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Localization of DNA in *Coxiella burnetii* by Post-embedding Immunoelectron Microscopy

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INTRODUCTION

Coxiella burnetii, the etiological agent of Q fever, is a mildly acidophilic obligate intracellular bacterium which grows only in the phagolysosomes of eukaryotic cells.[1] Growth of *C. burnetii* within the phagolysosomes leads to the formation of distinctly different morphological cell types, namely, the spore, the small cell variant (SCV), and the large cell variant (LCV).[2] Ultrastructural studies have provided supportive evidence that *C. burnetii* multiplies by transverse binary fission.[2-5] In addition to a typical bacterial growth cycle, *C. burnetii* undergoes sporogenic differentiation.[2] An asymmetrical cellular division of the cytoplasm of the mother cell leads to the formation of the spore, which resembles the SCV after release of this cell from the mother cell.[6] The two morphological cell types are therefore members of a developmental cycle that consists of both vegetative growth and sporogenic cellular differentiation.[2,6-8]

Sporogenic cellular differentiation in *C. burnetii* is initiated by asymmetrical septation.[6] Morphological observations of the events in the process of sporogenesis in *C. burnetii* indicate that the DNA is segregated into unequal compartments and that the mother cell is killed during the development of the spore.[6] In a typical endospore former, *Bacillus subtilis*, each of the two unequal compartments receives a chromosome generated by the last cycle of vegetative DNA replication as a consequence of differential gene expression.[9,10] To demonstrate the presence of DNA in *C. burnetii*, we employed post-embedding immunoelectron microscopy and monoclonal antibodies against DNA. The specificity of the antibody to DNA was tested by exposing the sections to enzymatic digestion with RNAse and DNAse prior to immunolabeling. Since DNA-binding of histone-like proteins has been identified in prokaryotes,[11] enzymatic digestion with proteinase K was also carried out to maximize the digestion of nucleic acids with RNAse or DNAse. Our work demonstrates that DNA is segregated into two different compartments as a

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result of asymmetrical septation. Our conclusion is that sporogenic cellular differentiation must be considered as a necessary and vital event in the life cycle of *C. burnetii*.

MATERIALS AND METHODS

Bacterial Strains

Coxiella burnetii (Nine Mile strain phase I, clone 7) was purified from infected, antibiotic-free, hen egg yolk sacs by isopycnic Renografin gradient centrifugation.[12]

Electron Microscopy

Purified and viable *C. burnetii* were fixed for 3 h in 1.25% glutaraldehyde and 0.2% picric acid in 0.066 *M* sodium cacodylate buffer, pH 6.8, at 4°C. Pre-embedding in 2% Difco agar was followed by a 15-min rinse in the same buffer. The dehydration and embedding procedures were performed with L R White resin as previously described.[13] Thin sections (60–100 nm) were mounted onto uncoated nickel grids and subjected to enzymatic digestions prior to immunolabeling.

Preparation of Enzymes for Digestions

DNAse (RQ1, RNAse free; Promega, Madison, WI) was diluted in buffer (40 m*M* Tris-HCl, pH 7.9, 10 m*M* NaCl, 6 m*M* $MgCl_2$) at a final concentration of 100 μg/ml. Pancreatic RNAse, from an original stock solution of 2 mg/ml, was diluted in buffer (50 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$), to a final concentration of 100 μg/ml. Proteinase K was reconstituted in double-distilled water at a final concentration of 100 μg/ml.

Enzymatic Digestions

DNAse. The grids containing the sections were washed by floating on drops of buffer (40 m*M* Tris-HCl, pH 7.9, 10 m*M* NaCl, 6 m*M* $MgCl_2$) for two 5-min periods at 22°C. The grids were sequentially transferred to (i) DNAse (100 μg/ml) for 1 h at 42°C, (ii) buffer (Tris-HCl, NaCl, $MgCl_2$) for two 5-min periods, and (iii) demineralized and distilled water for two 5-min periods. The grids were then left to air-dry before immunolabeling.

RNAse. The steps were similar to those described for DNAse digestion. The buffer, however, was replaced by Tris-HCl, pH 7.5, containing 5 m*M* $MgCl_2$. RNAse was used at a concentration of 100 μg/ml.

Proteinase K. The grids were washed by floating on drops of double-distilled water for two 5-min periods at 22°C. The grids were then sequentially transferred to proteinase K (100 μg/ml) solution for 1 h at 22°C and double-distilled water for three 5-min periods. The grids were then left to air-dry before immunolabeling. Some grids were subsequently subjected to DNAse digestion, either alone or followed by RNA digestion, before immunolabeling.

Primary Antibody and Colloidal Gold Labels

Monoclonal antibody (Boehringer-Mannheim Biochemicals) reactive with both single-stranded (ss) DNA and double-stranded (ds) DNA was used as primary antibody. This anti-DNA monoclonal antibody reacts with native and denatured DNA isolated from various organisms and does not recognize RNA. The lypholized antibody was reconstituted in distilled water to final concentrations of 10 and 5 μg/ml in buffered solution. Goat anti–mouse IgM, colloidal gold conjugate (5 and 10 nm), was obtained from Janssen Life Sciences Products, Piscataway, NJ. The conjugate was diluted 1 : 10 in a solution (PBS/BSA) containing phosphate-buffered saline (pH 7.5) and bovine serum albumin (2 mg/ml).

Immunolabeling

The grids were washed in PBS/BSA and transferred sequentially to (i) primary antibody in PBS/BSA (2 h at 22°C), (ii) PBS/BSA (two 5-min rinses), (iii) diluted gold conjugate (1 h at 22°C), and (iv) PBS/BSA (two 5-min rinses). Negative controls were prepared without pre-incubation with primary antibody. Enzymatic digestion with either DNAse; RNAse; proteinase K; a combination of proteinase K and DNAse; or a combination of proteinase K, DNAse and RNAse was also carried out to determine the specificity of the immunolabeling.

Examination of Electron Microscopy Specimens

Sections were stained with 0.5% uranyl acetate in 50% ethanol (30 s). Some sections were additionally stained with lead citrate (30 s), as indicated in figure legends. The specimens were examined in a JEOL 100CX transmission electron microscope operated at 80 kV.

RESULTS AND DISCUSSION

Post-embedding Immunolabeling Techniques

We used post-embedding immunocytochemistry to localize the DNA of *C. burnetii*. The major advantage of this technique is that any antigen exposed on the cut surface of the section, whether internal or external to the cells, will be exposed to the immunological reagents.[14–17] Colloidal gold probes[16,18–20] were selected for the labeling. The hydrophilic L R White resin (a polyhydroxyl aromatic acrylic) was chosen as the embedding medium primarily because (i) the resin is highly hydrophilic, allowing good penetration of the antibodies,[17,21] and (ii) heat-curing at 50°C minimizes denaturation of the antigens during embedding and polymerization.[17,21]

The main problem in the adoption of post-embedding techniques is the retention of ultrastructural details.[19,22] In our procedure, ultrastructure and contrast were compromised for increased sensitivity of the immunocytochemical method

by avoiding post-fixation with osmium tetroxide and *en-bloc* staining with uranyl acetate. The omission of these stains decreased the ability of ultrastructural differences between the SCV and LCV to be recognized. The conditions of fixation and embedding collapsed the nucleoid of the LCV and the SCV. Fortunately, the collapse of the nucleoid did not interfere with the immunolabeling of DNA.

Morphology of C. burnetii *(phase I)*

The LCV revealed DNA fibrils radiating and criss-crossing randomly across the nucleoid to the ribonucleoprotein material, which was displaced to the periphery of the cell (FIG. 1a). Most of the nucleoid had an electron-lucent background, and, at the center of the nucleoid, the fibrils formed bundles (FIG. 1a). This appearance of the nucleoid was expected, since the morphology of the nucleoid depends on the effects of preparative procedures and relates to the immobilization of the protoplasmic elements during fixation.[23,24]

The SCV had a very dense central nucleoid with confined individual filaments which were not easily resolved (FIG. 1b). The collapse of the nucleoid of the SCV was not as substantial as that of the LCV. Therefore, the electron-lucent background seen in the LCV was not so obvious in the SCV. Also, the ribonucleoprotein material remained tightly packed around the nucleoid. Under the conditions of fixation and staining used, the SCV did not show clearly the characteristic layers of membranes (FIG. 1b). Instead, the space occupied by these layers of membranes appeared as an electron-lucent area devoid of any recognizable structures.

Spores revealed a dense central core surrounded by a cell coat consisting of both electron-lucent and electron-dense layers (FIG. 1c).

DNA Localization in the SCV and the LCV

In the LCV, the 10-nm gold label was mostly confined in the central nucleoid region (FIG. 2a). Some label was also scattered just beyond the periphery of the nucleoid in the region of the ribonucleoprotein material. Although the electron-lucent area was not labeled, the DNA fibrils which criss-crossed at the periphery of the cell were labeled.

To visualize gold label of smaller size (5 nm, FIG. 2b) on the electron-dense ribonucleoprotein material and nucleoid, the sections were stained with uranyl acetate alone. Despite poor structural contrast, labeling of the central nucleoid and the DNA within the granular ribonucleoprotein just beyond the periphery of the electron-lucent area of the LCV could be seen (FIG. 2b).

Regardless of the size of the gold label, labeling of the SCV DNA was confined to the electron-dense nucleoid and the nucleoid periphery (FIG. 2c).

The presence of the label in the nucleoid periphery was not unexpected, as the monoclonal antibody against DNA recognized both ssDNA and dsDNA. A recent study on immunolabeling of DNA distributed in prokaryotic nucleoids showed that dsDNA is present throughout the nucleoid whereas ssDNA is specifically located on the nucleoid periphery.[25] We did not attempt to differentiate the ssDNA from the dsDNA of *C. burnetii*.

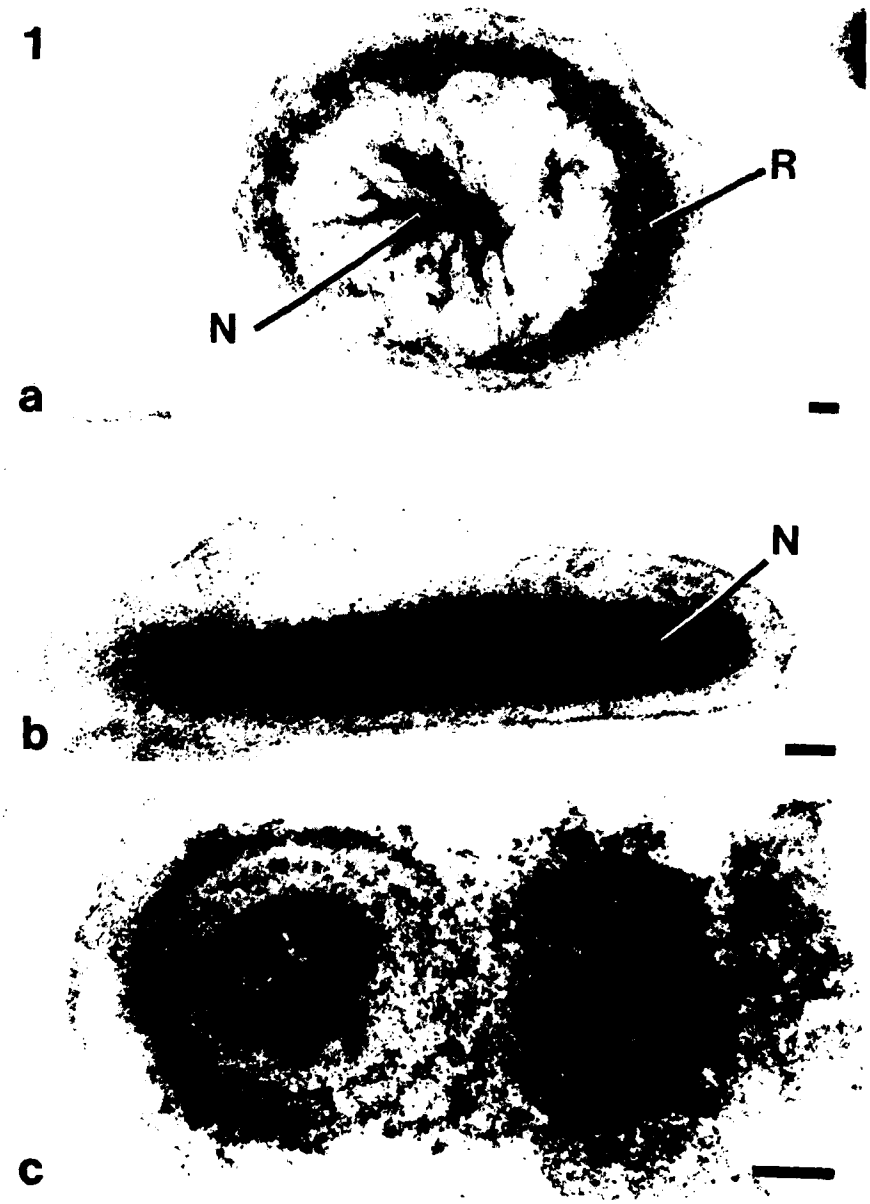

FIGURE 1. Ultrastructure of *C. burnetii* (phase I) embedded in L R White resin. **(a)** Large cell variant, **(b)** small cell variant, and **(c)** spore. N, nucleoid; R, ribonucleoprotein. *Bars* = 100 nm.

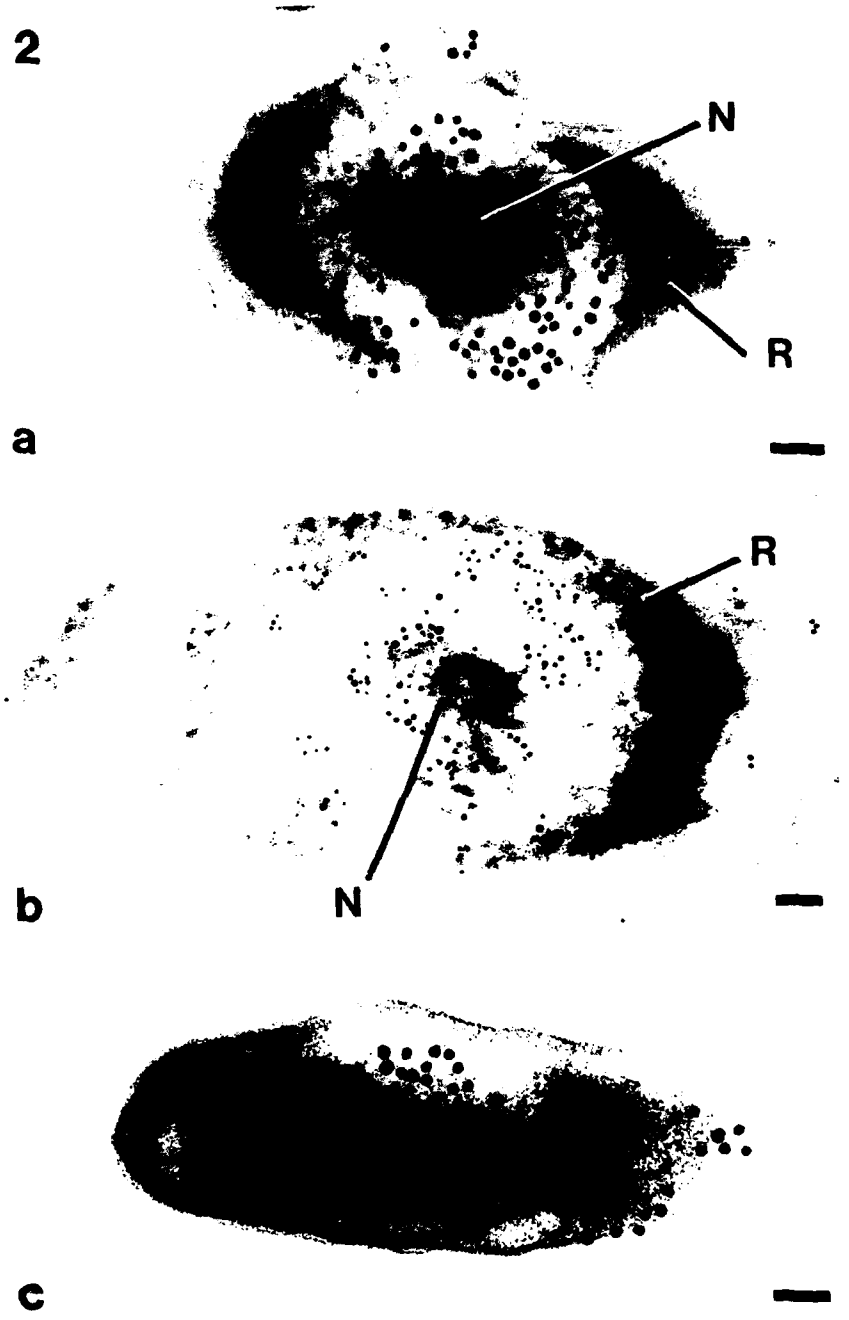

FIGURE 2. Labeling of DNA with murine monoclonal antibodies against ssDNA and dsDNA and with anti-mouse IgM conjugated to colloidal gold, 10 nm **(a, c)** and 5 nm **(b)**, in *C. burnetii* cells. **(a)** Large cell variant, **(b)** large cell variant stained only with uranyl acetate, **(c)** small cell variant. N, nucleoid; R, ribonucleoprotein. *Bars = 100 nm.*

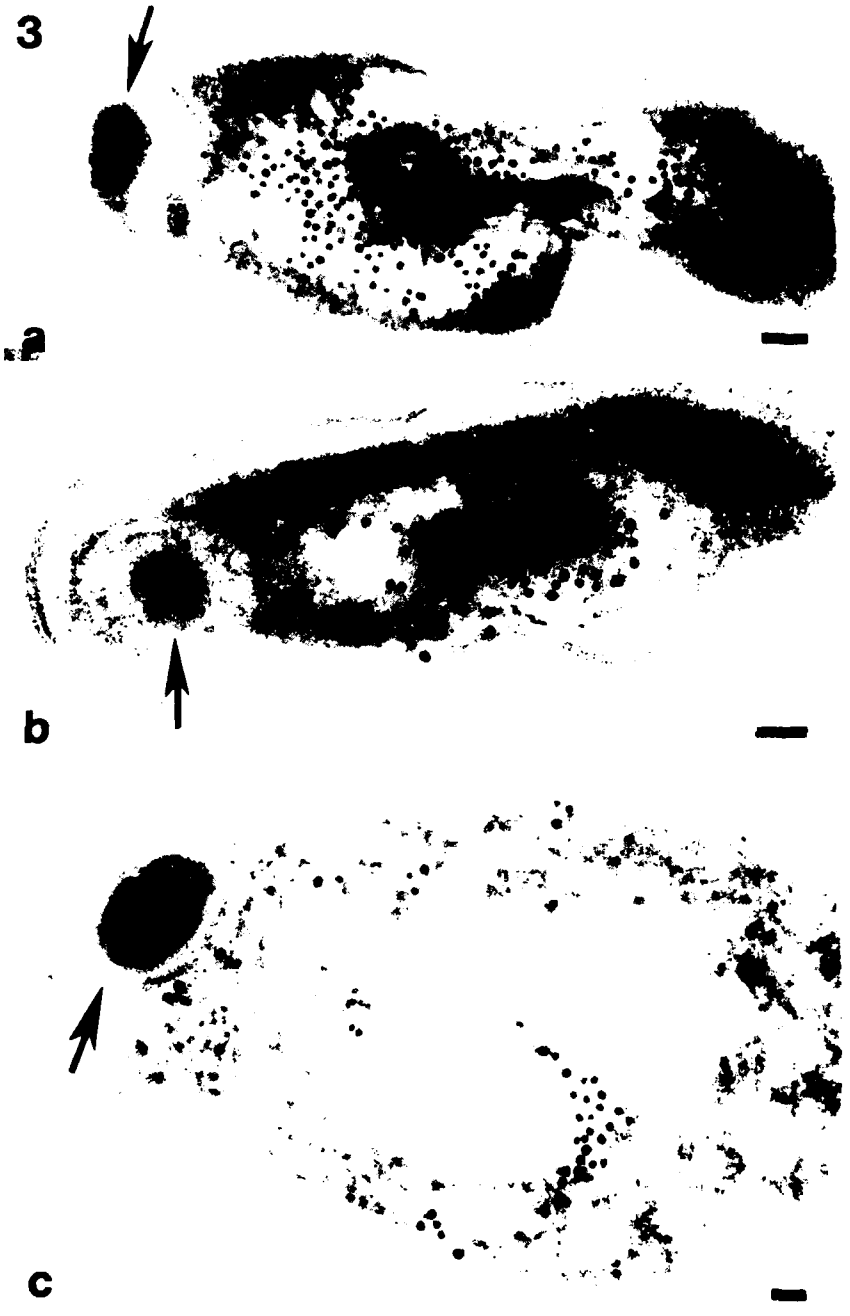

FIGURE 3. Localization of DNA in the spore (*arrows*) and the LCV mother cell of *C. burnetii*. Note the degradation of the nucleoid of the LCV mother cell at the terminal stage of development (c). *Bars* = 100 nm.

DNA Localization in the Spore

The DNA of the spore was clearly immunolabeled (FIG. 3). In the early stages of differentiation, the nucleoid of the mother cell was heavily labeled by the anti-DNA antibody (FIG. 3a). The labeling still persisted as the nucleoid of the mother cell became more dispersed near the terminal stage of differentiation (FIG. 3b). When the morphological integrity of the mother cell was lost at the terminal stage of spore development, the nucleoid DNA was also dispersed. In a mother cell showing prominent labeling of the interior of the spore, the poorly organized nucleoid of the mother cell was not labeled (FIG. 3c). The results clearly demonstrated that DNA was segregated into two compartments, the spore and the mother cell, as a result of sporogenic differentiation. Importantly, each compartment had undergone a separate developmental fate: the mother cell showed a poorly organized nucleoid with marked reduction of the labeling for DNA during the terminal stage of differentiation, while the spore remained labeled.

Affinity of the Antibody Label for DNA

Thin sections were treated with enzymes prior to immunolabeling to determine the specificity of the label for DNA in the spore, the SCV, and the LCV. After treatment of sections with proteinase K, the labeling was confined to the nucleoid of both the LCV (FIG. 4a) and the SCV (not shown). The labeling along the periphery of the nucleoid appear to be reduced (FIG. 4a). This was not surprising, because DNA-binding, histone-like proteins in prokaryotes have a higher affinity for ssDNA than for dsDNA, and these proteins stabilize dsDNA against thermal denaturation.[11] Therefore, digestion of the proteins by proteinase K in the nucleoid region may promote the leaching of the ssDNA along the nucleoid periphery in both the SCV and the LCV.

The labeling of the central core of the spore was also reduced after proteinase K treatment (FIG. 4b). Such a reduction implies that the DNA of the spore exists in double- and single-stranded forms in association with binding proteins.

After DNAse treatment of sections, the label was drastically reduced in the spore (not shown), the SCV (not shown), and the LCV (FIG. 4c). Furthermore, treatment of sections with proteinase K and DNAse decreased the label on the spore, the SCV, and the LCV to a greater degree than did the DNAse treatment alone (FIG. 5a). Removal of the DNA-binding proteins by digestion with proteinase K was thus necessary to permit complete digestion of the DNA with DNAse.

Treatment of sections with RNAse did not eliminate immunolabeling of the DNA. Despite some reduction of the label along the nucleoid periphery of both the SCV (not shown) and the LCV (FIG. 5b), label remained confined to the nucleoid of these cells. Label in the core of the spore was reduced by RNAse treatment (FIG. 5c).

After treatments of sections with proteinase K, DNAse, and RNAse, no label was found on the spore, the SCV, and the LCV. The combination of all three enzymes apparently etched the nucleoids of all of the cell types (data not shown).

In conclusion, post-embedding immunolabeling of DNA effectively demonstrated that DNA of the nucleoid is indeed segregated in both the spore and the mother cell, thus confirming the original proposal that *C. burnetii* undergoes sporogenesis as well as transverse binary fission, both of which constitute the developmental cycle.

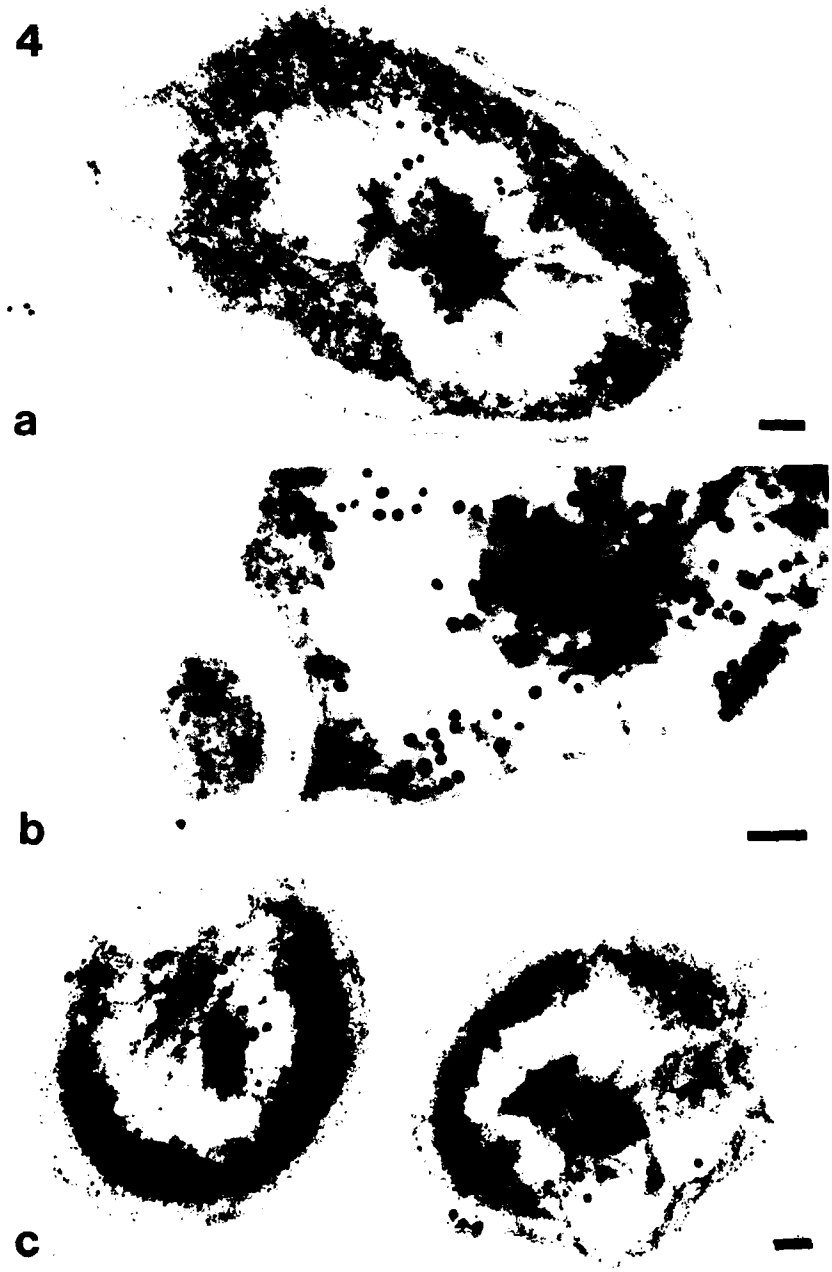

FIGURE 4. Effect of enzymatic digestions on thin sections of *C. burnetii* cells prior to immunolabeling of DNA. Effect of **(a)** proteinase K on the LCV, **(b)** proteinase K on the spore, and **(c)** DNAse on the LCV. *Bars* = 100 nm.

FIGURE 5. Effect of enzymatic digestions on thin sections of *C. burnetii* cells prior to immunolabeling of DNA. Effect of **(a)** proteinase K and DNAse on the spore (*arrow*), SCV (S), and LCV (L); **(b)** RNAse on the LCV; and **(c)** RNAse on the LCV mother cell and spore (*arrow*). *Bars* = 100 nm.

SUMMARY

The deoxyribonucleic acid of *Coxiella burnetii* was detected with monoclonal antibodies against single-stranded and double-stranded DNA by post-embedding immunoelectron microscopy. The antibody labeled the nucleoid of the spore, the small cell variant (SCV), and the large cell variant (LCV). The DNA was segregated completely in both the spore and the mother cell. At the terminal stage of development of the spore, the loss of nuclear morphology in the mother cell was related to the reduction in the labeling of the nucleoid. The mother cell was therefore killed for the development and release of the spore.

REFERENCES

1. HACKSTADT, T. & J. C. WILLIAMS. 1981. Proc. Natl. Acad. Sci. USA **78:** 3240–3244.
2. MCCAUL, T. F. & J. C. WILLIAMS. 1981. J. Bacteriol. **147:** 1063–1076.
3. ANACKER R. L., K. FUKUSHI, E. G. PICKENS & D. B. LACKMAN. 1964. J. Bacteriol. **88:** 1130–1138.
4. BURTON, P. R., J. STUECKEMANN & D. PARETSKY. 1975. J. Bacteriol. **122:** 316–324.
5. NERMUT, M. V., S. SCHTAMEK & R. BREZINA. 1968. Acta Virol. **12:** 446–452.
6. MCCAUL, T. F., H. A. THOMPSON & J. C. WILLIAMS. Ultrastructure of the sporulation process in *Coxiella burnetii:* Asymmetrical septation and the segregation of DNA into the endogenous spore. Manuscript submitted.
7. AITKEN, I. D., K. BOGEL, E. CRACEA, E. EDLINGER, D. HOUWERS, H. KRAUSS, M. RADY, J. REHACEK, H. G. SCHIEFER, N. SCHMEER, I. V. TARASEVICH & G. TRINGALI. 1987. Infection **15:** 323–327.
8. SCHMEER, N., H. KRAUSS & H. G. SCHIEFER. 1987. Dtsch. Med. Wochenschr. **112:** 184–188.
9. STRAGIER, P., B. KUNKEL, L. KROOS & R. LOSICK. 1989. *Science* **243:** 507–512.
10. KROOS, L., B. KUNKEL & R. LOSICK. 1989. Science **243:** 526–529.
11. DRLICA, K. & J. ROUVIERE-YANIV. 1987. Microbiol. Rev. **51:** 301–319.
12. WILLIAMS, J. C., M. G. PEACOCK & T. F. MCCAUL. 1981. Infect. Immun. **32:** 840–851.
13. MCCAUL, T. F., J. C. WILLIAMS & N. BANERJEE-BHATNAGAR. Antigenic differences between *Coxiella burnetii* morphological cell types revealed by post-embedding immunoelectron microscopy and immunoblotting. Manuscript submitted.
14. BEESLEY, J. E. 1985. R. Microsc. Soc. Proc. **20:** 187–197.
15. BEESLEY, J. E. 1986. J. Microsc. **143:** 177–186.
16. BEESLEY, J. E. 1984. *In* Immunolabelling for Electron Microscopy. J. M. Polak & I. M. Varndell, Eds.: 289–303. Elsevier Science Publishers B. V. Amsterdam, The Netherlands.
17. NEWMAN, G. R. & B. JASANI. 1984. *In* Immunolabelling for Electron Microscopy. J. M. Polak & I. M. Varndell, Eds.: 53–70. Elsevier Science Publishers B. V. Amsterdam, The Netherlands.
18. ROTH, J. 1986. J. Microsc. **143:** 125–137.
19. ROTH, J. 1983. *In* Techniques in Immunocytochemistry. G. R. Bullock & P. Petrusz, Eds. Vol. 2: 217–284. Academic Press. London.
20. HORISBERGER, M. 1984. *In* Immunolabelling for Electron Microscopy. J. M. Polak & I. M. Varndell, Eds.: 17–26. Elsevier Science Publishers B. V. Amsterdam, The Netherlands.
21. CAUSTON, B. E. 1984. *In* Immunolabelling for Electron Microscopy. J. M. Polak & I. M. Varndell, Eds.: 29–36. Elsevier Science Publishers B. V. Amsterdam, The Netherlands.
22. ROTH, J., M. BENDAYAN, E. CARLEMALM, W. VILLIGER & M. GARAVITO. 1981. J. Histol. Cytol. **29:** 663–671.

23. HOBOT, I. A., W. VILLIGER, J. ESCAIG, M. MAEDER, A. RYTER & E. KELLENBERGER. 1985. J. Bacteriol. **162:** 960–971.
24. WOLDRINGH, C. L. & N. NANNINGA. 1985. *In* Molecular Cytology of *Escherichia coli*. N. Nanninga, Ed.: 161. Academic Press. London.
25. HOBOT, J. A., M-A. BJORNSTI & E. KELLENBERGER. 1987. J. Bacteriol. **169:** 2055–2062.