Fine Structure of *Rickettsia canada* in Tissues of *Dermacentor andersoni* Stiles

LYLE P. BRINTON AND WILLY BURGDORFER

Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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Preliminary observations on growth and developmental fine structure of *Rickettsia canada* in various organs and tissues of the hard tick, *Dermacentor andersoni* Stiles, are reported. *R. canada* is typically rod-shaped, being delimited with a three-layered wall having a velvety coating adsorbed to its exterior surface. A finely reticulated cytoplasmic matrix containing prominent ribosomes is delimited with a three-layered unit membrane. Average length and width of these organisms are 1.6 by 0.4 μm . Although *R. canada* produces a generalized infection in *D. andersoni*, hypodermal and muscle tissues experience heaviest growth. Three morphologically distinct rickettsial forms were observed in individual hypodermal cells: (i) typical growth forms with a finely reticulated cytoplasmic matrix and distinct ribosomes; (ii) atypical forms with lightly to densely staining cytoplasm and a coagulated appearance in which ribosomes cannot be distinguished from the matrix; and (iii) forms with crystalline bodies that have a striated to beaded lattice structure and, at times, a fibrillar body in the cytoplasm as well. Occasional finger-like to irregular invaginations of the plasma membrane are noted. Intranuclear growth was demonstrated by electron microscopy in gut epithelial cells only. Growth and development of *R. canada* were manifest in all tissues examined.

Rickettsia canada, a member of the typhus group, was originally isolated (17) from pools of *Haemaphysalis leporispalustris* collected from indicator rabbits in the Richmond area of Ottawa, Canada. Its relationships to various species of tick vectors, *H. leporispalustris*, *Dermacentor andersoni*, *D. variabilis*, and *Amblyomma americanum*, as well as its behavior in a variety of suspected host animals, have been and still are subjects of intensive studies (5, 7). The present paper concerns preliminary observations on the fine structure of *R. canada* during its development in tissues of experimentally infected Rocky Mountain wood ticks, *D. andersoni*.

MATERIALS AND METHODS

As reported previously (5), strain 2678 of *R. canada*, the only isolate available so far, produces a generalized infection of all tissues after ingestion by *D. andersoni*. It has since been established (Burgdorfer and Brinton, unpublished data) that this agent is passed transovarially to 100% of filial ticks. For electron microscopy, hemolymph preparations, hypodermal and muscle tissues, gut and gut diverticula, Malpighian tubules, and ovarian tissues of ticks from the fourth filial generation were used.

Ticks were dissected in Neisenheimer's saline (KCl, 0.1 g; NaCl, 7.5 g; CaCl₂, 0.2 g; NaHCO₃, 0.2 g; 1 liter of water, pH 7.2), and the various organs were processed at 0 to 4 C. They were fixed in 3% glutaraldehyde

in 0.1 M phosphate buffer at pH 7.4 for 1 hr. Tissues were washed in two changes of phosphate-buffered sucrose (0.2 M) at pH 7.4 for 2 hr followed by post fixation in 1.0% osmium tetroxide in 0.1 M phosphate buffer with 0.20 M sucrose at pH 7.4 for 1 hr. Tissues were rapidly dehydrated at 5-min intervals in an ethanolic series with the last two changes in absolute ethanol at room temperature (21.5 C). Two 10-min changes in propylene oxide were followed by infiltration in a propylene oxide-araldite 502 mixture. Embedding and polymerization followed as recommended by Luft (16). Silver-gold (ca. 60 nm) to gray sections were cut with a Porter-Blum MT-1 ultramicrotome and placed upon Formvar-coated, carbon-stabilized grids. Sections were stained in uranyl acetate and lead citrate (20) and then examined in a Hitachi HU11-E electron microscope at 75 kv with a 50- μm objective lens aperture.

Hemocytes were obtained by amputating the tibiotarsal segments of one or more legs and allowing the tick to bleed directly into glutaraldehyde by a modification of the method of Granados et al. (9). The number of ticks, generally 5 to 10, necessary for obtaining a hemocyte pellet was determined by their state of engorgement. Hemocytes were processed as above with centrifugation in a refrigerated centrifuge at 100 $\times$ g for 5 min before each solution change. Immediately before dehydration, the cells were transferred to bovine serum albumen (22) or 1.5% agar and centrifuged to a pellet.

RESULTS

R. canada exhibits morphology characteristic

for most rickettsiae. Externally it is delimited by a three-layered cell wall (Fig. 1), approximately 7-nm thick, with a velvet-like coating (Fig. 2) attached or adsorbed to the exterior surface. All layers appear of equal thickness, although the internal layer occasionally is thickest and stains most densely (Fig. 1). The cell plasma membrane also is three-layered and of typical unit membrane construction; it may be pulled away from the cell wall or may follow it very closely. The cytoplasm is finely reticulated and may contain unevenly distributed ribosomes (Fig. 3), depending on the metabolic state or stage of development of the organisms. Electron-lucent vacuolar bodies are infrequently observed in the cytoplasm (Fig. 13). Average length and width of *R. canada* is 1.6 by 0.4 μm . Larger, mostly pre- or early-phase division forms measure up to 2.5 by 0.4 μm . Multiplication is by binary fission with sister cells averaging 1.2 μm in length. Occasionally organisms are found with finger-like (Fig. 4) to irregular (Fig. 2) indentations of the plasma membrane protruding into the cytoplasm.

Hypodermal tissues. In hypodermal tissues, *R. canada* occurs as three morphological types (Fig. 5-7) each exhibiting a preference for growth in different areas of its host-cell cytoplasm. Type I (Fig. 6) is found in the cytoplasm adjacent to cell surfaces lined with microvilli and exposed to the hemocoel. These rickettsiae contain distinct ribosomes unevenly distributed throughout their cytoplasm. Density of the cytoplasm and general integrity of the plasma membrane with the cell wall suggest that these rickettsiae are in an active growth phase. Type II (Fig. 5), markedly different in appearance, is located adjacent to the plasma membrane at the opposite side of the host cell, whose surface is exposed to the basement membrane and endocuticle of the integument. Although rod-shaped, these organisms exhibit a cytoplasm that is "coagulated" and finely granular with irregularly defined densely and lightly staining areas. Typical ribosomes are not observed but may be represented by some of the dense-staining material. Division forms occur among these rickettsiae which also exhibit great propensity for clonelike growth. Type III (Fig. 7) generally is found distributed in host-cell cytoplasm between areas occupied by types I and II. The cell cytoplasm is reduced to amorphous particles and is absent around most rickettsiae. Three morphological characteristics distinguish type III from the other two described forms: (i) Presence of crystalloid bodies, generally one and occasionally two, randomly located in the rickettsial cytoplasm. Grossly, these bodies are ovate to oblong or irregularly rectangular. They have a crystalline, lattice-like fine structure which is variably striated from banded (Fig. 8) to semibanded

(Fig. 9) in organization. Banded subunits are paired and of approximately 16 nm in diameter. Beaded subunits measure about 9 nm. (ii) Presence of fine fibrillar reticulated areas, often in the same cytoplasm and closely associated with crystalloid bodies. (iii) Consistent "pulling away" of the protoplast from its wall, suggesting shrinkage of the cytoplasm, or, conversely, a stretching or extension of the wall. The longest organism of this type measured 2.7 μm ; its protoplast was approximately 1.9 μm . Although some ribosomal bodies are discernible, they are not as numerous as in type I rickettsiae.

Intracellular organization of infected hypodermal tissue cells invariably shows considerable disruption, especially where numerous rickettsiae are present. This breakdown of host-cell cytoplasm is manifested first by a clear space or "halo" around most of the rickettsial organisms preceding a general deterioration of the cytoplasm (Fig. 17).

Gut tissue. In gut tissue, development of *R. canada* is not confined to specific cell types but occurs throughout the epithelium, including cellular processes and nuclei (Fig. 10; see also reference 7). Clear areas or "halos" devoid of stainable material surround many rickettsiae (Fig. 1). Intracytoplasmic rickettsiae frequently possess plasma membranes closely and uniformly associated with the cell wall (Fig. 1 and 2); in other cases, the plasma membrane may show areas of detachment from the cell wall. Such forms are predominant in nuclei (Fig. 10) of epithelial cells. Ribosomes of these rickettsiae are irregularly distributed throughout the cytoplasm, and the matrix consists of a finely granular to fibrillar meshwork. Occasionally, invaginations of the plasma membrane irregularly protrude into the cytoplasm (Fig. 2). Relatively small densely stained patches of material frequently occur in intracytoplasmic rickettsiae (Fig. 2) but have yet to be noted in intranuclear forms.

Rickettsiae are also frequently observed free in blood residue of the gut lumen. Their cell walls are typically more collapsed or irregular than those of intracellular organisms, and their cytoplasmic matrix and ribosomes are not as well organized, varying from granular to semifibrillar. A narrow clear zone around them may be indicative of some metabolic activity.

Malpighian tissues. Morphology of *R. canada* in Malpighian tissues varies with cellular organization of host-cell cytoplasm which differs between the two sexes of *D. andersoni* (Fig. 11 and 12). Rickettsiae in tubule cells of unfed males are dispersed and vary in concentration from heavy to light among high concentrations of dark- to light-staining glycogen-like bodies (Fig. 11). Prominent halos surround individual organisms,

FIG. 1. *R. canada* in a gut epithelial cell of *D. andersoni*. Note three-layered wall and cell membrane; also evident is breakdown of host-cell cytoplasm.

FIG. 2. Long specimen of *R. canada* in a gut epithelial cell. Three-layered wall is well-defined with velvety coating adsorbed to its external surface. Arrow points to irregular invagination of cell membrane.

FIG. 3. *R. canada* in a hypodermal cell of female *D. andersoni*. Distinct "halo" around organisms suggests exogenous digestion.

FIG. 4. Finger-like invagination (arrows) of cell membrane into cytoplasmic matrix of *R. canada*.

FIG. 5. *R. canada* in the cytoplasm of a hypodermal cell, adjacent to the basement membrane and integument. These atypical type II growth forms, including division form, have a "coagulated" appearing cytoplasm and apparently are devoid of ribosomes.

FIG. 6. Type I growth forms of *R. canada* at opposite side of same host cell as in Fig. 5, adjacent to cell surface exposed to hemocoel. Portions of microvilli (arrows) extend from this surface of the host cell.

FIG. 7. Type III atypical growth forms of *R. canada* in same cytoplasm as Fig. 5 and 6, approximately midway between types I and II. Note distinct crystalloid bodies (arrows) with parallel lattice-like structures. Fibrillar zones are also variably located in cytoplasm.

FIG. 8. Banded type (arrows) of crystalloid inclusion in cytoplasm of *R. canada* in hypodermal cell of male *D. andersoni*.

FIG. 9. Beaded type (arrows) of crystalloid inclusion in cytoplasm of *R. canada* in hypodermal cell of female *D. andersoni*.

FIG. 10. *R. canada* in nucleus of gut epithelial cell.

FIG. 11. *R. canada* in Malpighian tubule cells of male *D. andersoni*. Each organism has a well-defined "halo" surrounding it. Cytoplasm of tubule cell contains numerous glycogen-like particles.

FIG. 12. *R. canada* in Malpighian tubule cell of unfed female 1 month after molting. Rickettsiae are clustered adjacent to microvillar lined luminal surface of cell.

and at their cytoplasmic boundaries some granules of glycogen-like material have apparently undergone partial digestion (Fig. 11). Grossly, these rickettsiae are slightly elliptical in longitudinal section. The well-defined three-layered wall and unit membrane enclose a weakly reticulated cytoplasmic matrix containing unevenly distributed ribosomes. In unfed female ticks, a narrow band of electron-dense cytoplasm is primarily concentrated around the periphery of Malpighian tubule cells (Fig. 12). High concentrations of small and large (0.08 to 1.0 μm) light- to gray-staining vacuoloid bodies are dispersed throughout the remainder of the cytoplasm. This vacuolar material gives the cytoplasm an alveolate appearance. Rickettsiae are characteristically localized in clonelike groups within masses of these small nonstaining bodies adjacent to the luminal surface of tubule cells (Fig. 12). At the opposite sides of these cells, where cell boundaries are exposed to the hemocoel, only occasional, isolated rickettsiae are observed; they become more numerous in the direction of the luminal surfaces of these cells. Only poorly defined to incomplete "halo" spaces are evident around organisms, which possess a distinct, rodlike morphology. Their sinuate cell walls and membranes surround a dense cytoplasmic matrix containing unevenly distributed ribosomes.

Salivary glands. In salivary glands, rickettsiae have thus far been observed in types II and III alveoli (25) and in higher numbers in cells associated with their secretory ducts (Fig. 13), where they are in juxtaposition to duct walls. In alveolar cells, they are found in the ribosome-rich cytoplasm among secretion granules (Fig. 14). Thus far, no organisms have been noted intranuclearly or within cytoplasmic secretory granules. An irregular rod-shaped morphology with a moderate to dense reticulated cytoplasmic matrix characterizes *R. canada* in these cells. Ribosomes vary in number, being greater with increased density of the matrix (Fig. 13). Clear spaces of variable diameter also surround these rickettsiae, being larger in diameter in cells associated with secretory ducts than in secretory alveolar cells.

Ovarian tissues. Ovarian tissues of unfed females, approximately 1 month after molting, regularly showed heavy infection by *R. canada*. Organisms are found individually in interstitial cells or in clonelike groups in oocytes. In contrast to what is seen in hypodermal tissue, cytoplasmic vacuolation in the presence of *R. canada* is absent. Occasionally, rickettsiae are found in immediate proximity to *Wolbachia* sp. present in interstitial cells at this time (Fig. 15). Clear spaces surrounding *R. canada* in both cell types are of variable diameter and, in contrast, are generally

absent around *Wolbachia* sp. These rickettsiae usually have sinuate cell walls with plasma membranes following less sinuously. Density of the cytoplasmic matrix is variable, as is ribosomal distribution. Division forms in ovarian tissue have a sharply constricting plasma membrane and wall like those observed in other tissues (Fig. 16); the constricting wall apparently pinches off the cytoplasm, in a manner somewhat similar to that reported for most gram-negative bacteria under normal conditions (24).

Hemocytes. Hemocytes from *D. andersoni* adults infected with *R. canada* often reveal considerable numbers of organisms, especially in granular hemocytes of the spherule subtype. In spherule cells they occur among spheres, while in typical plasmatocytes they are found variously associated with the rough endoplasmic reticulum and free ribosomes. As yet, intranuclear forms have not been detected by electron microscopy. Peripheral or surface morphology of individual organisms varies from smooth to sinuate and is generally surrounded by typical clear spaces. Often the cytoplasmic matrix is unevenly reticulated and ribosomes are relatively sparse. Division forms have a cytoplasmic matrix and ribosome complement of slightly increased density (Fig. 16). In one instance, a cytoplasmic bridge at the site of constriction during cell division contained a membrane-like partition transversely oriented across its width (Fig. 16).

DISCUSSION

In general, the subcellular appearance of *R. canada* in various tissues of experimentally infected *D. andersoni* is similar to that of certain other rickettsial agents (*R. rickettsi*, *R. prowazeki*, *R. orientalis*) pathogenic to man (6, 10, 19). Its three-layered plasma membrane is typical of unit membranes in biological systems including rickettsiae, and its three-layered cell wall appears very similar to that of gram-negative bacteria (8, 24). During binary division, both plasma membrane and cell wall constrict sharply at the site of cytoplasmic cleavage. Significance of the membrane-like partition observed in a division form in a hemocyte is not known. This partition may represent final division of the plasma membrane, which in turn could serve as a line of communication for final cleavage of the cell wall, or a template upon which new wall material is elaborated. Localization of *R. canada* in hemocytes is, in general, similar to *R. prowazeki* in hemocytes of *Hyalomma dromedarii* (4).

The reticulated cytoplasmic matrix of *R. canada* very likely represents deoxyribonucleic acid distribution in this procaryotic organism. In typical growth forms, reticulation of the matrix is

FIG. 13. *R. canada* in cytoplasm of cell surrounding secretory duct wall of salivary alveolus.

FIG. 14. Alveolar cell of salivary gland with *R. canada* in densely basophilic cytoplasm among gray-staining granules.

FIG. 15. *R. canada* in an interstitial cell of ovarian tissues in close association with the rickettsia-like symbiotes, *Wolbachia* sp. (arrows). Both kinds of organisms contain free, distinct ribosomes and have a finely reticulated cytoplasmic matrix.

FIG. 16. Division form of *R. canada* in a granular hemocyte. Constricting wall and membrane appear to be pinching cytoplasm into two sister cells. An indefinite membranous strand (arrow) extends to each point of constriction.

FIG. 17. Survey photomicrograph of *R. canada* in hypodermal tissue of male *D. andersoni* revealing extensive cytoplasmic deterioration. Longitudinal forms are typically rod-shaped, whereas cross sections are coccoid.

generally consistent, and is similar to that reported for *R. prowazeki* (3, 12) and *R. rickettsi* (3). A heavy reticulum shown for *R. prowazeki* (2) was not observed in *R. canada*. Ribosomal distribution is similar to that reported for *R. senetsu* and *R. prowazeki* (3) but differs from the parallel chains of ribosomes occasionally noted in the Breinl strain of *R. prowazeki* (23). Finely granular areas associated with early development of *R. prowazeki* in L cells in vitro (14) were not seen with *R. canada* in *D. andersoni*, nor was *R. canada* confined to membrane-limited vacuoles as reported for *Coxiella burnetii* (15) in testicular tissue of guinea pigs. A centrally located densely staining mass, like that reported for *C. burnetii* (1, 18, 21, 26), was not observed. The occasionally detected irregular and finger-like invaginations of the plasma membrane are similar to those reported for *R. quintana* (11) and may represent simplified mesosomal organelles. Electron-lucent vacuolar bodies infrequently observed in the cytoplasm may be cross sections of these invaginations. Since such invaginations in *R. canada* were infrequent, it is not possible to correlate metabolic or functional significance to their structure.

The nature and function of crystalloid bodies are unique; they have not been observed in other rickettsiae and their nature and function are unknown.

Although infection of *R. canada* in *D. andersoni* involves all organs and their tissues, intensity and pattern of rickettsial development varies in different organs. Hypodermis and muscle tissues are most heavily infected, indicative perhaps of a more nearly optimal intracellular environment for rickettsial development. Thus, disruption of cellular structures, especially in hypodermis, is undoubtedly a direct effect of massive rickettsial infection. The halos around most *R. canada* may be interpreted as digested cytoplasmic matrix, and the velvety coating on the outer surface of the three-layered wall may represent adsorbed digested host-cell cytoplasm—a phenomenon known for bacterial agents.

The degree to which a group of cells or even a single cell can influence physiological behavior and morphological appearance of a foreign agent is difficult to ascertain. Yet, the presence of three morphologically different growth forms in individual hypodermal cells strongly suggests that the organisms respond to differing intracellular activities (e.g., pH, metabolic processes) or perhaps to stimuli from surrounding tissues. Kordová and Rěhácěk (13) noted that different forms of *R. prowazeki* appear in tissue culture during the course of infection and that these changes are similar to those observed in organs of ticks.

From these initial observations on the growth and developmental fine structure of *R. canada* in *D. andersoni*, it is clear that a true parasitic relationship exists with attendant histopathology of tissues and organs involved.

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