JOURNAL OF BACTERIOLOGY, Aug. 1978, p. 633-640
0021-9193/78/0135-0633$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 135, No. 2
Printed in U.S.A.

Morphological and Cell Association Characteristics of *Rochalimaea quintana*: Comparison of the Vole and Fuller Strains

BRUCE R. MERRELL,* EMILIO WEISS, AND GREGORY A. DASCH
Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014

Received for publication 10 March 1978

The vole and Fuller strains of *Rochalimaea quintana* were grown on monolayers of mouse L cells irradiated 7 days previously and examined by light microscopy and scanning and transmission electron microscopy. Most of the bacteria of both strains were shown to adhere to the L cells but remained in an extracellular location. Cell division was frequently seen among the extracellular bacteria. The few intracellular bacteria seemed to be within vacuoles and did not multiply. Attachment to the eucaryotic cell did not seem to involve pili or other bacterial surface structures. The dimensions of the bacteria were approximately 0.45 μm in width by 1.0 to 1.7 μm in length. The cell envelope consisted of the usual trilaminar cell wall and plasma membranes separated by a layer of low electron density, as found in other gram-negative bacteria. No significant differences between the vole and Fuller strains either in morphology or relationship to eucaryotic cells were encountered.

In a previous communication (12) we described some properties of the vole agent, isolated by Baker (1) in 1943, which led us to the surprising identification of this agent as a strain of the trench fever rickettsia, *Rochalimaea quintana*. A detailed comparison of the vole agent with an established strain of *R. quintana* became therefore desirable for purposes of confirmation and determination of variation within the species. It was shown in recent experiments (unpublished data) that the vole agent grows quite well in a modified Evans medium (10) used for the Fuller strain of *R. quintana*. One of the more interesting observations, however, was that in the absence of an adequate axenic medium the vole agent was capable of growing in close association with eucaryotic cells (12). A similar close relationship exists between human strains of *R. quintana* and the gut epithelium of lice (3, 15), but their interaction with mammalian cells has not been studied in detail (11). Thus, this study had the dual purpose of comparing the fine structure of the vole agent to the well-known structure of the Fuller strain (3), as well as to explore the interaction of both strains with mammalian cells.

MATERIALS AND METHODS

Preparation of cultures. The passage history of the vole agent was described by Weiss et al. (12). The experiments were performed with chicken embryo passages 44 and 46. The Fuller strain of *R. quintana* was obtained from the American Type Culture Collection and passaged five times on Vinson agar (11) prior to inoculation of cell cultures. The mouse LM_3 cells were irradiated 7 days prior to inoculation, as in a previous study (12), and cultivated on plastic cover slips of Leighton tubes (Costar), 16-ounce (ca. 475 ml) plastic flasks, or tissue culture eight-chamber slides (Lab-Tek Products). The multiplicity of infection was approximately 0.05 to 0.5 viable microorganism per eucaryotic cell in the case of the vole agent and somewhat less in the case of the Fuller strain. At intervals the flask cultures were examined with a Leitz Diavert phase-contrast microscope, and the slide cultures were fixed with Carnoy fixative and stained with Giemsa.

TEM. For transmission electron microscopy (TEM), the cell culture monolayers on plastic cover slips in Leighton tubes were washed gently in three changes of phosphate-buffered saline. They were fixed in 2% glutaraldehyde in 0.03 M HEPES (*N*-2-hydroxyethyl piperazine-*N*′-2′-ethanesulfonic acid) buffer, followed by postfixation in 2% OsO_4, also in HEPES buffer. Because of the solubility of the cover slips in both propylene oxide and acetone, embedment in Mollenhauer Epon-Araldite mixture no. 1 (6) was accomplished without a transitional solvent. The dehydrated monolayers were infiltrated overnight in a 1:1 mixture of 100% ethanol and complete Epon-Araldite embedding medium and two changes, maintained for 2 h each, in 100% complete embedding medium. Polymerization was achieved by incubating the specimens at room temperature for 96 h, instead of the recommended 12 h at 80°C (6), to prevent deformation of the plastic cover slips.

The specimens were sectioned perpendicularly to

the cell sheet, without removing the cover slip plastic, with a diamond knife (DuPont) on a Sorvall MT-2 ultramicrotome. The block face was oriented in such a way that the knife edge first encountered the Epon-Araldite before the softer cover slip plastic.

The sections were placed on uncoated 300-mesh grids and stained with both uranyl acetate and lead citrate. Several of the grids were also floated on a drop of a solution of latex spheres of 0.312-μg diameter (Ernest F. Fullam, Inc., Schenectady, N.Y.) and air dried. All material was examined with a Siemens 1A electron microscope.

In addition to the study of sectioned material, samples of medium from infected L cells were stained by negative staining in 2% phosphotungstic acid adjusted to pH 4.5 with 1 N KOH.

SEM. For scanning electron microscopy (SEM), the cell culture monolayers were fixed and dehydrated as described above for TEM. Following two 10-min changes in 100% ethanol, the cover slips were critical-point dried (Denton Vacuum Co.) in CO_2 and glued to aluminum studs. They were then sputter-coated with gold-palladium and examined with an AMR 1000 scanning electron microscope (American Metals Research) at 20 kV.

RESULTS

The growth of the Fuller strain on L cells appeared to be comparable to that of the vole agent described previously (12), but somewhat slower. When L cells were inoculated with a high multiplicity of either strain and examined by phase-contrast microscopy or following Giemsa staining, the majority of bacteria appeared to adhere to the L cells within a short time after infection. By comparing L cells inoculated with the vole agent 3 and 9 h previously, the doubling time for cell-associated bacteria was calculated to be about 4 h, and infection was quite heavy by 48 h, but many of these bacteria were associated with L-cell debris or seemed to fill the gaps left by exfoliated L cells. Bacteria of the Fuller strain were seen in small numbers for about 48 h, but eventually growth became as profuse as in the case of the vole strain. Differences between L cells moderately or heavily infected with the vole or Fuller strains were not apparent, with the possible exception that the vole agent might have caused greater destruction of the marginal cytoplasm.

Infection with the vole agent or the Fuller strain could be readily recognized in unstained preparations by phase microscopy even at low magnification by what appeared to be masses of bacteria adhering to the surface of the L cells. Infection with an obligate intracellular bacterium, such as *Rickettsia typhi,* does not change the appearance of the L cells, until the terminal stage of disintegration of the monolayer (14). Figure 1 illustrates a Giemsa-stained cell infected 80 h previously with the Fuller strain.

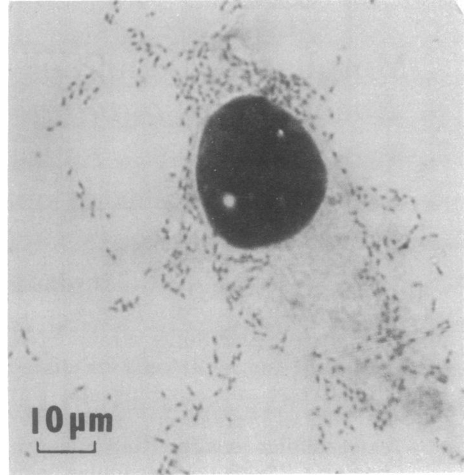

FIG. 1. *Light microscopy of L cell infected 80 h previously with the Fuller strain, fixed with Carnoy fixative and stained with Giemsa.*

The cell is almost completely surrounded by bacteria, which also appear to cover a good portion of the cytoplasm. Infection of Giemsa-stained L cells with the vole agent was shown in a previous publication (12).

The fine structure of the vole and the Fuller strains was well preserved by the HEPES-buffered fixation method. The results were comparable to those obtained by the bacterial fixation technique of Ryter and Kellenberger (9), but the HEPES-buffered fixation method had the advantage of being particularly satisfactory for the preservation of the surface structures of the irradiated L cells.

Figure 2 illustrates SEM of heavily infected L cells. The cells appeared to be completely covered with the Fuller (Fig. 2A) or vole strains (Fig. 2B). In many areas of the surface the bacteria appeared to be several layers thick, but few bacteria were seen in the spaces surrounding the L cells. At higher magnification (Fig. 2C) the vole agent can be seen in close association with the plasma membrane of the L cells.

The intimate association of the bacteria to the plasma membrane of an L cell is illustrated in TEM of thin sections (Fig. 3). There seems to be no preferred orientation of the bacteria on the surface of the L cells, but rather a random colonization. This is also evident in the SEM photographs (Fig. 2). Occasionally granular electron-dense material was seen between the outer cell wall of the bacteria and the plasma membrane of the L cell, but no evidence of pili or flagella was seen in any of the preparations, including those that were negatively stained (Fig. 4). The furrows seen in Fig. 4 were probably

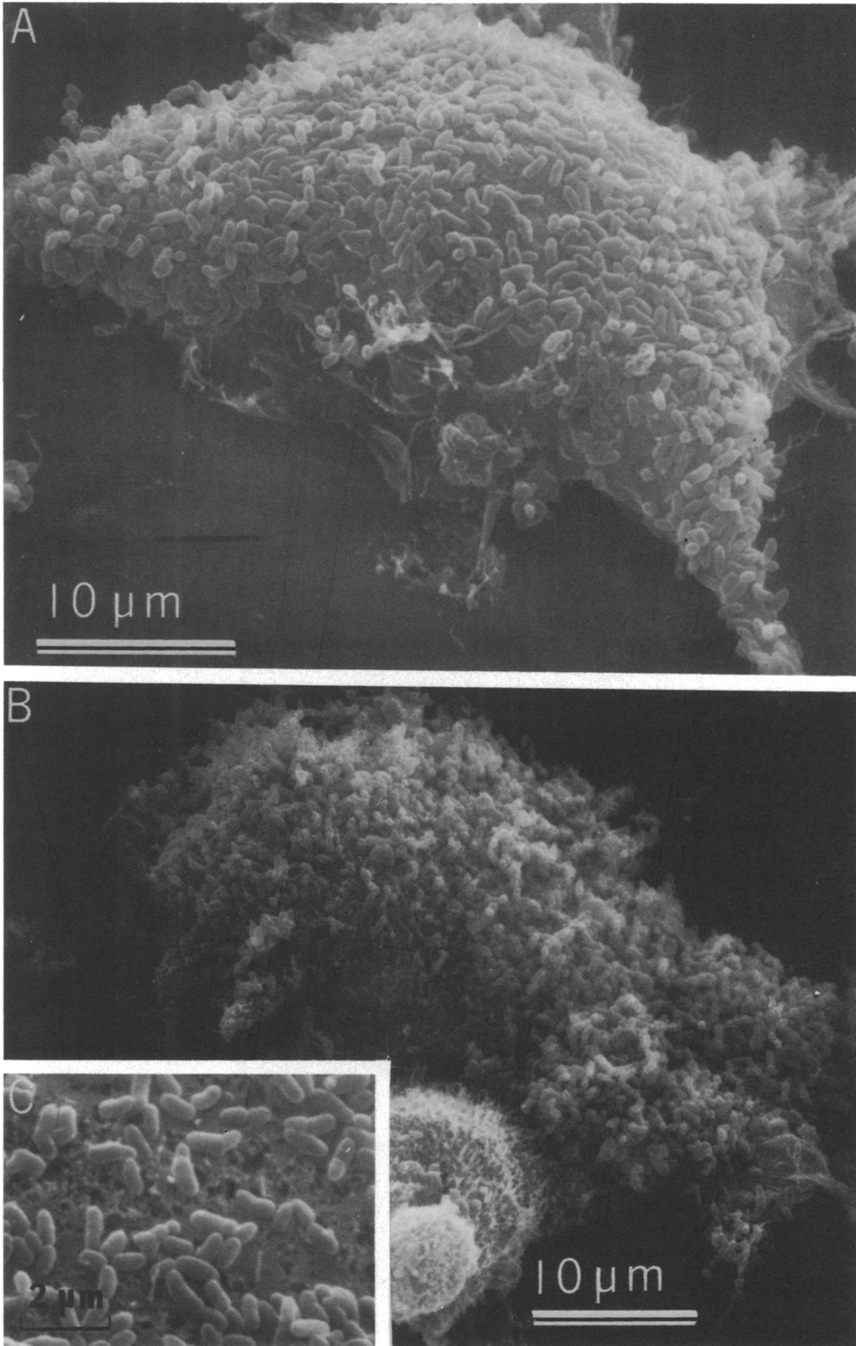

FIG. 2. *SEM of infected irradiated mouse L cells. (A) Fuller strain, 96 h after infection. (B) Vole agent, 48 h after infection. (C) Higher magnification of (B).*

artifacts from the drying and flattening of the bacteria during preparation.

Bacteria undergoing division were seen frequently (Fig. 5). In these bacteria there was a constriction perpendicular to the long axis of the cell. Various stages of division were observed, including those with shallow constrictions, representing early stages (Fig. 5A), and those in

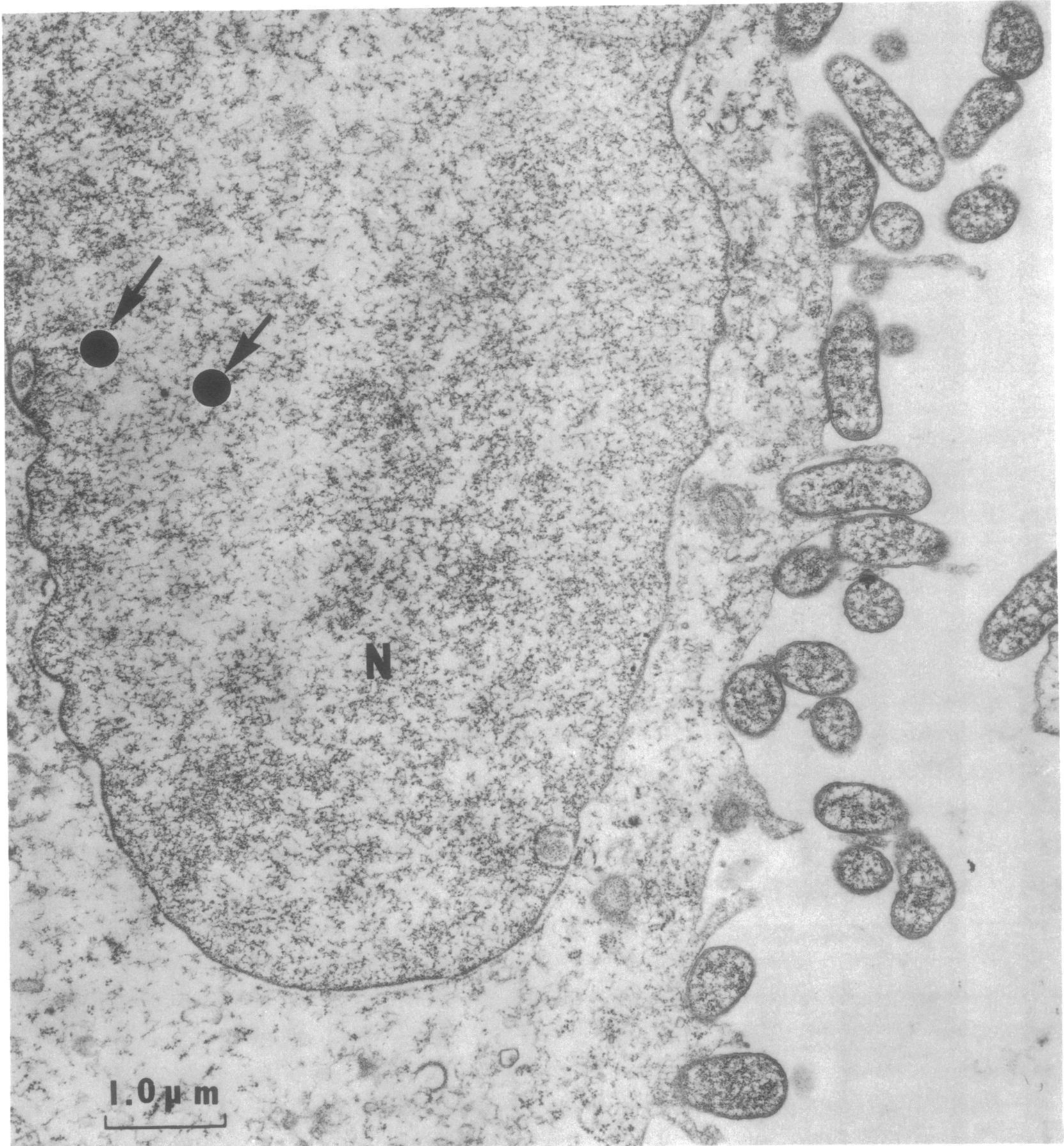

FIG. 3. *TEM of irradiated L cell infected 48 h previously with the vole agent: N, nucleus; arrows, uniform 0.312-μm latex spheres.*

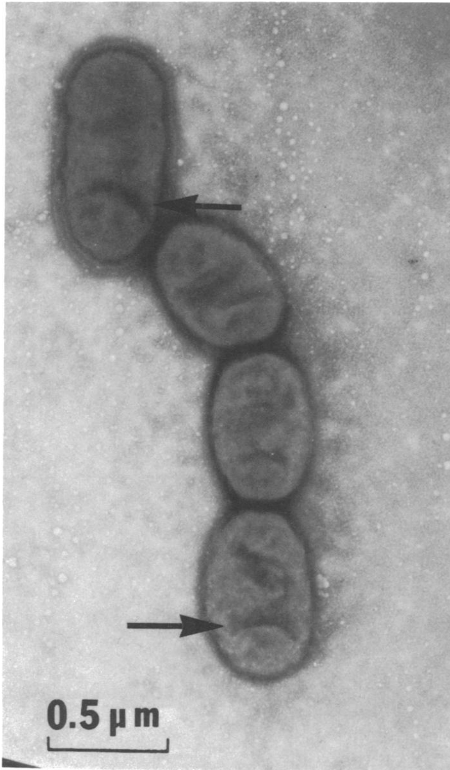

FIG. 4. *Negative staining of vole agent from the fluid of a 48-h culture. Arrow, Furrow on the surface of the bacterium.*

which there was an almost complete cell wall and membrane between the two organisms, representing advanced stages of division (Fig. 5B). There seemed to be no alteration to the ultrastructure of the dividing bacteria other than the constriction furrow, although division appeared sometimes to be unequal (Fig. 5B). Dividing forms were also seen by SEM (Fig. 2C). All the dividing forms appeared to be extracellular.

Several TEM fields from cultures infected with either the vole or the Fuller strains appeared to have L cells containing intracellular bacteria. The sections usually did not contain an L-cell nucleus and might have represented planes close to the surface, suggesting that some of the bacteria were possibly lying in surface depressions. Others, either single or in small groups, were surrounded by a eucaryotic cell membrane (Fig. 6). None of these bacteria were seen in the process of cell division.

The fine structure of the vole and Fuller strains was similar to that of other gram-negative bacteria. In thin sections the bacteria appeared to be either circular or rod-shaped, from 0.3 to 0.5 µm wide (average, 0.45 µm), when

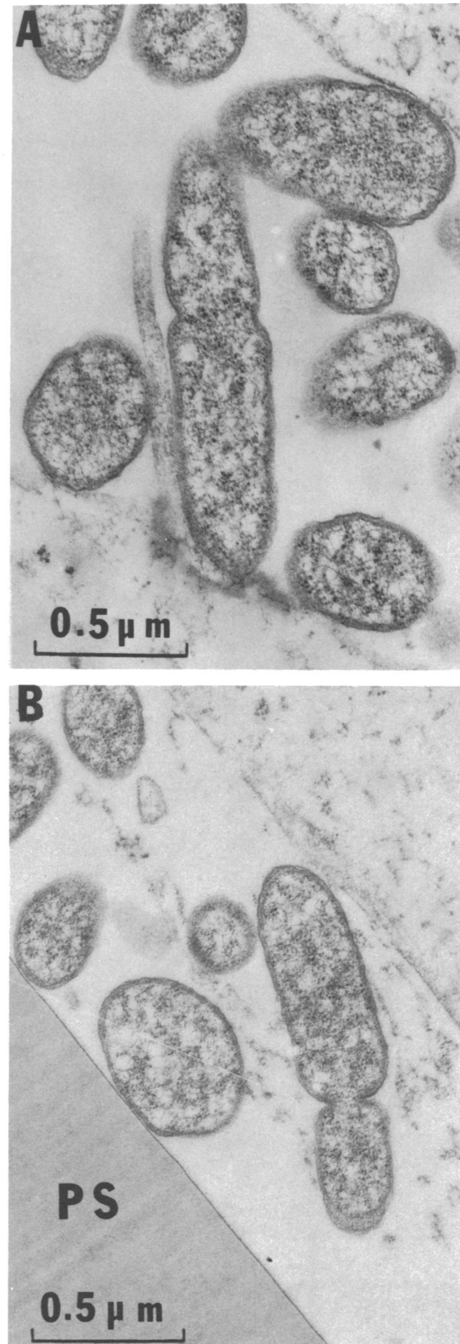

FIG. 5. *TEM of dividing bacteria. PS, Plastic substrate. (A) Vole agent, 52 h postinfection. (B) Fuller strain, 96 h after infection.*

compared with uniform latex spheres 0.312 µm in diameter (Fig. 3). Because of their random orientation, their length was more difficult to

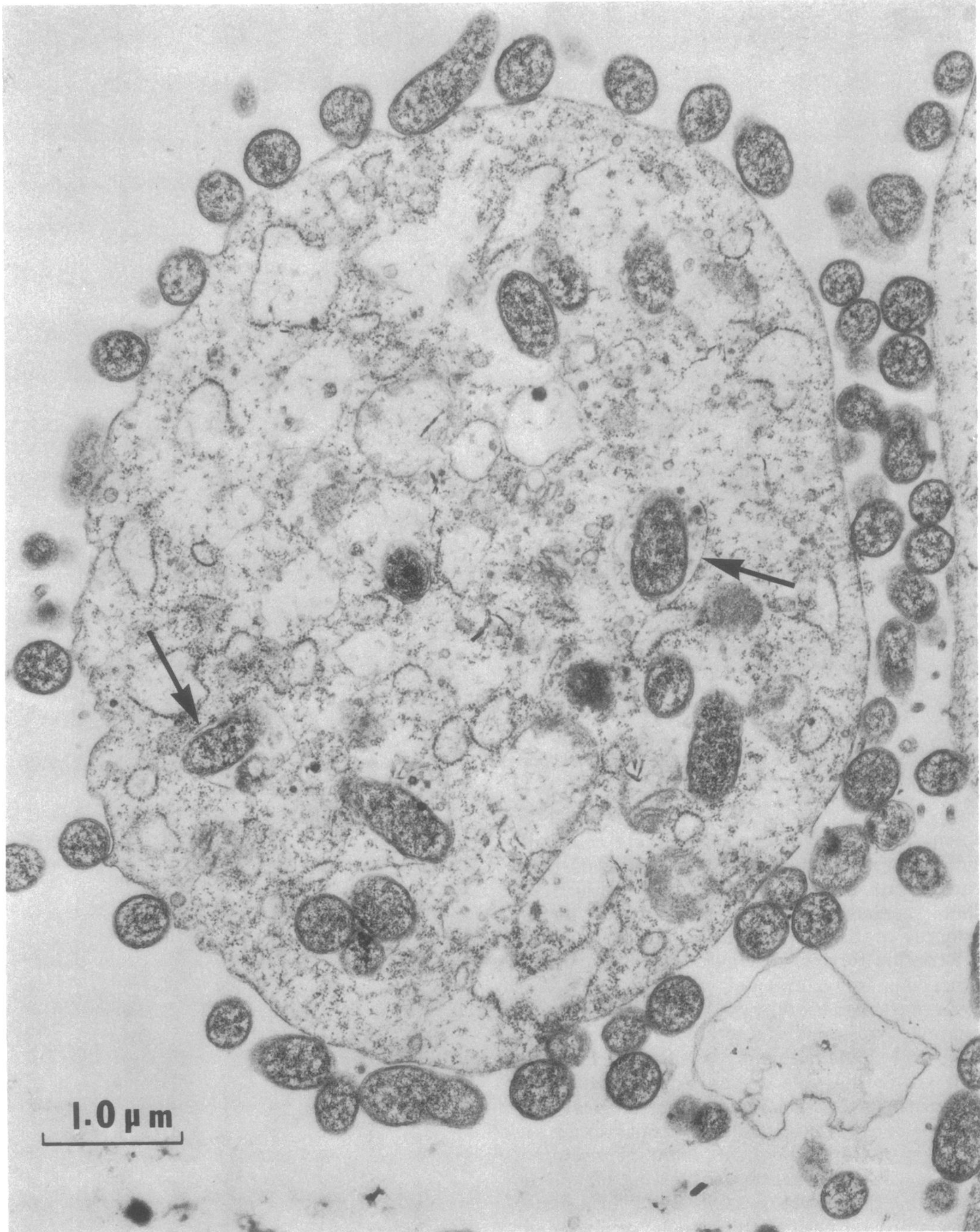

FIG. 6. *TEM of marginal section of L cell infected 52 h previously with the vole agent. Arrows, Membranes surrounding bacteria.*

determine, but it is estimated to have averaged 1.2 μm. The length seldom exceeded 1.7 μm in a nondividing cell. The bacteria are enclosed by three clearly defined structures (Fig. 7). The outermost is a trilaminar outer membrane approximately 8 to 9 nm thick. The second is a homogeneous layer of low electron density, varying in thickness from 6 to 13 nm, which separates

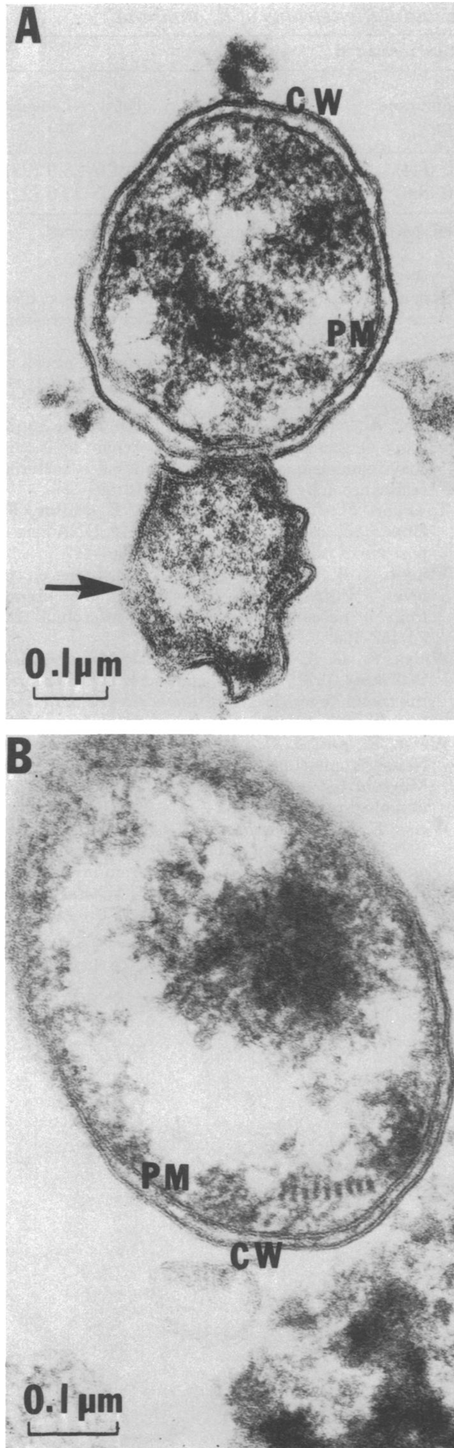

FIG. 7. *TEM of R. quintana. CW, Cell wall; PM, plasma membrane; arrow, aberrant form. (A) Vole agent, 48 h postinfection. (B) Fuller strain, 96 h postinfection.*

the outer membrane from the inner trilaminar plasma membrane, approximately 7 nm thick. The total thickness of the three layers is about 21 to 28 nm. Both membranes of *R. quintana* have the typical unit membrane structure consisting of two electron-dense layers separated by an electron-translucent layer as described by Robertson (8). Similar structures were also seen in negatively stained preparations (Fig. 4). Aberrant forms were occasionally seen (Fig. 7), but they could be artifacts.

The cytoplasm of the bacteria also contained coarse aggregations of electron-dense granules enmeshed in a network of fine filaments (Fig. 7). The granules probably represent the ribonucleoprotein and the filaments the deoxyribonucleic acid component, demonstrated in other bacteria (9), although in this case neither the classic Ryter-Kellenberger fixation (9) nor the "Versene test" (5) were employed.

No differences of any significance were noted in the fine structure of the vole and Fuller strains (Table 1).

DISCUSSION

The vole agent is a small, relatively typical gram-negative bacterium, indistinguishable from the Fuller strain, as described by Ito and Vinson (3). No pili or other surface bacterial structures were observed in the study by Ito and Vinson (3) or in this study, although the methods used have demonstrated pili on *Vibrio parahaemolyticus* and *Escherichia coli* (4). Adherence to mammalian cells is a property of numerous pathogens. *Neisseria* adhere to cells by means of pili (13). Oral streptococci adhere to tooth surfaces by means of small fibrils and/or dextrans (2). The mechanism of association of the two strains of *R. quintana* to L cells remains unknown. Any cytopathic effect on the L cells must have been small, because very large numbers of bacteria accumulated before the L cells disintegrated. The observation made by light microscopy that the vole but not the Fuller strain causes exfoliation of bits of marginal cytoplasm has been suggested but not confirmed by electron microscopy. The few bacteria that gained an intracellular location appeared to have been engulfed and in the process of being digested. The requisite for both strains for an extracellular location was demonstrated by the observation that all dividing forms were extracellular.

It remains to be determined whether or not the adherence of the two strains to L cells might be associated with a pathogenic role. The Fuller strain is the known agent of trench fever in man, but it is not virulent for laboratory animals or lice (3, 11, 15). The vole agent is not virulent for

TABLE 1. *Measurements of structures of vole and Fuller strains of R. quintana*[a]

Strain	Structure measured				
	Whole cell (μm)		Outer membrane (nm)	Plasma membrane (nm)	Total cell envelope (nm)
	Width	Length			
Vole	0.3–0.5 (0.45)	≤1.7	8.0–9.0 (8.4)	6.8–7.1 (7.0)	21.0–28.0 (23)
Fuller	0.3–0.5 (0.44)	≤1.7	8.0–9.0 (8.6)	6.9–7.3 (7.1)	21.0–27.0 (22)

[a] Based on comparison with uniform 0.312-μm latex spheres. Mean, where applicable, in parentheses.

laboratory animals, and its virulence for man is unknown.

ACKNOWLEDGMENTS

We are indebted to Martha L. Hale for the cultures of the Fuller strains.

This work was supported by the Naval Medical Research and Development Command Work Units MR04105.0007 and MR0000101.1178.

LITERATURE CITED

1. **Baker, J. A.** 1946. A rickettsial infection in Canadian voles. J. Exp. Med. **84**:37–51.
2. **Gibbons, R. J., and J. Van Houte.** 1975. Bacterial adherence in oral microbial ecology. Annu. Rev. Microbiol. **29**:19–44.
3. **Ito, S., and J. W. Vinson.** 1965. Fine structure of *Rickettsia quintana* cultivated in vitro and in the louse. J. Bacteriol. **89**:481–495.
4. **Joseph, S. W., B. R. Merrell, M. Sochard, and W. P. Brown.** 1978. Visualization of the attachment of *Vibrio parahaemolyticus* in the rabbit ileum by electron microscopy, p. 727-732. *In* O. Johari (ed.), Scanning electron microscopy/78/II. Scanning Electron Microscopy, Inc., Chicago.
5. **Kellenberger, E., A. Ryter, and J. Séchaud.** 1958. Electron microscopic study of DNA containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
6. **Mollenhauer, H. H.** 1963. Plastic embedding mixtures for use in electron microscopy. J. Stain Technol. **39**:111–114.
7. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. **17**:208–242.
8. **Robertson, J. D.** 1959. The ultrastructure of cell membranes and their derivatives. Biochem. Soc. Symp. **16**:3–43.
9. **Ryter, A., and E. Kellenberger.** 1958. Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique. I. Les nucléoides des bactéries en croissance active. Z. Naturforsch. **13**:597–599.
10. **Tyeryar, F. J., Jr., E. Weiss, D. B. Millar, F. M. Bozeman, and R. A. Ormsbee.** 1973. DNA base composition of rickettsiae. Science **180**:415–417.
11. **Vinson, J. W., and H. S. Fuller.** 1961. Studies on trench fever. I. Propagation of rickettsia-like microorganisms from a patient's blood. Pathol. Microbiol. Suppl. **24**:152-166.
12. **Weiss, E., G. A. Dasch, D. R. Woodman, and J. C. Williams.** 1978. The vole agent identified as a strain of the trench fever rickettsia, *Rochalimaea quintana*. Infect. Immun. **19**:1013-1020.
13. **Weiss, E., and J. C. Hill.** 1975. Immunization against Neisseria infection, p. 423–440. *In* E. Neter and F. Milgrom (ed.), 4th International Convocation of Immunologists, Buffalo, N.Y., 1974. Karger, Basel.
14. **Weiss, E., L. W. Newman, R. Grays, and A. E. Green.** 1972. Metabolism of *Rickettsia typhi* and *Rickettsia akari* in irradiated L cells. Infect. Immun. **6**:50–57.
15. **Wolbach, S. B., J. L. Tood, and F. W. Palfrey.** 1922. The etiology and pathology of typhus. Harvard University Press, Cambridge.