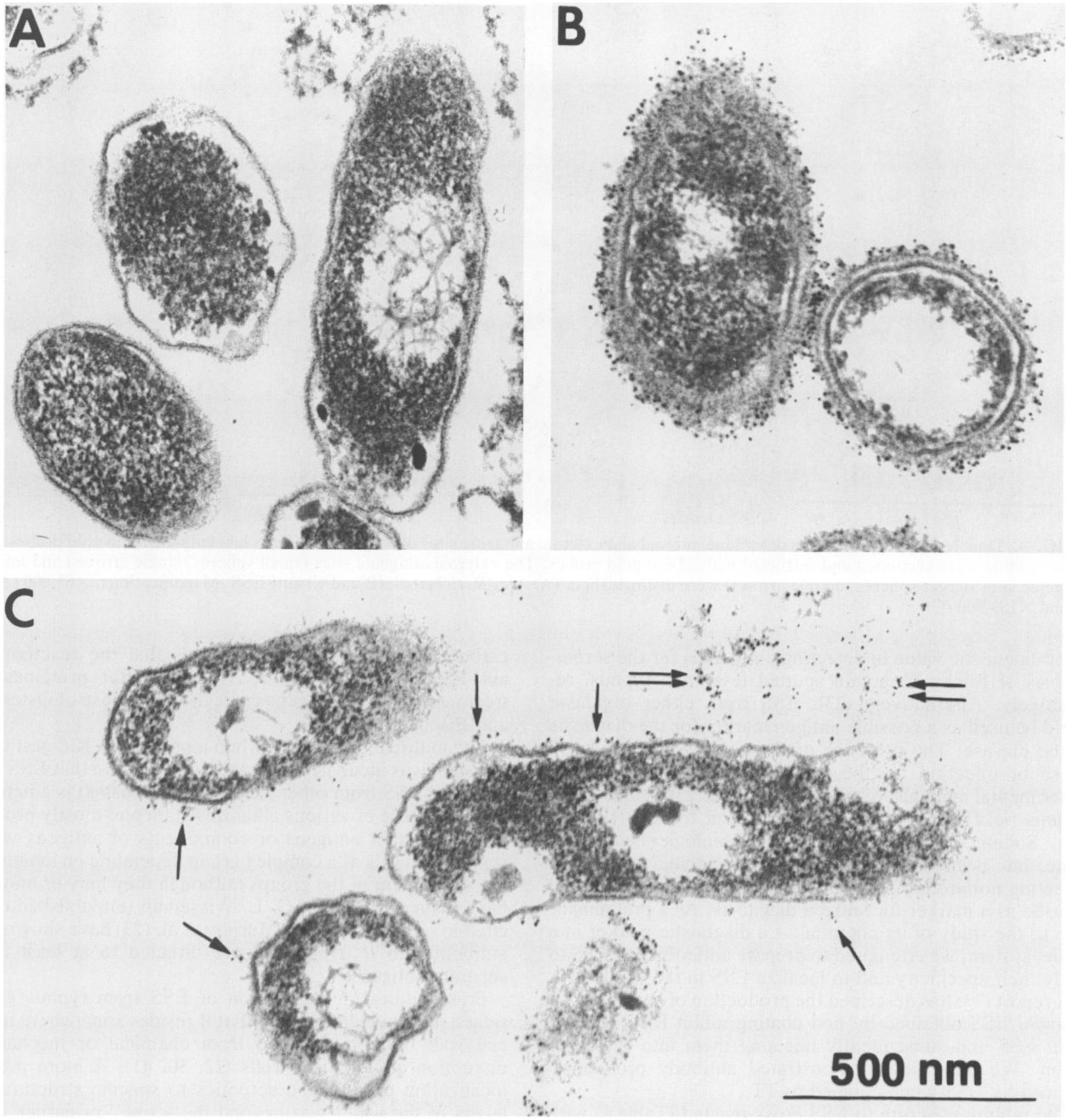

FIG. 3. Immunolabeling prior to embedding to localize external antigenic sites of *R. rickettsii*. Control antiserum (A), anti-*R. rickettsii* (B), and anti-ESS (C, arrows) were incubated with *R. rickettsii*. The ESS sites could be seen emanating from the cell surface (double arrows). Magnification, ×225,000.

serum was incubated with whole organisms before embedding, extensive labeling occurred at the cell surface (Fig. 4B). When this serum was applied after sectioning, labeling was distributed over the entire cell (Fig. 4A). When anti-ESS serum was applied before embedding, the initial label was scattered on the cell surface, with some of the antigenic sites appearing to emanate away from the surface (Fig. 3C). When this labeled material was then sectioned and exposed to anti-ESS, the new label was restricted to the region of the cytoplasmic membrane, extending to the outer cell surface (Fig. 4B and C).

Since the size of the 15-nm immunogold complex may have exceeded the diameters of the cell envelopes (29), we eliminated the possibility that the label observed with anti-ESS and the 15-nm probes on the inner peripheries of the cells may in fact have been surface label. This was done by labeling sections of previously unlabeled cells with small 5-nm probes; the results confirmed that there were internal ESS sites located in the region extending from the periphery of the cytoplasm to the cell surface (Fig. 5).

DISCUSSION

ESSs from *R. rickettsii* and *R. typhi* coated on biologic (RBC) and nonbiologic (latex) particles have been shown to

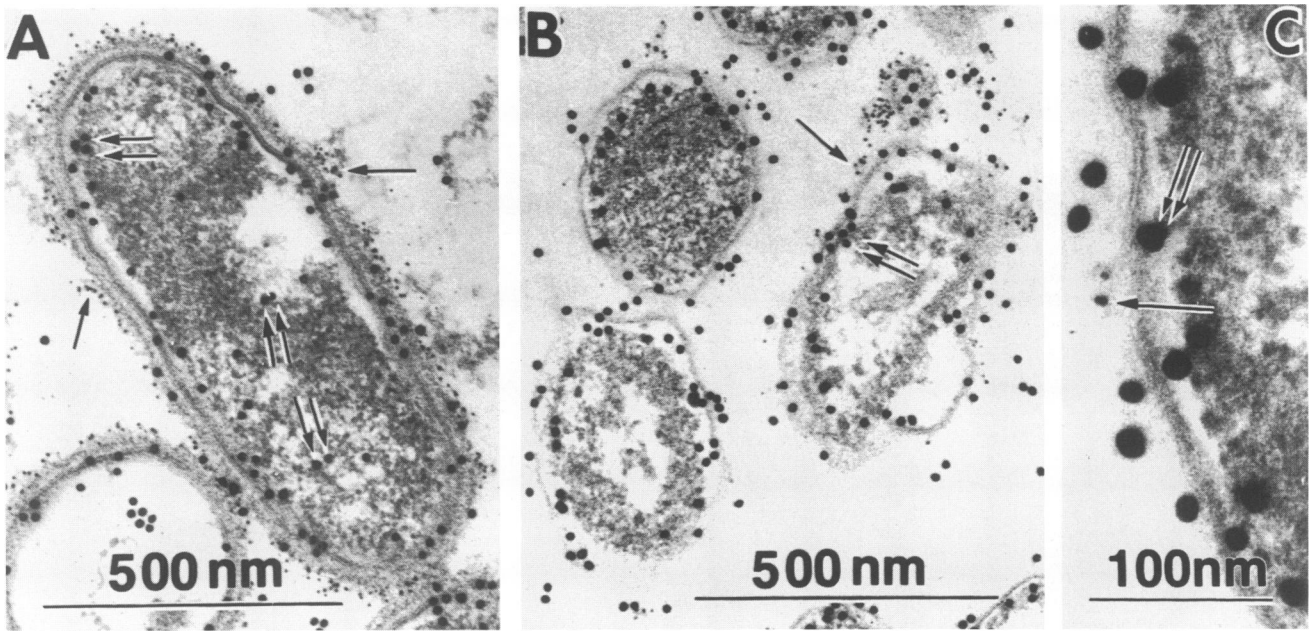

FIG. 4. Double immunolabeling to determine internal and external antigenic sites of *R. rickettsii*. Cells labeled with 5-nm gold probes prior to embedding were sectioned and relabeled with 15-nm gold probes. The external antigenic sites (small spheres, single arrows) and internal antigenic sites (larger spheres, double arrows) were distinguished. (A) Anti-*R. rickettsii*; (B and C) anti-ESS. Magnifications, ×63,000 (A and B) and ×189,000 (C).

be of diagnostic value in detecting antibodies for the serodiagnosis of Rocky Mountain spotted fever and typhus, respectively. Alternatively (23), ESS from either organism could be used as a possible antigen marker for the diagnosis of the diesase. The presence of *R. typhi* ESS in the early phase of infection has been shown in biologic fluids of experimental animals (cited in reference 23). ESSs from various rickettsial organisms are resistant to NaOH digestion. Sodium hydroxide digestion of biologic fluid, e.g., urine, has been used to minimize nonspecific reaction in detecting nonprotein antigens in the fluid; thus, ESS could be used as a marker for antigen diagnosis. As a preliminary step to the study of its potential as a diagnostic marker in a model system, we attempted to prepare antibodies to ESS to study their specificity and to localize ESS in the whole cell.

A report (23) has described the production of antibodies to *R. typhi* ESS obtained by first coating rabbit RBC with *R. typhi* ESS and subsequently injecting them into the same rabbit. We have now demonstrated antibody production induced by ESS when coupled to TT.

The rabbit antiserum to ESS cross-reacted (Table 1) with *R. conorii*, a member of the spotted fever group rickettsiae. This was expected since cross-reaction within rickettsial groups is extensive. This antiserum did not cross-react with *R. typhi*, a member of the typhus group rickettsiae. The rabbit antisera also did not cross-react with the unrelated *Proteus* and *Legionella* antigens. Cross-reaction of rickettsial sera from humans with the unrelated *Proteus* organisms has long been known (39); however, cross-reaction to *Legionella* antigens has only recently been reported (33; D. Raoult, G. A. Dasch, and D. M. Hussong, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, E75, p. 115).

Osterman and Eisemann (25) have shown that the haptenic activity of ESS is destroyed upon exposure to periodate, and ESS does attach to untanned sheep or human RBC, as does NaOH-treated lipopolysaccharide (LPS) from *Escherichia coli* (24), indicating that the hapten fraction of ESS may be a carbohydrate material. Our findings that the reactivity of anti-ESS with ESS and *R. rickettsii* after proteinase K treatment of these antigens correlate with those of Osterman and Eisemann.

The multiple bands seen when a proteinase K digest of *R. rickettsii* was incubated with anti-ESS indicate that ESS (and probably ESS from other rickettsial organisms) is a heterologous mixture of various alkali-resistant and mostly proteinase K-resistant antigens or components of antigens which separate on gels as a complex group depending on length and the attachment of the groups (although they may or may not have a single epitope). C. L. Wisseman (unpublished data, cited in reference 23) and Murphy et al. (23) have shown that antibodies to *R. typhi* ESS are directed to at least three separate antigens.

Prior studies of localization of ESS from typhus group rickettsiae have determined that it resides somewhere in the cell wall fraction obtained from chemical or mechanical disruption of rickettsial cells (12, 30, 41). A more precise localization of antigenic activities to specific structures or layers of the wall "was beyond the scope" of earlier work (12). Colloidal gold complexed to an antiglobulin as a second antibody is a useful immunocytochemical marker for electron microscopy. With anti-ESS, no cytoplasmic labeling was found and surface labeling was scattered and was associated with material which appeared to be coming away from the cell surface. This location of ESS may correlate with the findings of Anacker et al. (2) regarding the presence of an LPS-like material on the cell surface and as part of the slime layer described by Silverman (32). The additional label found between the cytoplasmic and outer membranes may indicate that components of ESS other than those on the surface are present or that ESS is a part of the inner layer of the cell wall. Alternatively, this may be the site of synthesis and/or assembly of ESS.

This study shows that it is possible to produce antibodies to ESS by coupling it to TT. These antibodies are directed

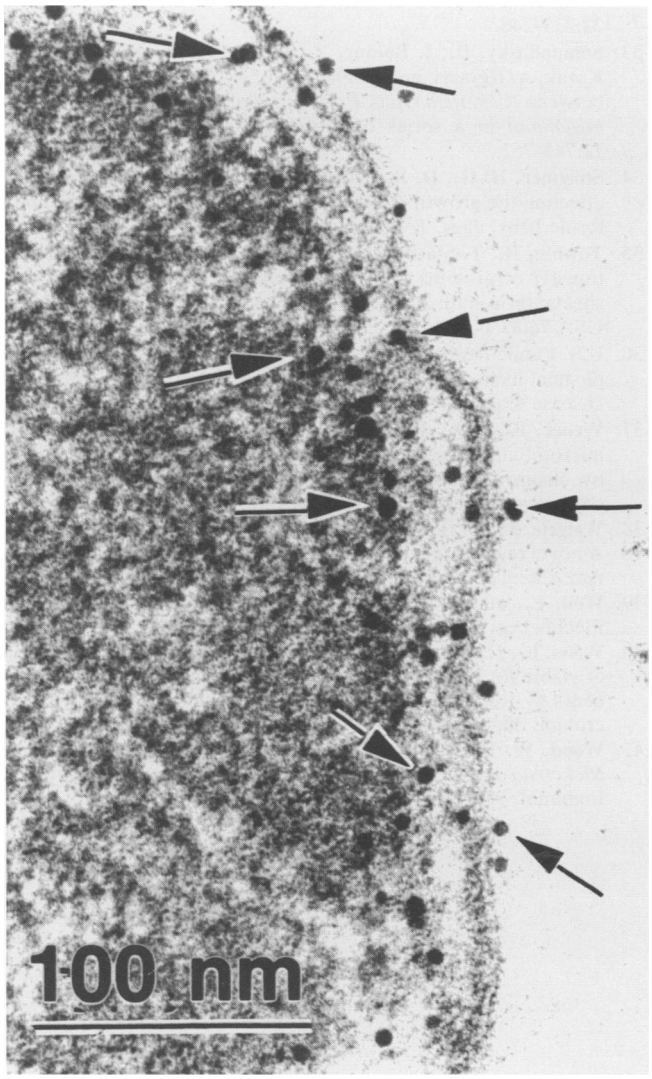

FIG. 5. Single immunolabeling of ESS sites after sectioning. Previously unlabeled cells were sectioned and exposed to anti-ESS, followed by a 5-nm gold probe. The ESS sites were located in the region extending from the cytoplasmic membrane to the outer cell surface (arrows). Magnification, ×225,000.

against a proteinase K-resistant fraction of *R. rickettsii*, which suggests, although indirectly, that ESS is a carbohydrate and/or LPS-like material (2, 25). We have also localized ESS outside the cytoplasm and on the cell surface regions rich in carbohydrate and LPS. The usefulness of these antibodies in detecting ESS as a diagnostic marker is being evaluated.

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