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External Layers of *Rickettsia prowazekii* and *Rickettsia rickettsii*: Occurrence of a Slime Layer

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Using a simple specific-antibody stabilization procedure on organisms gently liberated from their host cells, we have demonstrated by electron microscopy that *Rickettsia prowazekii* and *Rickettsia rickettsii* possess a coat of variable thickness, external to the outer leaflet of the cell wall and the structure designated by others as a "microcapsule," which corresponds most closely to the slime layer of certain other bacteria. Reactions in the methenamine silver and ruthenium red staining procedures and the failure to be visualized by standard procedures suggest that the slime layer is largely polysaccharide in nature. It is postulated that this slime layer accounts in large part for the large, electron-lucent, halo-like zone which is found by electron microscopy to surround organisms of the typhus and spotted fever groups in the cytoplasm of their host cells, that it may be the locus of some major group-specific antigens, and that it may function as an antiphagocytic mechanism, as an aid for attachment of rickettsiae to potential host cells, or both. Moreover, because the attenuated E strain of *R. prowazekii* has been shown to possess a substantial slime layer, the basis for attenuation is not likely to be a simple smooth-to-rough variation.

For some time, we have had the strong impression that typhus rickettsiae probably possess a substantial amount of antigenic material adjacent to the exterior portion of their cell wall or outer envelope.

In our experience, mostly with the typhus group of organisms, we have been influenced by a series of observations: (i) the remarkable reduction in the quantity of "soluble" antigen which can be freed by ether treatment after gradient purification of yolk sac-grown rickettsiae as compared with crude rickettsial preparations (C. L. Wisseman, Jr., unpublished data); (ii) the observation of what appears to be a "capsule" surrounding intracellular typhus rickettsiae by phase-contrast, immunofluorescence, and immunoperoxidase microscopy (Wisseman, unpublished data); (iii) the substantial thickness of the outer layer of *Rickettsia prowazekii* as well as an external fibrillar structure on intracellular organisms demonstrable by the freeze-etch technique (D. T. Brown, et al., manuscript in preparation); and (iv) the marked differences in brilliance and morphology of purified versus intracellular typhus and spotted fever group rickettsiae stained with corresponding fluorescein-conjugated antibodies (C. L. Wisseman, Jr., et al., manuscript in preparation).

In addition, scattered observations by others lend support to this hypothesis: (i) the demonstration of appreciable quantities of antigen in the rickettsia-free supernatants of infected-tissue homogenates (5); (ii) the liberation of soluble antigen and morphologically distinct surface material by treatment of rickettsial suspensions with ethyl ether (5, 31, 34, 35); (iii) the suggestion of an easily removed layer exterior to the cell wall in yolk sac-grown organisms (1); and (iv) the regular occurrence of an electron-lucent zone surrounding rickettsial bodies in ultrathin sections of infected cells (2, 10, 32, 33).

Collectively, these observations suggest that the typhus group, and probably also the spotted fever group, of rickettsiae possess a layer (in addition to the so-called "microcapsular" layer) external to the cell wall which (i) is substantial in size, (ii) is easily removed by ordinary laboratory procedures applied to extracellular organisms, (iii) is electron-lucent in conventional preparations, (iv) is probably complex in structure and composition, and (v) is the locus of major group-specific antigens.

The present study yields electron microscopic evidence of a substantial complex layer of rickettsia-specific material external to the cell wall of typhus and spotted fever group rickettsiae. This has been possible through application of two technical principles. The first is the liberation of rickettsiae from host cells with minimal manipulation. The second is the stabilization of

this extracellular material by reaction with specific antibody immediately upon liberation of the organism from its host cell, an important principle demonstrated recently by Bayer with the polysaccharide layer surrounding certain *Escherichia coli* strains (7, 8) and by others (4, 17, 20, 22, 23, 25, 30, 33) for a variety of both gram-positive and gram-negative organisms.

MATERIALS AND METHODS

The virulent Breinl and attenuated E strains of *R. prowazekii,* and the Sheila Smith strain of *R. rickettsii,* all plaque purified (Wisseman, et al., manuscript in preparation), were grown in secondary chicken embryo fibroblasts in tissue culture as previously described (39, 40) in the presence of half-strength Dulbecco modification of Eagle basal medium with Earle salts containing 0.1% glucose (Grand Island Biological Co.) and 10% fetal calf serum. The chicken embryo cells, infected in suspension (Wisseman, et al., manuscript in preparation), were grown as monolayers on 75-cm^2 plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.) at 32°C in an atmosphere of 5% CO_2 in air. After approximately 48 h of incubation, the infected chicken embryo cells were removed from the substrate with 0.05% trypsin–0.02% ethylenediaminetetraacetic acid, washed by centrifugation in fresh medium, resuspended in 1 to 2 ml of fresh medium, and transferred to a tight-fitting Dounce homogenizer. Either human convalescent antityphus serum, human or guinea pig convalescent anti-Rocky Mountain spotted fever serum, normal human serum, or rabbit anti-yolk sac serum was added to the washed suspension of infected chicken embryo cells. These cells were gently disrupted by about 40 strokes of the Dounce homogenizer. Examination by phase-contrast microscopy revealed that between 50 and 70% of the cells were broken. After an incubation period of 30 to 60 min either at ambient temperature or in crushed ice to allow the antiserum to react (the temperature appeared to be unimportant), the suspension was fixed by adding 5 to 10 volumes of a solution of 5% acrolein and 0.25% glutaraldehyde (both from Polysciences, Warrington, Pa.) prepared in 50 mM cacodylate (Na) buffer, pH 7.3, either for 1 h at 22°C or overnight (18 h) at 4°C. The material was washed once in cacodylate buffer by centrifugation at 17,000 × *g* and was postfixed in 1% osmium tetroxide (in 50 mM cacodylate) containing 1% tryptone and 0.5% NaCl for 1 h at 22°C. The organisms were suspended in 2% Noble agar, cut into 1-mm^3 blocks, stained for 2 h with a 2% aqueous solution of uranyl acetate, dehydrated in an ascending ethanol series, and embedded in Epon 812 by the method of Luft (24). Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome with a DuPont diamond knife, picked up on carbon-coated collodion grids, and stained with uranyl acetate and lead citrate. Specimens were viewed in a Siemens Elmiskop 1A electron microscope operating at 80 kV.

In other experiments, various special procedures were performed on typhus rickettsiae. (i) After treatment with specific antiserum, the disrupted cells were fixed as described above in the acrolein-glutaraldehyde fixative. However, in these experiments, ruthenium red (Ladd Research Industries) was added to the fixative to a concentration of 0.35 mg/ml. After a 1-h incubation at 22°C in this solution, the cells were washed 1× in 50 mM cacodylate buffer and postfixed in 1% osmium tetroxide containing 1% tryptone, 0.5% NaCl, and 0.35 mg of ruthenium red per ml for 1 h at 22°C. After postfixation the cells were processed for electron microscopy as described above. (ii) Ultrathin sections of Dounce-liberated, antibody-stabilized rickettsiae were stained by the silver-methenamine technique described by Dhir and Boatman (16).

RESULTS

Studies on *R. prowazekii.* Ultrathin sections through chicken embryo cells in tissue culture which had been infected with either the Breinl or attenuated Madrid E strain of *R. prowazekii* consistently revealed an electron-lucent zone of 25 to 130 nm surrounding the organisms in the host cell cytoplasm (Fig. 1). This zone failed to stain with the conventional methods used in electron microscopy, i.e., osmium tetroxide, uranyl acetate, and lead citrate. Suspecting that this electron-lucent zone contained material synthesized and secreted by the rickettsiae, we approached the problem as did Bayer for *E. coli* (7, 8).

The capsular material surrounding *E. coli,* which also did not stain by conventional methods, could be rendered visible in ultrathin sections when the organisms were exposed to specific anticapsular antiserum before fixing and processing. Accordingly, we applied this principle to *R. prowazekii* by gently disrupting infected chicken embryo cells in the presence of potent anti-typhus antibody. We hoped that by using minimal mechanical force we would avoid washing away rickettsial surface material, possibly stabilize this material by reaction with antibody, and, finally, render it visible as in the case of *E. coli* referred to above. This procedure was successful in the first attempt and was reproducible. Figure 2 shows a large fibrous layer of electron-opaque material surrounding an organism of the virulent Breinl strain of *R. prowazekii.* Similar preparations in which fetal calf serum, normal human serum, or rabbit anti-yolk sac serum was substituted for the typhus-immune serum failed to show this material (Fig. 3a and b).

The cell-associated materials external to the outer membrane of the rickettsiae as revealed by antibody treatment appeared complex. Antibody-treated rickettsiae regularly possessed the following structures external to the cell wall when examined by thin-sectioning techniques: (i) a well-defined zone of 15 to 20 nm directly adjacent to the outer membrane (described in

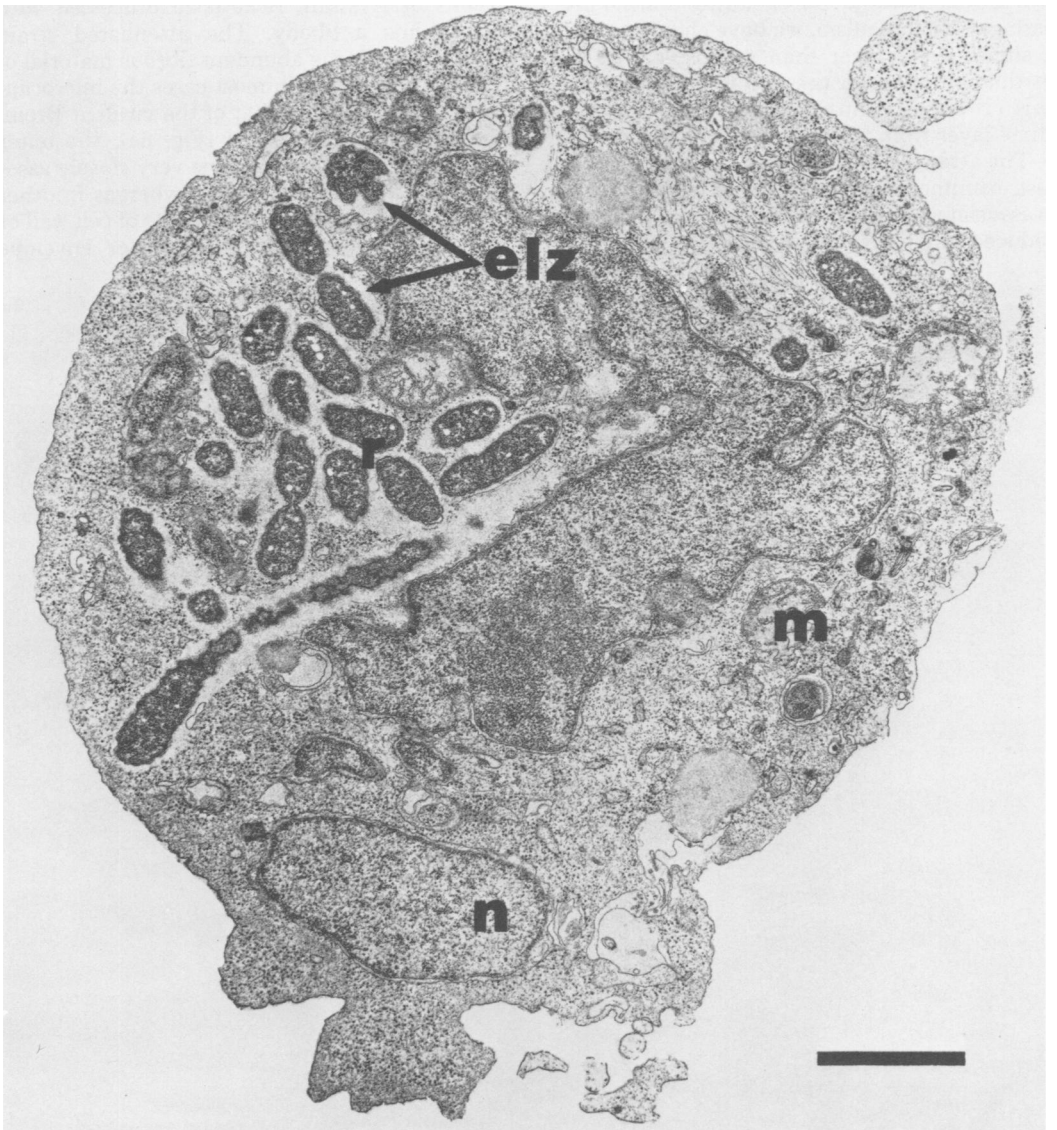

FIG. 1. *Ultrathin section through chicken embryo fibroblast infected with the virulent Breinl strain of R. prowazekii. elz, Electron-lucent zone; m, mitochondrion; n, host cell nucleus; r, rickettsiae. Bar = 1 μm.*

more detail below) and (ii) a large, electron-dense fibrous layer which extended up to 300 nm from the outer region of the zone in (i) above. Occasionally, depending on the sectioning plane, the 15 to 20 nm zone was absent, and the dense fibrous layer appeared to originate directly from the outer membrane as shown in Fig. 2. The two zones described above may correspond, at least in part, to the thick layer shown by freeze-etching of intracellular organisms and to the fibrous material seen associated with the external surface (Brown et al., manuscript in preparation). The smaller zone on rickettsiae has been described by others as a microcapsular layer (1, 5, 29).

Occasionally, very large outer layers can be demonstrated on the surface of free, antibody-treated typhus rickettsiae (Fig. 4). Because the outer layer on free organisms is sometimes much larger than the electron-lucent zone surrounding intracellular organisms, it is possible that the host cell cytoplasm acts as a limiting boundary

for the fibrous layer. Because of the ease with which this material is lost during routine processing of the organism, we have chosen to call it a slime layer rather than a capsule, although distinction between the two is based solely on this criterion. Figure 5 shows diffusion of the slime layer from the surface of the organism.

The attenuated E strain of *R. prowazekii* was also examined to determine whether attenuation is associated with either a lack of or a drastically reduced amount of the materials on the outer membrane. Figures 6a and b are electron micrographs of E strain rickettsiae processed with anti-typhus antibody. The attenuated strain possesses both the abundant fibrous material of the slime layer and in most cases the microcapsular layer similar to that of the virulent Breinl strain. In some instances (Fig. 6a), the outer portion of the cell envelope is very closely associated with the fibrous layer, whereas in other areas, a zone containing fragments of cell wall or cell membrane separates the outer envelope

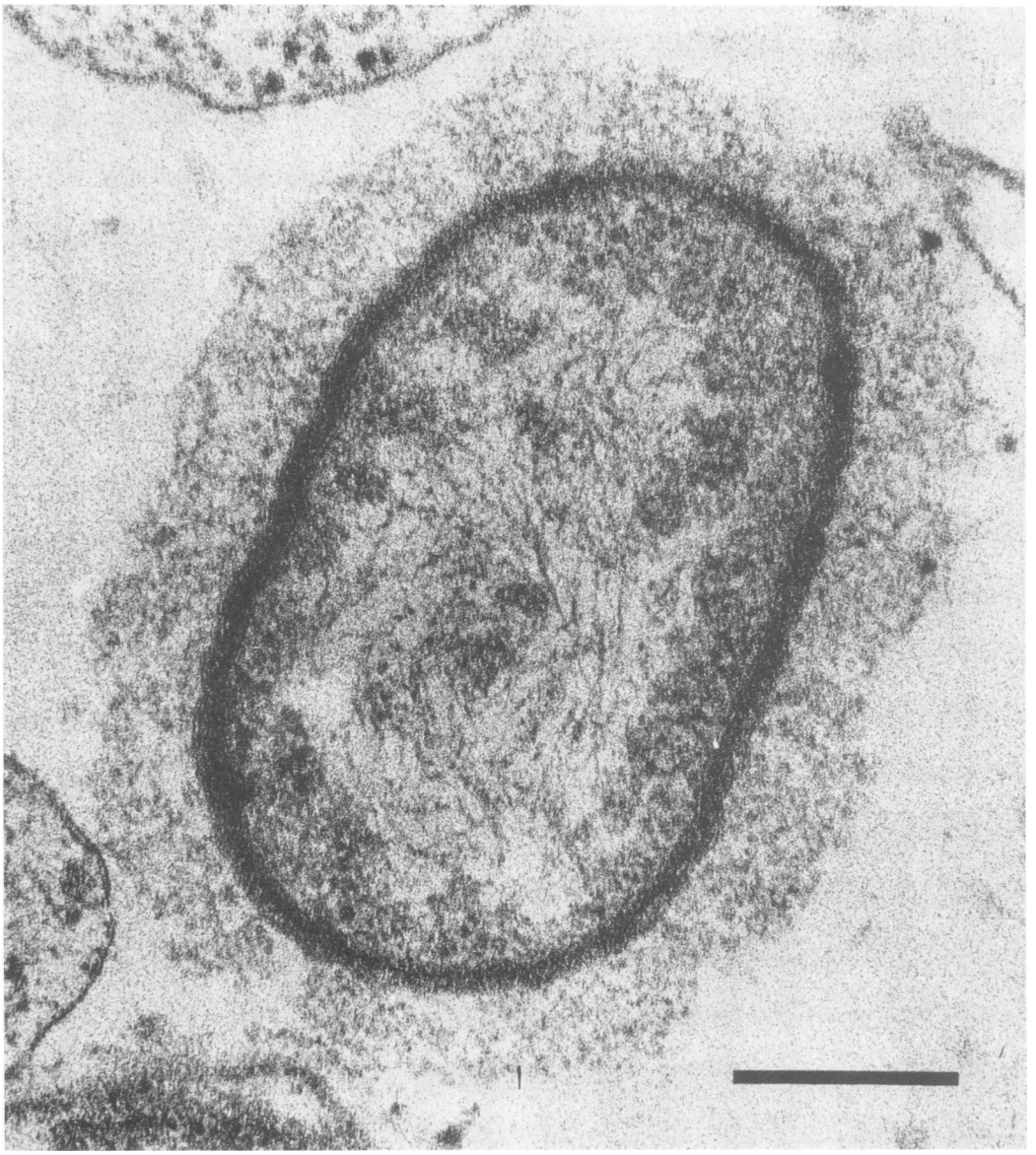

FIG. 2. *Thin section of the Breinl strain of R. prowazekii released from chicken embryo host cell in the presence of specific antityphus serum. Bar = 0.2 μm.*

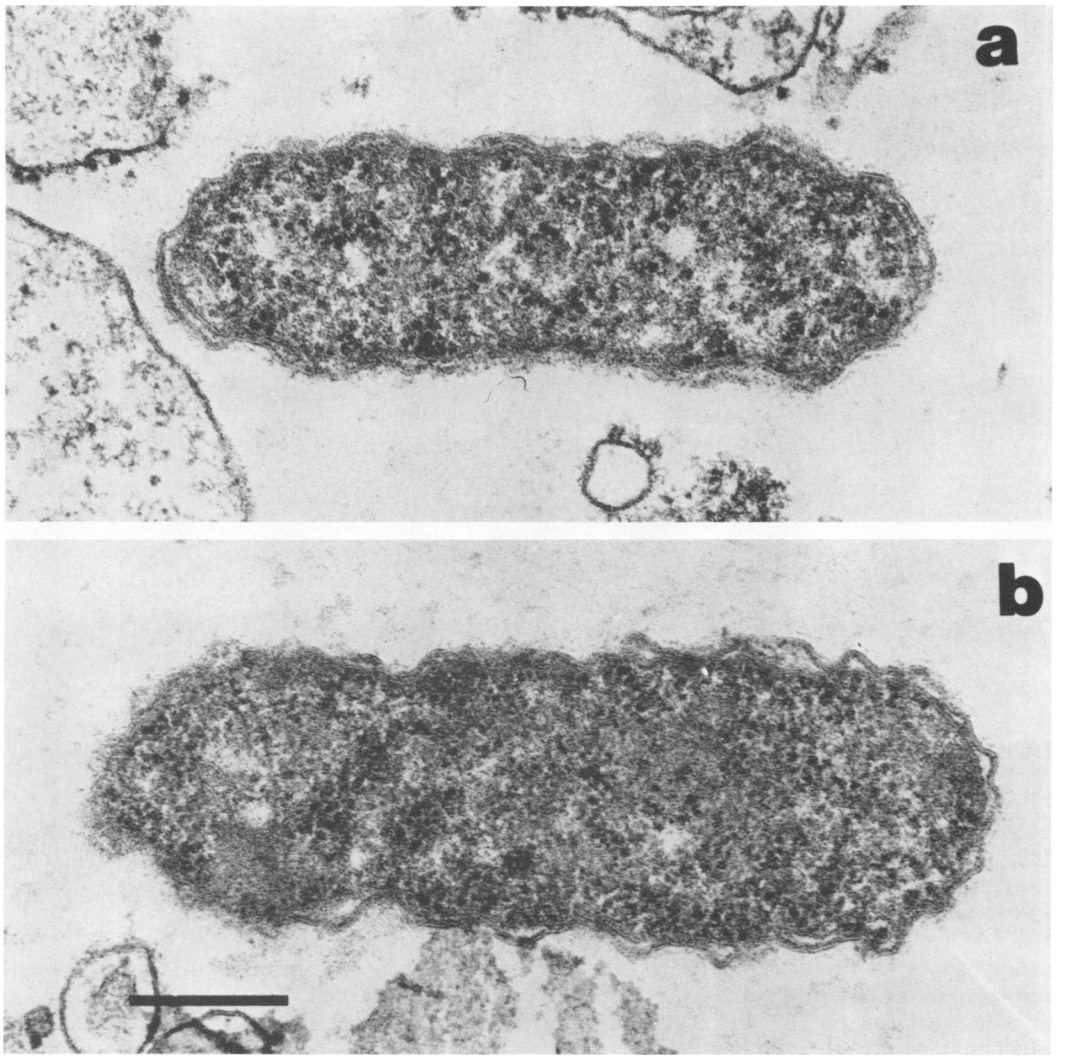

FIG. 3. *(a) Thin section of the Breinl strain of R. prowazekii released from chicken embryo host cell in the presence of normal human serum. Bar = 0.25 μm. (b) Thin section of the Breinl strain of R. prowazekii released from chicken embryo host cell in the presence of rabbit anti-yolk sac serum. Bar = 0.25 μm.*

from the fibrous layer. The latter observation was rare and was not seen in thin sections of the Breinl strain or in members of the spotted fever group of rickettsiae. In addition, evaginations of the cell wall appear within this zone, suggesting that this cell is undergoing autolysis. Concentric layering of the fibrous material is also evident around this cell, although this was a rare observation. Figure 6b, also a section through the Madrid E strain, shows diffusion of the fibrous layer from the cell wall.

Because of the likelihood that the slime layer and possibly the microcapsule consist of, or are rich in, polysaccharides, two methods for demonstrating cell surface polysaccharides were applied to gently released, antibody-stabilized *R. prowazekii:* (i) ruthenium red (12, 13, 19, 21) and (ii) silver methenamine (16).

Ruthenium red is capable of binding to mucopolysaccharides, but when used in conjunction with osmium tetroxide, it may also react with some of the more polar lipids and with proteins (19). Studies in our laboratory on *R. prowazekii* with ruthenium red showed the following staining pattern (Fig. 7): (i) the microcapsular layer (described previously) which did not stain uniformly with ruthenium red, but rather showed small, electron-dense projections with a 13-nm

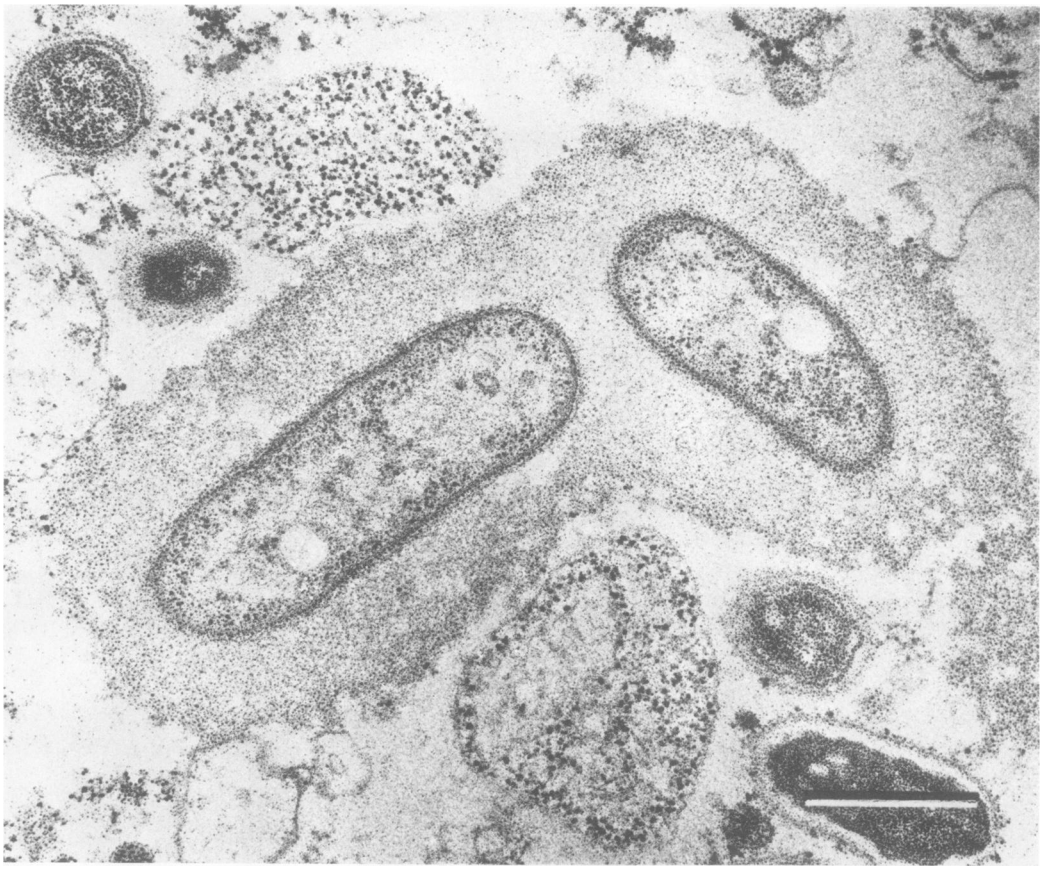

FIG. 4. *Thin section of the Breinl strain of R. prowazekii similar to that shown in Fig. 2. Two rickettsiae appear to be embedded in the large, slime-like layer. Bar = 0.5 μm.*

periodicity (27–29) radiating outward from the surface of the cell wall; and (ii) a layer of variable thickness external to the microcapsular layer which stained densely with ruthenium red and presumably represents the slime layer.

Further studies to determine the nature of the slime layer were carried out by the silver methenamine technique to stain polysaccharide-containing structures. Figures 8a and b show cells of *R. prowazekii* released from chicken embryo cells in the presence of specific antibody as described above and stained by this method. In Fig. 8a, a 30- to 40-nm clear (non-silver staining) region completely surrounds the rickettsiae and separates two distinct areas that react positively with the silver stain, the cell wall and an outer layer. The outer layer, although quite small in this cell, presumably is the same layer which stains with ruthenium red (Fig. 7). In the cell in Fig. 8b, both the clear zone and a larger outer layer are present. On certain areas of the cell, there is little evidence of the microcapsular layer (possibly a function of the sectioning plane), and the outer layer appears to be contiguous with the rest of the cell.

Studies with *R. rickettsii*. Electron-lucent zones have also been observed by us and by others (2) surrounding *R. rickettsii* in ultrathin sections through infected cells. Accordingly, when *R. rickettsii* in chicken embryo cells was subjected to the same gentle disruption-antibody treatment, with either a *R. rickettsii* convalescent guinea pig serum or the serum of a patient recently convalescent from Rocky Mountain spotted fever, both outer fibrous and inner microcapsular areas similar to those demonstrated with *R. prowazekii* were observed (Fig. 9b). Neither normal serum nor anti-yolk sac serum stabilized the slime-like material on the surface of the wall.

R. rickettsii actively escapes from infected host cells by passage through the plasma membrane during the early stages of the infection cycle and accumulates in the culture medium where it is again capable of infecting new cells (36). We took advantage of this phenomenon to

determine whether *R. rickettsii*, which had escaped from host cells by natural processes and was capable of infecting other host cells, carried a substantial amount of the slime layer with it in the process. The medium from 72-h *R. rickettsii*-infected flasks of chick embryo cells was replaced with culture medium containing 5 to 10% Rocky Mountain spotted fever convalescent serum. The culture was reincubated overnight. On the next morning the antibody-containing medium was removed, and the rickettsiae which had escaped into the medium overnight were sedimented by centrifugation and fixed.

Rickettsiae bearing substantial quantities of the slime layer were readily found (Fig. 9a).

The culture supernatant fluid also contained rickettsiae associated with host cell fragments (Fig. 10). In some instances the rickettsiae appeared to be completely enclosed in vesicles formed by host cell membrane (Fig. 10a) and, in this instance, the membrane prevented the antibody in the medium from reacting with the slime layer. Only the microcapsule is partially visible. In other instances (Fig. 10b), host cell membrane fragments were closely applied to the outer surface of the slime layer and, in effect, partially surrounded the organism. Antibody in the medium appeared to have access to the slime layer, perhaps to stabilize it and to render it electron dense.

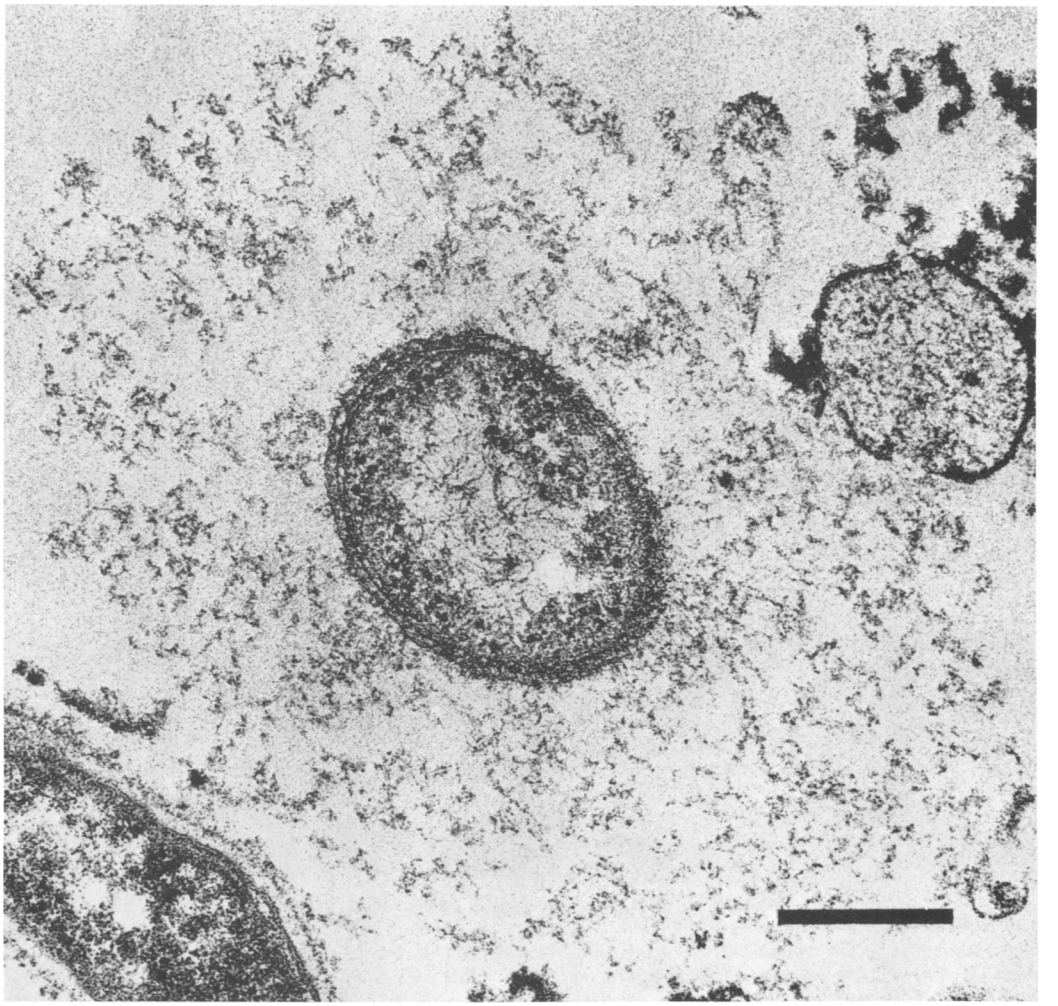

FIG. 5. *Thin section of Breinl strain of R. prowazekii in which the fibrous layer around the cell is being dispersed into the surrounding milieu. Bar = 0.25 μm.*

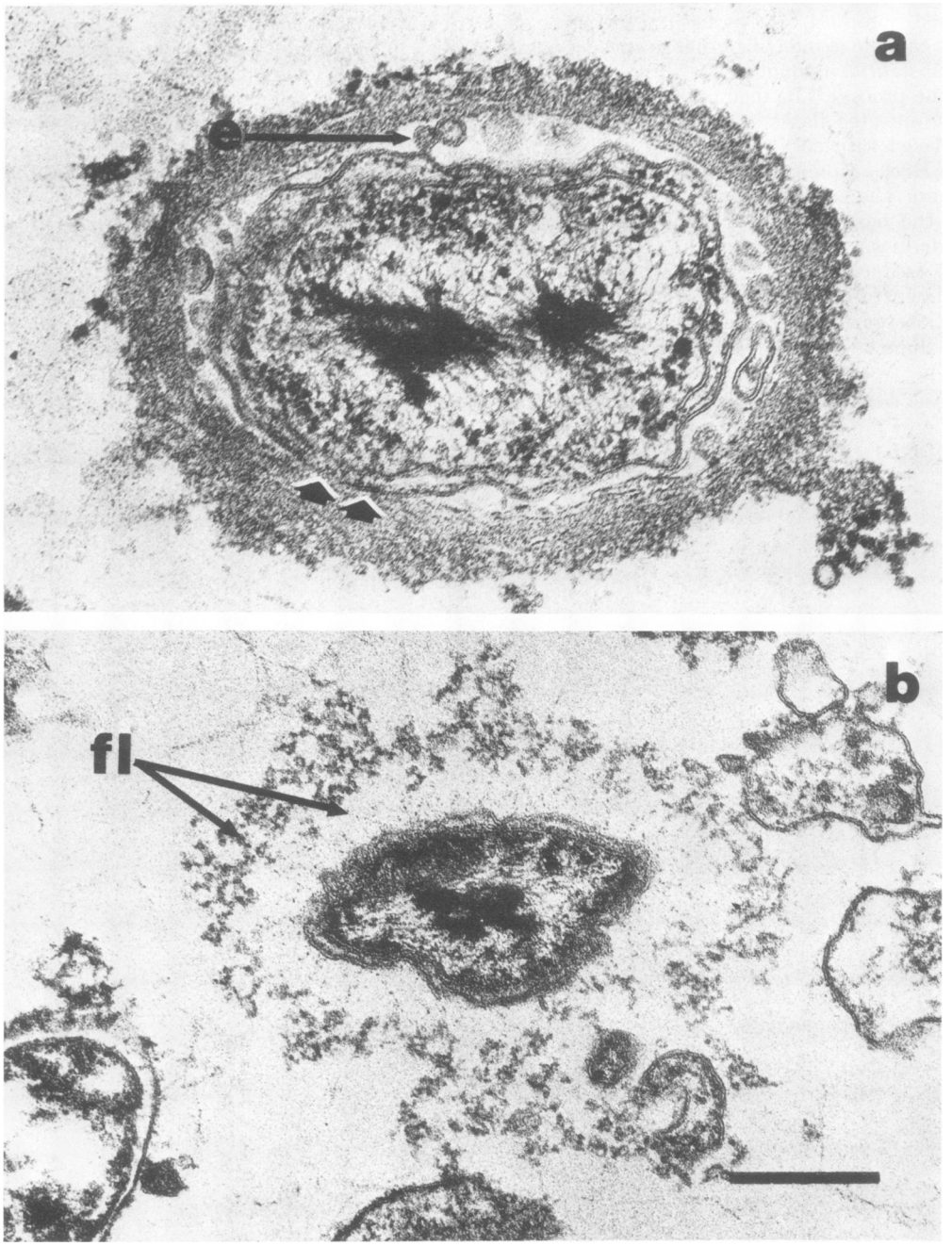

FIG. 6. *(a) Ultrathin section of the avirulent Madrid E strain of R. prowazekii released from cultured chicken embryo cells in the presence of specific anti-typhus serum. Note fibrous slime layer which appears to circumscribe the cell in a concentric manner, and also the electron-lucent area in which fragments of cell wall or cell membrane are seen. e, Evagination of cell wall; close adhesion of slime layer and cell wall (short arrows). (b) Thin section similar to that in (a). Slime layer (arrows) seems to be sloughing from the outer envelope of the rickettsia. fl, Fibrous layer. Bar = 0.25 μm for (a) and (b).*

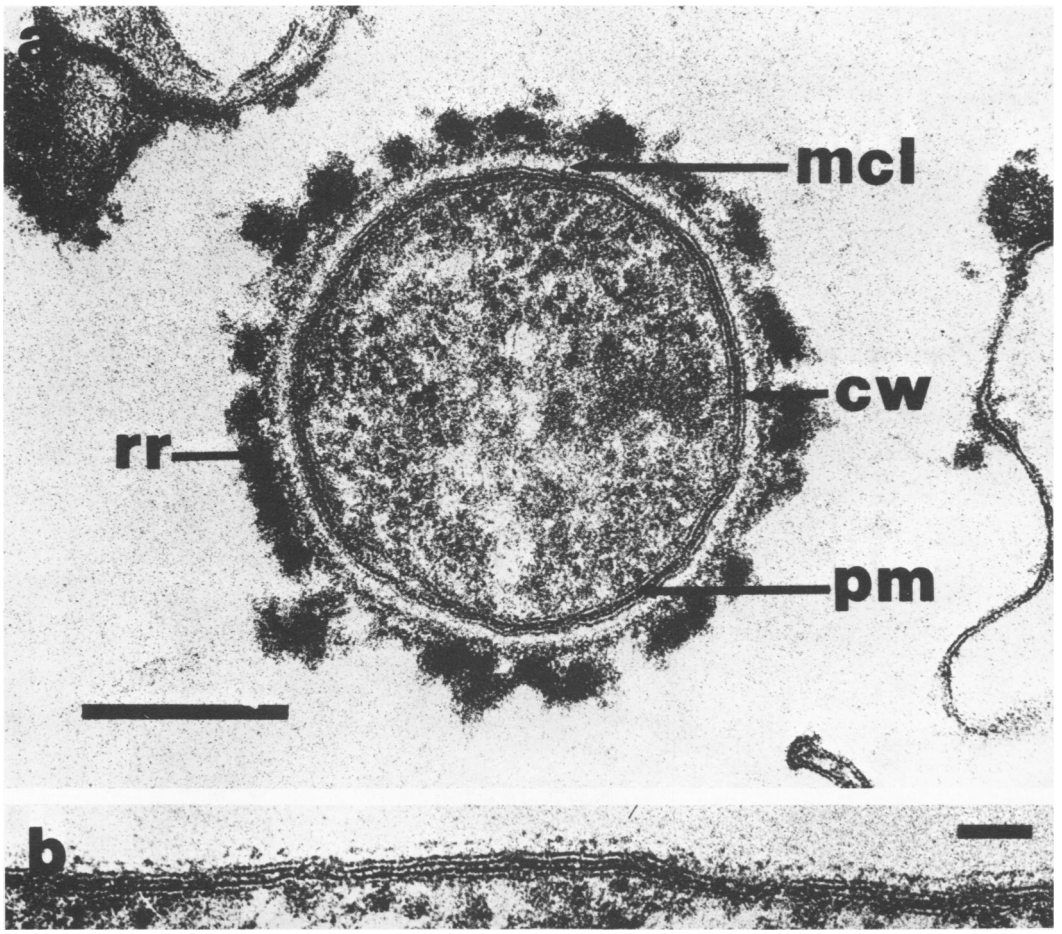

FIG. 7. *(a) Ultrathin section of the Breinl strain of R. prowazekii from a preparation which was stained during the fixation period with ruthenium red. rr, Ruthenium red staining material; mcl, microcapsular layer; cw, cell wall; pm, plasma membrane. Bar = 0.2 μm. (b) Ruthenium red-stained preparation of the Breinl strain of R. prowazekii showing microcapsular layer with projections radiating outward from the cell envelope. Bar = 50 nm.*

DISCUSSION

Morphologically, the structure of the typhus and spotted fever group of rickettsiae conforms to that traditionally described for a variety of gram-negative organisms (9, 11, 26). Members of these two groups possess the characteristic five-layered cell wall complex typical of gram-negative bacteria, and, in addition, a microcapsular layer directly adjacent to the outer envelope (1, 2, 29; Brown et al., manuscript in preparation).

We have demonstrated in this series of experiments that, in addition to the above structural features, these rickettsiae possess a large slime layer closely apposed to the external surface. Although we have suspected the presence of a slime layer on the surface of these organisms for some time, it has been only recently that we have found suitable methods for staining this structure so that it could easily be visualized. The slime layer appears to be a very labile entity in that it is easily removed during routine processing of the organisms. Only when specific antiserum is added to the suspending medium into which the rickettsiae are to be released from infected host cells can this highly labile structure be retained on the cell surface. Attempts at stabilizing the slime layer with either normal, nonimmune sera or anti-yolk sac sera were not successful.

The demonstration of a slime layer on the surface of rickettsiae lends support to our hypothesis that the large electron-lucent zone surrounding the organisms in the cytoplasm of var-

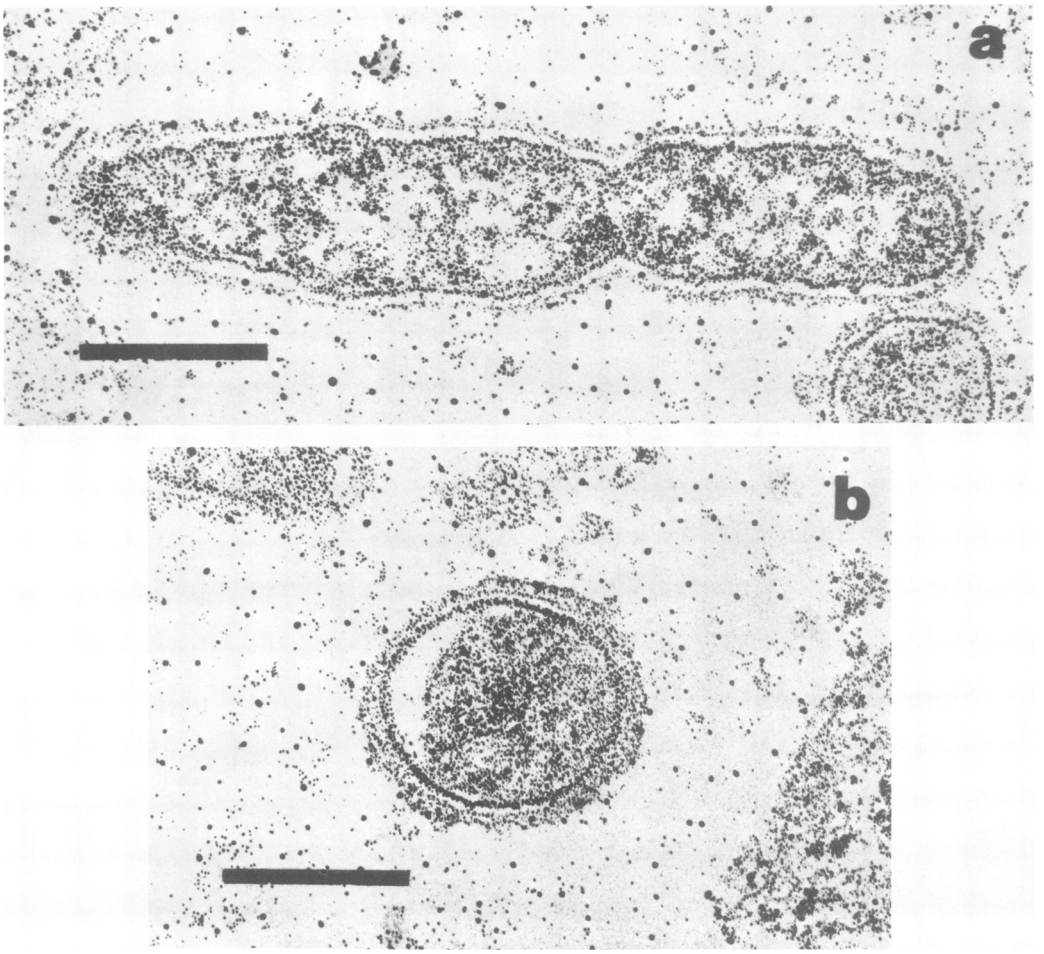

FIG. 8. *Ultrathin section of the Breinl strain of R. prowazekii released from chicken embryo host cell in the presence of specific anti-typhus serum. The sections were hydrolyzed with periodic acid and stained with silver methenamine. Bar in (a) and (b) = 0.5 μm.*

ious host cell systems and in louse feces (2, 10, 32, 33) is not due solely to destruction of the host cell by the rickettsiae (10) or to a shrinkage artifact (2) as has been suggested, but, in fact, to a large extent to a very large fibrous structure physically similar to the slime layers and capsules of many other gram-negative and gram-positive bacteria (4, 8, 17, 20–23, 25).

Although chemical analysis of the slime material has not yet been completed, the electron micrographs suggest initially that the slime material is polysaccharide in nature. This hypothesis is based on the following observations: (i) the electron-lucent nature of the slime layer in infected host cells (the conventional fixatives glutaraldehyde and osmium tetroxide do not, with very few exceptions, react with polysaccharides and, thus, would not permit good visualization of this structure by electron microscopy); and (ii) ruthenium red and silver methenamine, both of which are used for selective staining of polysaccharides, reacted positively with the slime layer. Although these studies strongly suggest the polysaccharide nature of this material, further confirmatory studies will be carried out to determine the precise chemical composition. Because the virulence of bacteria such as the pneumococcus has unequivocally been attributed to the presence of a large extracellular capsular polysaccharide, we tested both the virulent Breinl and attenuated E strains of *R. prowazekii* to determine whether this phenomenon applied to this system. No detectable differences were found except on occasion the concentric layering of the slime material on the surface of E strain organisms. The significance

of this is not known. Nevertheless, it is clear that the attenuation of the E strain is not due to absence of slime layer and, hence, is not merely a "rough" strain comparable to those of certain bacteria (15) and Phase II of *Coxiella burnetii* (37).

In addition to the typhus organisms, we have examined *R. rickettsii,* reported here, as well as several other members of the spotted fever group of rickettsiae (Wisseman et al., unpublished data). Except for minor variations in size (an observation which might reflect the antibody

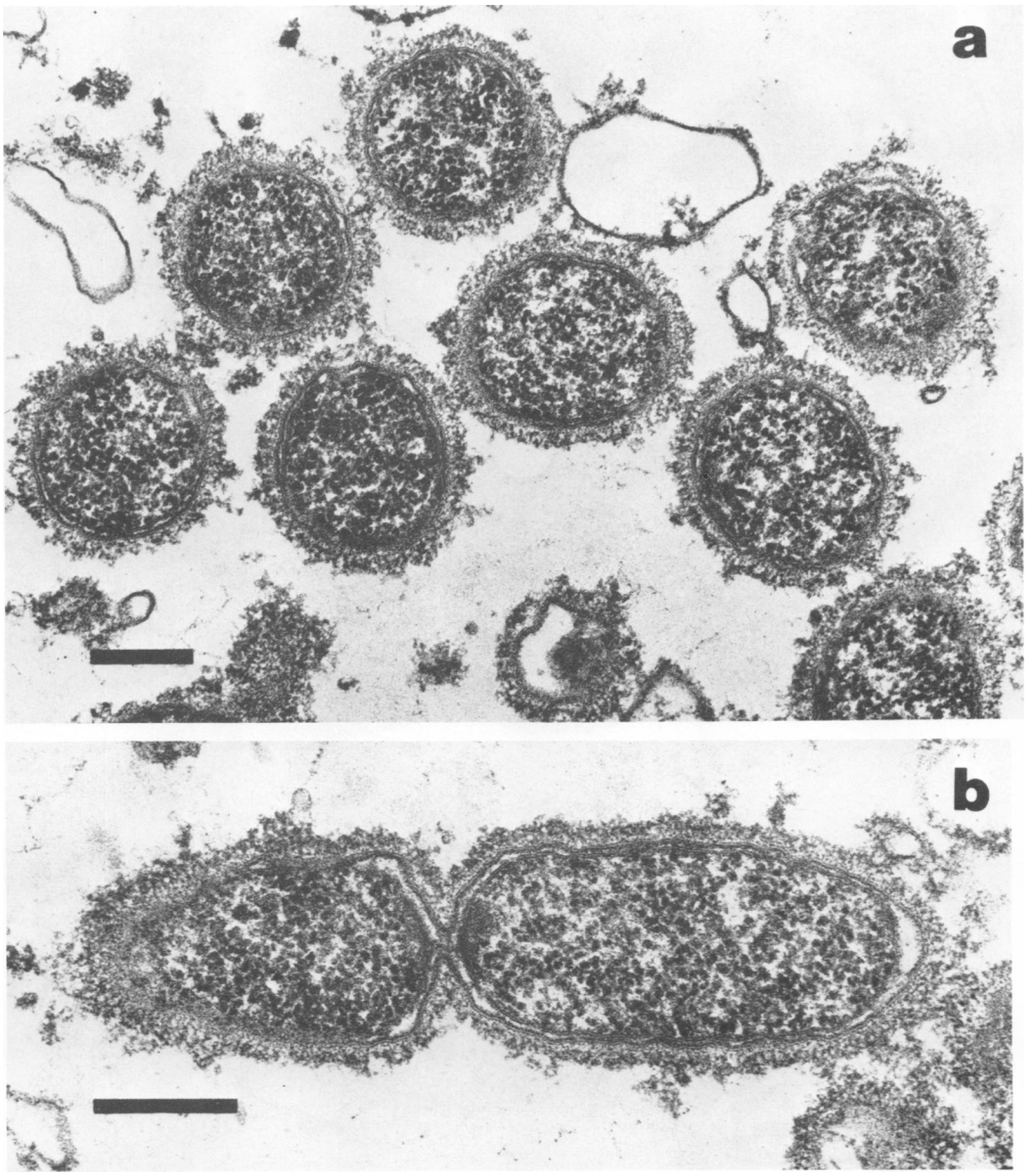

FIG. 9. *(a) Ultrathin section of R. rickettsii which had escaped from infected chicken embryo cells. The slime layer material was stabilized on the surface of the rickettsiae by the addition of Rocky Mountain spotted fever convalescent serum 18 h before harvesting the culture fluid. Bar = 0.25 μm. (b) Ultrathin section of R. rickettsii released from chicken embryo host cell in the presence of specific anti-Rocky Mountain spotted fever serum. Bar = 0.3 μm.*

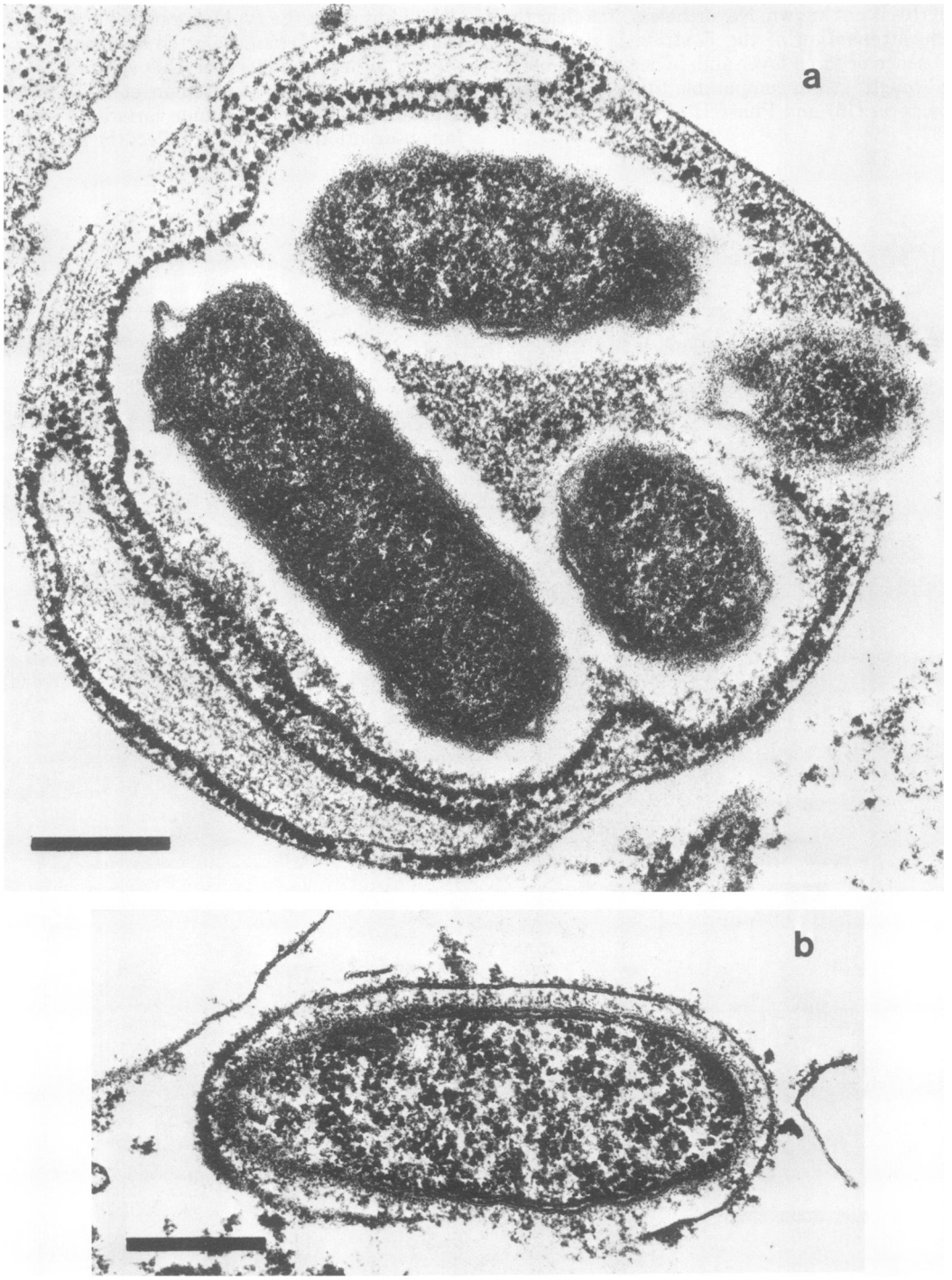

FIG. 10. *(a) Thin section through fragment of partially disrupted chicken embryo cell infected with R. rickettsii as in Fig. 9a and b. Note large electron-lucent zones surrounding the rickettsiae which were not exposed to the antiserum. Bar = 0.3 μm. (b) Thin section from same preparation as (a). Host cell membrane surrounds most of the organism, but is sufficiently damaged at one point to permit antiserum to gain entry and partially stabilize portions of the slime layer. Bar = 0.3 μm.*

concentration in the immune serum), all members examined had a fairly large slime layer on their cell surfaces.

To speculate on a possible function for the slime layer of the rickettsiae is hazardous at this time. However, two very realistic possibilities warrant consideration.

The antiphagocytic property associated with bacterial capsules such as the pneumococcus is well known. A similar function could be postulated for rickettsial slime layers. Although the opsonic effects of immune serum on purified or partially purified rickettsiae fed to human macrophages and to human and mouse polymorphonuclear neutrophils have been demonstrated (3, 18, 37), it would be of interest to determine whether the presence of appreciable amounts of slime material on the rickettsiae exhibits an antiphagocytic effect. Earlier studies (38) in these laboratories, performed with purified yolk sac-grown rickettsiae, which in the light of information presented here may have lost much of their slime layer, showed no unusual resistance to phagocytosis by polymorphonuclear neutrophils. On the other hand, the phase I polysaccharide antigen of *C. burnetii* was associated with a substantial antiphagocytic action with human polymorphonuclear neutrophils which was overcome by opsonization with antisera containing antibodies to phase I antigen (37).

Another potential but untested function of the slime layer of rickettsiae might be a role in attachment to host cells. Jones et al. (21) and more recently Costerton et al. (14) have shown that many bacteria in their natural environment possess a glycocalyx on their surfaces (a structure which appears morphologically similar to our slime layer) which is composed of fibrous polysaccharide strands. These authors suggest that, through this glycocalyx, bacteria attach to various substrates, cells, or other organisms, a concept of great biological significance with respect to the particular ecological niche to which the organism has adapted. The possibility that the slime layer of the rickettsiae may function in an analogous capacity, namely, the attachment to host cell in preparation for penetration (ET growth cycle), as an adaptation to obligate intracellular parasitism, is very appealing. Thus, the slime layer of typhus and spotted fever group rickettsiae may represent adaptations of a microorganism to its ecological niche which is of relevance to two domains of human scientific inquiry: (i) that of the medical microbiologist who is concerned with virulence (resistance to phagocytosis) and immunity (opsinization by antibodies) and (ii) that of the nonmedical microbiologist concerned with specialized mechanisms of surfaces important to the unique ecological niche occupied by the microorganism, whether it is a rock in a running stream (21); a cow's rumen (14), teeth (14), pharyngeal, or intestinal epithelium (14); or, in the current context, the cell membrane of a cell host to an intracellular parasite.

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