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Ultrastructural Investigations on Surface Structures Involved in *Coxiella burnetii* Phase Variation

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By using the cytochemical staining procedure with concanavalin A, horseradish peroxidase, and diaminobenzidine, no surface carbohydrates with terminal α-glucosyl or sterically closely related residues could be detected on the cell walls of *Coxiella burnetii* phases I and II. Using a polycationized ferritin derivative as a cytochemical probe, anionic binding sites were visualized in the electron microscope on cell membranes of *C. burnetii* phase II, but not on phase I organisms. The sites appeared to be masked in phase I particles. Anionic sites could be demonstrated on phase I organisms after treatment with $NaIO_4$ or dimethyl sulfoxide. A number of different biological properties of *C. burnetii* phases I and II may depend on the presence or absence of a net negative charge on the surface of the cell walls of these organisms.

In nature, *Coxiella burnetii* exists in antigenic phase I, whereas during passages in embryonated eggs, usually more than 10 (28), it converts to phase II, with an apparent loss of its phase I surface antigen. In animals, phase II strains convert rapidly, within one or several passages, to phase I. Linked with the antigenic status are certain other biological properties of phase I and II organisms.

Phase I particles, resembling "smooth" bacteria, are stable in purified suspensions; they do not agglutinate spontaneously and they are phagocytized only to a low degree in the absence of specific antibodies (6, 7, 11, 30). Organisms in phase I are much easier to purify than those in phase II (15). They manifest a low avidity for biological stains and react specifically in the indirect fluorescent antibody test (14). In general, phase I strains appear to be more virulent than phase II strains (21).

Phase II organisms resemble "rough" bacteria. They usually agglutinate spontaneously and are strongly agglutinated by normal serum (15, 19). They are readily phagocytized in the absence of specific antibodies (6, 7, 30). Phase II organisms have a remarkable avidity for certain stains, such as hematoxylin, basic fuchsin, and acridine orange, and they may react nonspecifically in the indirect fluorescent antibody test (14). The organisms also differ in their buoyant density (17).

Organisms in phase I differ in protein, nucleic acid, and polysaccharide content, as well as in polysaccharide composition, from particles in phase II. Glucuronic acid appears to be the sugar in greatest concentration in *C. burnetii* phase I cell envelopes, whereas only glucose and galactose could be demonstrated in phase II cell envelopes (18).

We have used cytochemical techniques to investigate relevant surface structures presumably involved in *C. burnetii* phase variation. Surface carbohydrate structures were studied by a cytochemical staining procedure (2, 22) with concanavalin A (ConA), horseradish peroxidase, and diaminobenzidine. The localization, distribution, and chemical nature of anionic sites on the surfaces of *C. burnetii* membranes were investigated by using a polycationized derivative of ferritin (10, 23).

MATERIALS AND METHODS

Organisms. *C. burnetii* phase I, strain Herzberg$^+$, in egg passage 2; strain Munich, in egg passage 4 (kindly provided by F. Weyer, Tropeninstitut Hamburg); *C. burnetii* phase II, strain Henzerling, in egg passage 18; and strain Nine Mile, in egg passage 56, were used.

Propagation and purification procedures. All strains were propagated in embryonated eggs. Infected yolk sacs were harvested and homogenized in 5× the volume of phosphate-buffered saline containing formaldehyde or glutaraldehyde (final concentration, 0.4 and 1%, respectively). The suspensions were kept at 4°C for 24 to 48 h. The inactivated organisms were purified by two cycles of fluorocarbon extraction, as described previously (24). Briefly, after addition of 20% Frigen 113 TR-T (Hoechst, Frankfurt), the suspensions were homogenized and centrifuged at 500 × *g* for 20 min. The organisms were extracted by repeated washing of the sediment and pelleted by centrifugation at 10,000 × *g* for 1 h.

The organisms were washed by resuspending and centrifuging once in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (0.002 M Tris, pH 7, in 0.145 M NaCl), once in Tris-buffered saline containing 0.1 M NH_4Cl to block any unreacted aldehyde groups of the membrane-bound formaldehyde or glutaraldehyde, and, finally, twice in Tris-buffered saline.

Cytochemical procedure. (i) ConA. The organisms were suspended in 5 ml of phosphate-buffered saline (0.002 M phosphate, pH 7.0, and 0.145 M NaCl) containing 50 μg of ConA per ml. The mixture was incubated for 30 min at 22°C. After centrifugation, the particles were thoroughly washed by resuspension in 5 × 5 ml of phosphate-buffered saline.

Coupling to the peroxidase was performed by suspending the organisms in 5 ml of phosphate-buffered saline containing 50 μg of horseradish peroxidase per ml. The reaction was allowed to proceed for 30 min at 22°C. The catalytic activity of the peroxidase coupled to ConA was revealed by the diaminobenzidine method (2, 22). *Mycoplasma mycoides* subsp. *capri* was used as a positive control (22).

(ii) Polycationized ferritin. The rickettsiae were suspended in Tris-buffered saline to a 20% cell suspension and allowed to react with the polycationized ferritin (Miles-Yeda Ltd., Rehovoth, Israel). Routinely, the final concentration of the polycationic ferritin was 0.75 mg/ml, which was allowed to react for 30 min at 22°C with the rickettsial suspension. The reaction was stopped by dilution, and the organisms were centrifuged and washed three times in Tris-buffered saline (24).

Chemical and enzymatic treatments of *C. burnetii*. Aldehyde-fixed organisms were treated with various chemical and enzymatic reagents: (i) dimethyl sulfoxide (Me_2SO), 20 h, 37°C (20); (ii) $NaIO_4$, 0.01 M, 4 h, 37°C (26); (iii) trichloroacetic acid, 0.5 N, 4 h, 0°C (1); (iv) phenol, 5 M, 10 min, 65°C (25); (v) Pronase P from *Streptomyces griseus* (Serva, Heidelberg), 20 μg in 1 ml of Tris-buffered saline, pH 7, 30 min, 22°C (23); (vi) hyaluronidase (Serva, Heidelberg), 1 mg/ml, 1 h, 37°C; (vii) lysozyme (Serva, Heidelberg), 1 mg/ml, 1 h, 37°C; (viii) chloroform-methanol, 2:1 (vol/vol), 30 min, 22°C ("lipid-extracted rickettsiae"); (ix) aqueous acetone (acetone-saline-NH_4OH, 9:1:0.05, vol/vol), 30 min, 22°C ("lipid-depleted [16] rickettsiae"); (x) anhydrous methanolic hydrochloric acid (0.1 N HCl in methanol), 20 h, 37°C. The chemically and enzymatically treated organisms were centrifuged, washed three times and cytochemically treated as described above.

Electron microscopy. After cytochemical treatment, the organisms were fixed for 2 h at 4°C with 1% osmium tetroxide in 0.025 M sodium cacodylate buffer, dehydrated in acetone, and embedded in Durcupan (Fluka, Basel, Switzerland). Thin sections were examined unstained in a Siemens Elmiskop 102 at 80 kV. Some studies were performed with suspensions of *C. burnetii*, which were treated with polycationized ferritin and directly applied to carbon-coated copper grids and investigated in the electron microscope without prior contrasting.

Fluorescence microscopy. Smears were prepared from a twofold dilution series of *C. burnetii* suspensions and stained with 0.2% acridine orange in phosphate-buffered saline for 5 min. Counting of red and green fluorescing particles was performed as described elsewhere (24).

CF reaction. Complement fixation (CF) tests were performed in microtiter plates, with 2 U of complement, using overnight incubation. Antisera directed against *C. burnetii* phase II antigen were obtained from guinea pigs 14 days after intraperitoneal infection with strain Herzberg; antisera against phases I and II were obtained 45 days after intraperitoneal infection with the same strain.

RESULTS

C. burnetii appeared to be well preserved after the purification procedure and the cytochemical reactions (Fig. 1 and 2). *C. burnetii* was not stained in either phase I or phase II by the cytochemical procedure (2, 22) with ConA, horseradish peroxidase, and diaminobenzidine. This negative result indicates that carbohydrate structures with terminal, nonreducing α-glucosyl or sterically closely related residues (27) are not exposed on the surface of *C. burnetii* phases I and II.

A remarkable difference between phase I and II organisms could be demonstrated, using polycationic ferritin as a cytochemical probe.

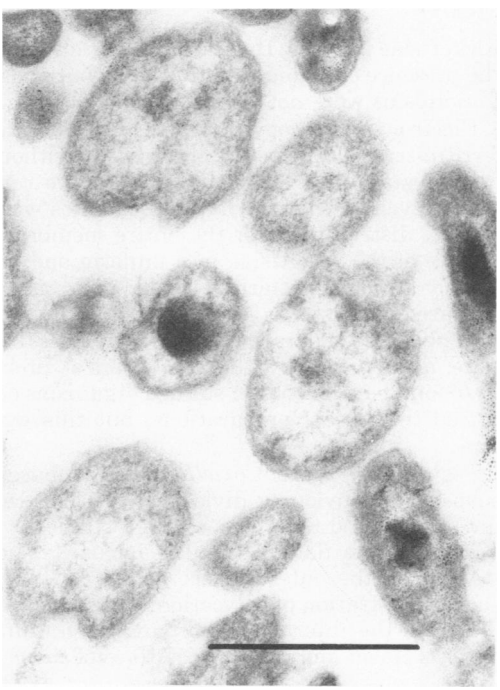

FIG. 1. *C. burnetii phase I, treated with cationic ferritin. Organisms are not stained by the cytochemical probe. Bar represents 300 nm in Fig. 1 through 8.*

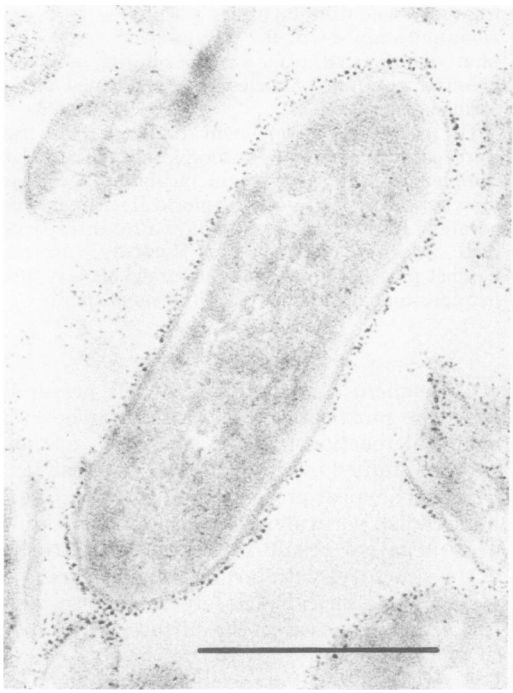

FIG. 2. *C. burnetii phase II, cytochemically treated with cationic ferritin. Accumulation of ferritin granules on cell membrane of the organism.*

Rickettsiae in phase II were labeled, indicating the presence of anionic binding sites, but phase I organisms were not (Fig. 1 and 2).

Electron microscopy of whole, polycationic, ferritin-treated phase II organisms, without further staining for negative or positive contrast, revealed that the ferritin granules were usually distributed over the entire membrane surface of the rickettsiae in a uniform and obviously random pattern (Fig. 3); they rarely appeared in clusters.

In phase II preparations, single particles that were not stained by the cytochemical probe were found; occasionally, stained organisms occurred in phase I preparations, but this was rare.

Figure 4 shows a *Coxiella* from a phase I suspension, obviously dividing, with one cell not stained and the other one well stained.

In Fig. 5, a damaged phase I particle, not stained on the outer surface of the cell wall, shows penetration of polycationic ferritin granules into the interior of the particle, staining the cytoplasm and the inner surface of the cell wall.

Influence of chemical and enzymatic treatments on the appearance of anionic binding sites on the surface of *C. burnetii*. The results of our studies on *C. burnetii* after prior treatment with various chemical and enzymatic reagents are given in Table 1. Anionic binding sites on phase II organisms were not removed or blocked by any treatment, but were reduced after treatment with methanolic HCl (Fig. 6). On phase I particles anionic binding sites appeared after treatment with Me_2SO (Fig. 7), sodium *m*-periodate, and methanolic HCl. Ferritin granules were attached to the surface of such particles in the same amount and distribution as on phase II organisms. After treatment with methanolic HCl, cationic ferritin deposits could be demonstrated on cell walls of phase I particles in a small amount. Lipid extraction with chloroform-methanol or lipid depletion with aqueous acetone (16), and treatment with the enzymes Pronase, hyaluronidase, and lysozyme, did not influence cationic ferritin-staining properties of phase I and II organisms.

In *C. burnetii* phase II preparations, after treatment with Pronase or aqueous acetone and labeling with cationic ferritin, we occasionally observed "ghosts," which carried anionic binding sites on both the outer and inner surface of what appeared to be the cell wall (Fig. 8).

Influence of cytochemical treatments on fluorescent staining of *C. burnetii*. After

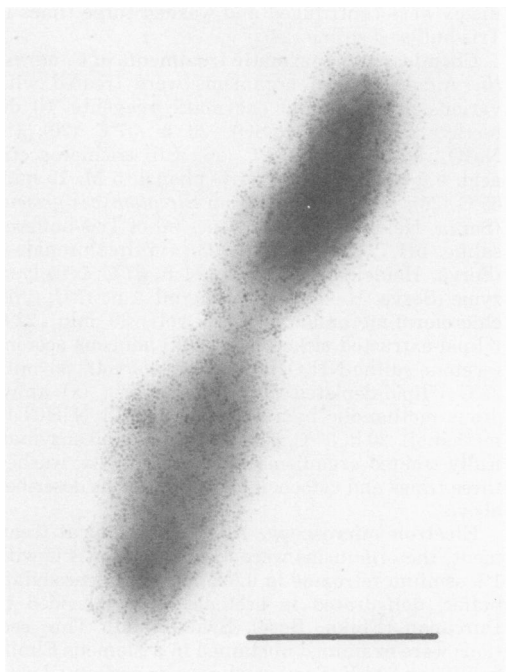

FIG. 3. *C. burnetii phase II, in binary fission, stained with cationic ferritin. Ferritin granules are distributed over the entire membranes of the organisms in a uniform and random pattern. Direct electron microscopic investigation without additional contrasting.*

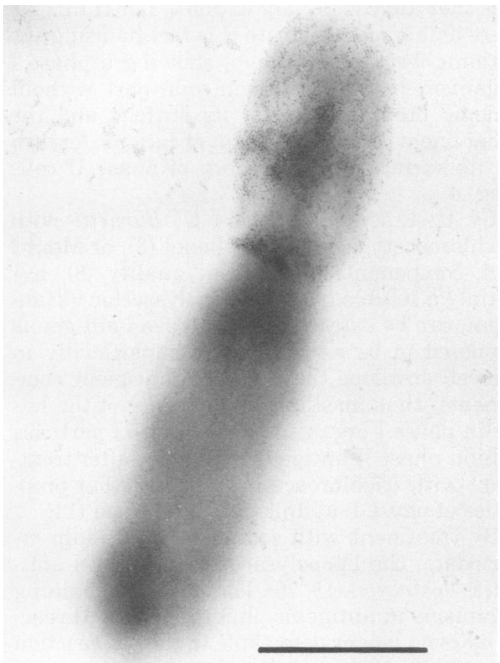

FIG. 4. *C. burnetii organisms from strain Munich in phase I, in binary fission, stained with cationic ferritin. Stained phase II daughter cell emerging from an unstained phase I organism. Direct electron microscopic investigation without additional contrasting.*

staining with the cationic dye acridine orange, fluorescence microscopy of aldehyde-fixed *C. burnetii*, phases I and II, revealed red and green fluorescing particles in different proportions. Untreated and Me_2SO-treated phase I suspensions contained more green than red fluorescing particles. Treatment with trichloroacetic acid or sodium *m*-periodate converted this proportion in phase I preparations. There were always more red than green stained organisms in untreated as well as treated suspensions in phase II (Table 2).

Effects of chemical treatments on antigenic reactivity of phase I and II *C. burnetii* in the CF reaction. "Soluble" phase I antigen could be extracted by treatment of phase I organisms with trichloroacetic acid, phenol, or Me_2SO. After treatment with sodium *m*-periodate, trichloroacetic acid, or Me_2SO, suspensions of phase I particles showed phase II reactivity; after treatment with phenol, no CF activity could be detected.

Antigen could usually not be extracted in measurable amounts from phase II organisms. In some trials a small amount of phase II antigen reactive in the CF test could be detected in the supernatant after treatment with Me_2SO. Antigenic reactivity of phase II particles was lost after phenol treatment.

DISCUSSION

The negative results of the cytochemical reaction with ConA, horseradish peroxidase, and

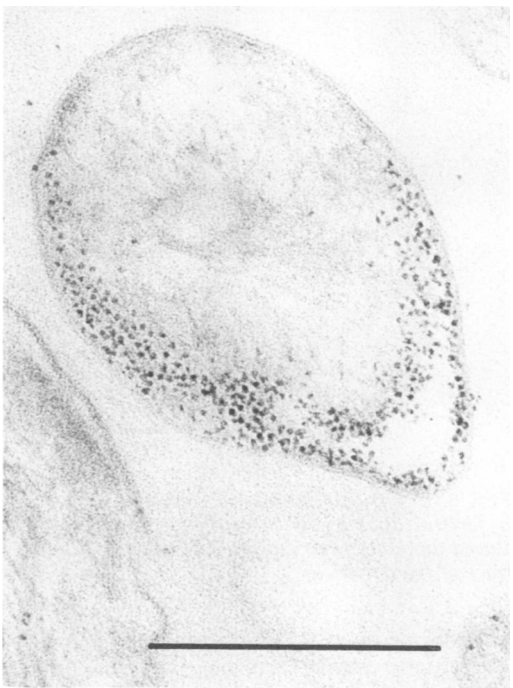

FIG. 5. *Damaged C. burnetii phase I organism from a suspension of strain Herzberg treated with cationic ferritin. No labeling of the outer surface of the cell membrane, but penetration of ferritin granules into the interior with staining of cytoplasm and inner surface of cell membrane.*

TABLE 1. *Electron microscopic demonstration of anionic binding sites on cell walls of C. burnetii phases I and II after chemical or enzymatic treatment*[a]

Treatment	Phase I	Phase II
Control	−	+++
Trichloroacetic acid	−	+++
Phenol	−	+++
Dimethyl sulfoxide	+++	+++
$NaIO_4$	+++	+++
Chloroform-methanol	−	+++
Aqueous acetone	−	+++
Methanolic HCl	+	+
Pronase	−	+++
Lysozyme	−	+++
Hyaluronidase	−	+++

[a] −, No deposition of ferritin granules on cell walls; +, slight deposition of ferritin granules on cell walls; +++, heavy deposition of ferritin granules on cell walls.

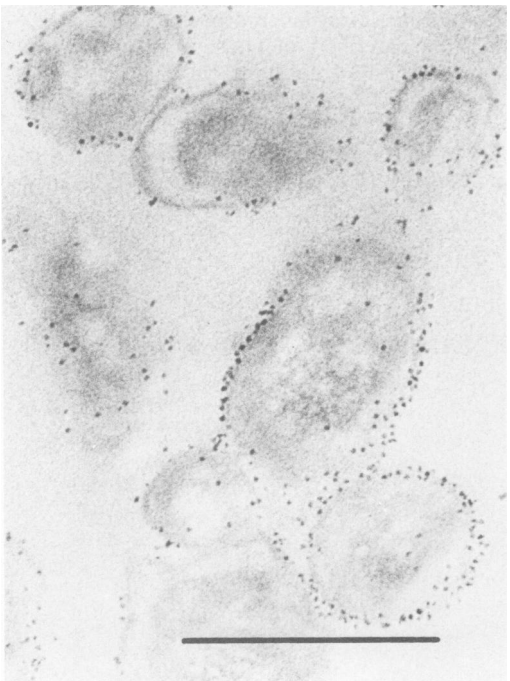

FIG. 6. *C. burnetii phase II cells stained with cationic ferritin after treatment with methanolic HCl. Reduced amount of ferritin granules attached to the surface of the particles.*

diaminobenzidine (2, 22) indicate that carbohydrate structures with terminal, nonreducing α-glucosyl or sterically closely related residues are not exposed on the surface of *C. burnetii* phases I and II.

Using polycationized ferritin as a cytochemical probe, we regularly could demonstrate anionic binding sites on the surface of phase II particles, but not on phase I organisms, of *C. burnetii,* thus providing a simple means for distinguishing our strains in either phase. The rare finding of single cells with anionic binding sites in phase I preparations and the occasional appearance of unstained organisms in phase II suspensions are in complete agreement with the fact that strains in either phase are always a mixture of organisms in phases I and II (13). Even the purest phase II preparation, after prolonged egg passage, contains small amounts of phase I material, as can be demonstrated serologically by hyperimmunization of guinea pigs, or rabbits, with such strains (3, 12).

It appears that a change of phase of a strain of *C. burnetii* does not represent a continuous increase or decrease of the amount of antigen I in the individual particles of the population but is actually a rise or fall of the number of particles that are completely in phase I or II (5). We were able to demonstrate this fact by using the cationic ferritin technique, showing a phase I organism in binary fission, one part without anionic binding sites on its surface and the other one with accumulation of cationic ferritin on its surface in the pattern of phase II cells (Fig. 4).

By treatment of phase I *C. burnetii* with trichloroacetic acid (1, 9), phenol (8), or Me_2SO (20), components of different quality (8), but mainly a soluble phase I lipopolysaccharide antigen, can be extracted. This phase I antigen is supposed to be situated more superficially in the cell envelope than phase II antigenic components, thus masking the presence of the latter in phase I organisms (12). Phase I particles exhibit phase II antigenic reactivity after treatment with trichloroacetic acid, but other properties of phase I organisms are retained (14).

By treatment with sodium or potassium *m*-periodate, the lipopolysaccharide phase I antigen is destroyed (15, 26), leaving the remaining organisms in antigenic phase II. Phase II reactivity is no longer detectable in the CF reaction after treatment with phenol.

In our experiments with cationic ferritin, conversion of phase I *C. burnetii* to phase II coincided with the appearance of anionic bind-

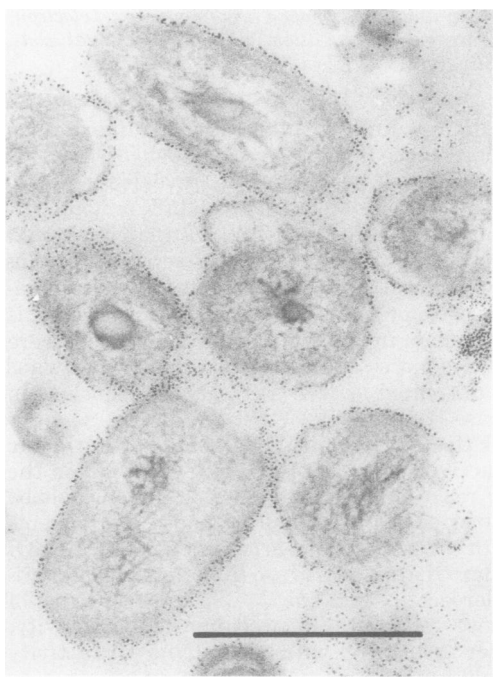

FIG. 7. *C. burnetii phase I, treated with Me_2SO and stained with cationic ferritin. Ferritin granules attached to the membranes of the organisms.*

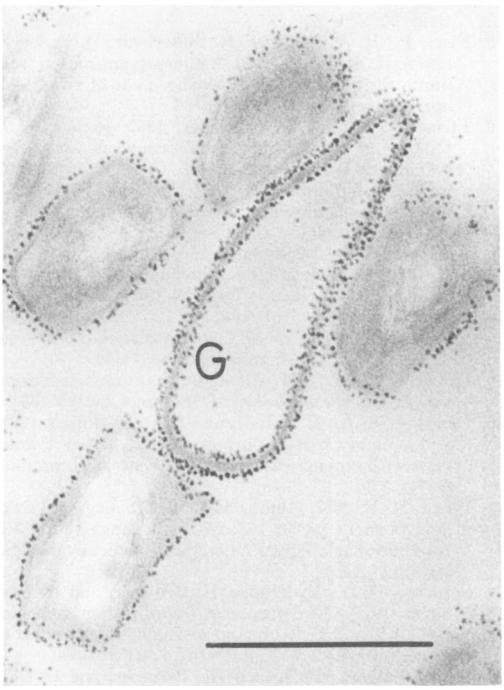

FIG. 8. *C. burnetii phase II, treated with Pronase and stained with cationic ferritin. G, "Ghost," with ferritin deposits on the outer and inner surface of the cell wall.*

TABLE 2. *Results of fluorescent staining of untreated and chemically treated phase I and II C. burnetii with the cationic dye acridine orange*

Treatment	Proportion of stained particles (red:green)	
	Phase I	Phase II
Control	1:1.26	1.63:1
Dimethyl sulfoxide	1:1.13	NA[a]
Trichloroacetic acid	2.50:1	7.06:1
$NaIO_4$	4.12:1	3.56:1
Phenol	NA	NA

[a] NA, Not assessable (clumping).

ing sites on the membrane surface of the particles after treatment with sodium *m*-periodate or Me_2SO. Anionic sites were not exposed (or even abolished) or coextracted with trichloroacetic acid or phenol. This indicates that absence of a net negative charge on the surface of phase I particles is not dependent on the phase I lipopolysaccharide surface antigen.

As a result of our investigations on mycoplasmas (23), we postulated that the anionic sites on their cell membranes are mainly lipid phosphate groups. The chemical nature of the anionic sites on *C. burnetii* phase II cell walls seems to be different from that of the anionic sites on mycoplasma membranes, since lipid extraction with chloroform-methanol and lipid depletion with aqueous acetone (16) did not abolish such sites on *C. burnetii*. The results of cytochemical and enzymatic treatments presented in this paper indicate that anionic binding sites on *C. burnetii* are carboxyl groups.

It can be assumed that at least some of the distinct biological properties of phase I and II cells should be directly connected with the electrochemical status of the cell wall of these particles, e.g., the net negative charge on phase II organisms, with their tendency to autoagglutination, and the fact that particles carrying such sites are easily phagocytized in the absence of specific antibody and show nonspecific reactivity in the fluorescent antibody technique. In contrast, the ease of preparation of purified suspensions of phase I organisms, their stability against autoagglutination, the fact that phase I organisms are phagocytized only to a low degree in the absence of specific antibody, their ability to react specifically in the indirect fluorescent antibody test, and their low avidity for biological stains like acridine orange, which is a "cationic" fluorescent stain, should at least depend on the fact that anionic sites are masked in phase I organisms.

With acridine orange, the color of fluorescent staining depends on the amount of stain bound by the organisms, higher concentrations resulting in red and lower in green fluorescence (29). Both types of particles existed in aldehyde-fixed *C. burnetii* phase I and II suspensions. In suspensions of organisms possessing anionic binding sites, e.g., phase II or trichloroacetic acid- and sodium *m*-periodate-treated phase I organisms, a higher proportion of particles stained red. In suspensions of particles lacking negative surface charges, i.e., suspensions of phase I organisms, the majority stained green.

Certainly other factors also play a role in the actual staining process (29). This may explain the different findings in electron and fluorescence microscopy of trichloroacetic acid- or Me_2SO-treated particles of *C. burnetii* in phase I; however, our results indicate that anionic binding sites on the cell walls of these rickettsiae influence the fluorescent staining properties of the organisms.

In our investigations with mycoplasmas (22), a striking membrane asymmetry was detected when isolated membranes of *M. mycoides* var. *capri* were incubated with the polycationic ferritin label. The probe was bound exclusively to only one, presumably the outer, surface. *C. burnetii* phase I was labeled not on the outer, but on the inner, surface of the cell wall if cationic ferritin had penetrated into the parti-

cles. Phase II "ghosts" were labeled on both surfaces.

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LITERATURE CITED

1. **Anacker, R. L., D. L. Lackman, E. G. Pickens, and E. Ribi.** 1962. Antigenic and skin-reactive properties of fractions of *Coxiella burnetii*. J. Immunol. **89**:145–153.
2. **Bernhard, W., and S. Avrameas.** 1971. Ultrastructural visualization of cellular carbohydrate components by means of Concanavalin A. Exp. Cell Res. **64**:232–236.
3. **Bobb, D., and C. M. Downs.** 1962. The phase antigens of Coxiella burnetii. Can. J. Microbiol. **8**:689–702.
4. **Bretton, R., W. Wicker, and W. Bernhard.** 1972. Ultrastructural localization of concanavalin A receptors in normal and SV_{40}-transformed hamster and rat cells. Int. J. Cancer **10**:397–410.
5. **Brezina, R.** 1968. New advances in the study of rickettsial antigens. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **206**:313–321.
6. **Brezina, R., and J. Kazar.** 1963. Phagocytosis of Coxiella burneti and the phase variation phenomenon. Acta Virol. **7**:476.
7. **Brezina, R., and J. Kazar.** 1965. Study of the antigenic structure of Coxiella burnetii. IV. Phagocytosis and opsonization in relation to the phases of C. burnetii. Acta Virol. **9**:268–274.
8. **Brezina, R., V. Pospisil, and S. Schramek.** 1970. Study of the antigenic structure of Coxiella burneti. VII. Properties of pheno-extracted phase I antigenic component. Acta Virol. **14**:295–301.
9. **Brezina, R., and J. Urvölgyi.** 1962. Study of the antigenic structure of Coxiella burnetii. I. Extraction of phase I antigenic component by means of trichloroacetic acid. Acta Virol. **6**:84–88.
10. **Danon, D., L. Goldstein, Y. Marikovsky, and E. Skudelsky.** 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. J. Ultrastruct. Res. **38**:500–510.
11. **Downs, C. M.** 1968. Phagocytosis of Coxiella burneti phase I and phase II by peritoneal monocytes from normal and immune guinea pigs and mice. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **206**:329–343.
12. **Fiset, P.** 1957. Phase variation of Rickettsia (Coxiella) burneti. A study of the antibody response in guinea pigs and rabbits. Can. J. Microbiol. **3**:435–445.
13. **Fiset, P.** 1959. Serologic diagnosis, strain identification and antigenic variation, p. 28–38. *In* J. E. Smadel (ed.), Symposium on Q fever. Med. Sci. Publ. no. 6, Walter Reed Army Institute of Research, Washington, D.C.
14. **Fiset, P., and R. A. Ormsbee.** 1968. The antibody response to antigens of Coxiella burneti. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **206**:321–329.
15. **Fiset, P., R. A. Ormsbee, R. Silberman, M. Peacock, and S. H. Spielman.** 1969. A microagglutination technique for detection and measurement of rickettsial antibodies. Acta Virol. **13**:60–66.
16. **Fleischer, S., and B. Fleischer.** 1967. Removal and binding of polar lipids in mitochondria and other membrane systems, p. 406–433. *In* R. W. Estabrook and M. E. Pullman (ed.), Methods in enzymology, vol. 10. Academic Press Inc., New York.
17. **Hoyer, G. H., R. A. Ormsbee, P. Fiset, and D. B. Lackman.** 1963. Differentiation of phase I and phase II Coxiella burnetii by equilibrium density gradient sedimentation. Nature (London) **197**:573–574.
18. **Jerrels, T. R., D. J. Hinrichs, and L. P. Mallavia.** 1974. Cell envelope analysis of Coxiella burneti phase I and phase II. Can. J. Microbiol. **20**:1465–1470.
19. **Ormsbee, R. A.** 1964. An agglutination-resuspension test for Q-fever antibodies. J. Immunol. **29**:159–166.
20. **Ormsbee, R. A., E. J. Bell, and D. L. Lackman.** 1962. Antigens of Coxiella burnetii. I. Extraction of antigens with nonaqueous organic solvents. J. Immunol. **88**:741–749.
21. **Pinto, M. R.** 1963. Alguns aspects da biologia da Coxiella burneti, p. 261–263. *In* Proceedings of the 7th international congress on tropical medicine and Malaria, vol. III.
22. **Schiefer, H.-G., H. Krauss, H. Brunner, and U. Gerhardt.** 1975. Ultrastructural visualization of surface carbohydrate structures on mycoplasma membranes by concanavalin A. J. Bacteriol **124**:1598–1600.
23. **Schiefer, H.-G., H. Krauss, H. Brunner, and U. Gerhardt.** 1976. Ultrastructural visualization of anionic sites on mycoplasma membranes by polycationic ferritin. J. Bacteriol. **127**:461–468.
24. **Schmatz, H. D., S. Schmatz, and H. Krauss.** 1976. Der Einfluss der Inokulationsdosis auf die Gewinnung von Coxiella burnetii-Antigen aus dem Dottersack embryonierter Hühnereier. Berl. Muench. Tieraerztl. Wochenschr. **89**:120–122.
25. **Schramek, S., and R. Brezina.** 1976. Characterization of an endotoxic lipopolysaccharide from Coxiella burnetii. Acta Virol. **20**:152–158.
26. **Schramek, S., R. Brezina, and V. Pospisil.** 1970. Improved method of preparing phase II Coxiella burneti antigen for the microagglutination test. Acta Virol. **14**:415.
27. **Sharon, N., and H. Lis.** 1972. Lectins: cell-agglutinating and sugar-specific proteins. Science **177**:949–959.
28. **Stoker, M. G. P., and P. Fiset.** 1956. Phase variation of the Nine Mile and other strains of Rickettsia burneti. Can. J. Microbiol. **2**:310–321.
29. **Strugger, S.** 1949. Fluoreszenzmikroskopie und Mikrobiologie. Verlag M. und H. Schaper, Hannover.
30. **Wisseman, C. L., Jr., P. Fiset, and R. A. Ormsbee.** 1967. Interaction of rickettsiae and phagocytic host cells. V. Phagocytic and opsonic interactions of phase I and phase II Coxiella burneti with normal and immune human leukocytes and antibodies. J. Immunol. **99**:669–674.