Cellular and Subcellular Organization of the 277F Agent, a *Spiroplasma* from the Rabbit Tick *Haemaphysalis leporispalustris* (Acari: Ixodidae)

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The cellular and subcellular ultrastructure of the helical 277F agent was studied in detail utilizing negative staining and ultrathin sections of fluids and/or tissues of infected chicken embryos and *Ornithodoros moubata* ticks. This organism, which lacks a cell wall and axial filaments, conforms closely in size and fine structural characteristics (plasma membrane, ribosomes, fibrillar reticulum, and thin glycocalyx-like coat on the cell surface) to the genus *Spiroplasma* of the order *Mycoplasmatales*.

In 1968, Pickens et al. (10) reported the isolation and preliminary characterization of a spirochete-like organism, referred to as the 277F agent, from the rabbit tick *Haemaphysalis leporispalustris* taken off two cottontail rabbits (*Sylvilagus nuttalli*) in western Montana. This organism, up to 30.0 μm long and 0.1 μm wide, was difficult to demonstrate by Giemsa stain, but under dark field it appeared uniformly and tightly coiled and exhibited typical corkscrew-like motility. It could regularly be maintained in embryonated chicken eggs, in which it was highly lethal, as well as in enriched liquid medium, and it passed through Berkefeld N but not Seitz EK filters.

One outstanding morphological feature of the 277F agent was a granule-like protuberance either at one end or anywhere along the length of the cell. There was evidence (resistance to heat treatment and passage through Berkefeld filters) that these granular products were infectious and capable of producing typical spirochete-like forms (10).

The cellular ultrastructure of this helical organism has been studied utilizing transmission electron microscopy. The results reported here suggest that the 277F agent is a mycoplasma-like organism closely similar to the corn stunt agent (1, 6, 7) or to *Spiroplasma citri*, the "Stubborn" disease agent of citrus (3, 4, 14).

MATERIALS AND METHODS

The 277F preparations examined consisted of infected allantoic and amniotic fluids of chicken embryos of the 14th passage (seed lot no. 3) and fluids and tissues of experimentally infected *Ornithodoros moubata* ticks, in which the 277F agent was found to grow profusely in all the tissues (W. Burgdorfer, unpublished data). These ticks originated from a colony of *O. moubata*, which proved free of the 277F agent. They were injected intracoelomically with

infected chicken embryo fluids of the 16th egg passage (seed lot no. 3). Passage of the 277F agent in chicken eggs was conducted as described previously (10).

For electron microscopy, allantoic and amniotic fluids of chicken eggs were harvested 5 and 6 days after inoculation. The fluids were pooled and differentially centrifuged at $480 \times g$ (10 min) and $9,750 \times g$ (30 min) to obtain partially purified pellets. These pellets, as well as the various tissues of ticks dissected 12 to 20 days after inoculation, were fixed at 2 to 4°C in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1.0 to 1.5 h and washed in two changes of phosphate-buffered 0.20 M sucrose (pH 7.4). Postfixation followed in phosphate-buffered (pH 7.4) 1% osmium tetroxide for 1 h. Cells and tissues were treated with a 0.5% uranyl acetate solution followed by rapid dehydration in an ethanolic series with three changes of 100% ethanol at room temperature. Two 10-min changes of propylene oxide preceded infiltration in 1:1 and 1:2 ratios of propylene oxide and Araldite 502 overnight with subsequent embedding in Araldite 502 (8).

After polymerization (8), silver-gold to gray sections were obtained on a Porter-Blum MT-1 ultramicrotome and placed on grids covered with carbon-stabilized Formvar films. Sections were stained in uranyl acetate and lead citrate (12) and examined in a Hitachi HU-11E electron microscope at 75 kV.

For negative staining, Formvar-coated grids were touched to the surface of coxal fluid exudates of infected *O. moubata*. After approximately 30 s, excess fluid was removed by touching grids to the edge of filter paper. A drop of 1.0% phosphotungstic acid (PTA) was immediately applied and the excess was removed after 10 to 15 s. The material was examined in the electron microscope soon after drying.

RESULTS

Negatively stained organisms in coxal fluids of *O. moubata* ticks clearly showed a spiral morphology (Fig. 1) and frequent branching (Fig. 2). Their diameters varied from approxi-

FIG. 1. Negative (PTA)-stained 277F specimen from coxal fluid of *O. moubata* tick. Note helical morphology, spheroidal body (b) and short branched arm. Magnification, $\times 66,000$.

FIG. 2. Helical 277F specimens exhibiting spheroidal bodies (b) located at sites of branching (PTA stained). Magnification, $\times 60,000$.

mately 53 nm to 91 nm, the average being about 70 nm. One or more spheroidal bodies or blebs of varying diameter (Fig. 2) were present in different locations on the spiral arms but were commonly found at sites where branching occurred. The diameters of these bodies varied from 88 by 108 nm to 373 by 433 nm; the mean diameter was 234 by 246 nm.

Ultrathin sections of the 277F agent from partially purified pellets of chicken embryo allantoic-amniotic fluids (Fig. 3) also revealed the helical morphology of the cells. In cross section they were principally ovoid, with diameters varying from 79 to 109 nm (mean diameter, 96 nm). All forms observed were delimited by a trilayered plasma membrane approximately 8 to 9 nm in diameter (Fig. 4). The cytoplasmic matrix varied in composition and organization from dense to light staining patches of amorphous material to an anastomosing meshwork of fine filaments (Fig. 4). Occasionally, small blebs were noted in which fine filaments were present. Larger spherical bodies (Fig. 5) exhibited budding or proliferation of membrane-limited cytoplasmic processes from their surfaces. The cytoplasmic contents of these bodies varied from dense to light staining and appeared to contain ribosomes and membrane-limited spherical bodies or vacuoles (Fig. 5).

Growth of the 277F agent in organs of *O. moubata* ticks was typically heavy in the central ganglion and coxal organs, whereas moderate growth was manifest in the Malpighian tubules. The organisms were localized primarily in extracellular spaces underlying the outer connective tissue sheath (neural lamella or basement membrane) and in close association with subjacent cells (Fig. 6). Their in situ gross morphology continued to be of general helical form, yet they also displayed greater individual morphological variability. This was manifest by irregular bending and variable diameters to their width (Fig. 6). In cross section, they varied from ovoid to irregularly ovoid shape (Fig. 7) and ranged in diameter from approximately 74 to 300 nm, with a mean diameter of >100 nm.

In each of the previously mentioned organs, the helical portion of these organisms were of essentially identical fine structure. They were delimited by a trilayered plasma membrane approximately 9.0 nm in width (Fig. 7). A velvety glycocalyx-like coating was present on the outer surface of this membrane. The cytoplasm was organized basically as a reticulum of fine filamentous strands (Fig. 8) containing patches of stainable amorphous material. Ribosomal-like particles were irregularly dispersed

throughout the helices and varied approximately 14 to 19 nm in diameter.

Organisms growing within the neural lamella of the central nerve mass were occasionally observed to develop spherical bodies or blebs (Fig. 9). These bodies were round to broadly ovoid in form and varied in diameter up to 740 nm. They were delimited by a trilayered plasma membrane, 9 to 10 nm in width. Their internal fine structure consisted of a very fine reticulum (Fig. 9) interspersed with amorphous material. Distinct ribosomal-like dense bodies, approximately 12 to 13 nm in diameter, were lightly dispersed throughout the intracellular reticulum. The peripheral cytoplasm of these bodies was of greater density, based on intensity of staining, than were the more interior regions.

Bacteriophage or other viral forms were not observed in association with the 277F agent.

DISCUSSION

Based in part on its gross helical morphology, "corkscrew" motility, and length (6 to 12 μm), the 277F agent was interpreted by Pickens et al. (10) to be a spirochete. Although the gross morphology of this agent is similar to that of *Treponema* spirochetes, specific structural and subcellular differences are distinct. *T. pallidum* is reported to be 5 to 20 μm long and approximately 0.2 μm in diameter and to possess a 15- to 20-nm trilaminar wall (5). In describing the fine structure of *T. reuteri*, Ryter and Pillot (13) clearly showed axial filaments between the cell wall and plasma membrane, which gave rise to both the spiral form and motility of these organisms. Cells of the 277F agent conformed closely in length to those of members of the genus *Treponema*; however, the width, with some exceptions in tick tissues, was usually half (100 nm or less) the diameter of treponemal spirochetes. The diameters of negatively stained 277F specimens from coxal fluids of *O. moubata* ticks were consistently less than half that of *T. pallidum*; this may be due, in part, to shrinkage during negative staining and subsequent drying. A consistently smaller mean diameter of the 277F agent, when observed in ultrathin sections of chicken embryo fluid concentrates and tick tissues, indicates that little shrinkage of negatively stained specimens occurred. The considerable variability in width of some 277F specimens in tick tissues is unaccounted for. In general, the width of negatively stained 277F specimens conformed closely with measurements reported for negatively stained specimens of *Spiroplasma citri* and with specimens of *S. citri* fixed with glutaraldehyde-sorbitol complete medium

FIG. 3. *Ultrathin section of 277F specimens from partially purified amniotic-allantoic chicken embryo fluids. Magnification, $\times 63,000$.*

FIG. 4. *Fine structure of 277F organisms from chicken embryo fluids reveals a limiting plasma membrane and cytoplasm containing anastomosing fibrils and amorphous material. Magnification, $\times 105,000$.*

FIG. 5. Ultrathin section through a spheroidal body with proliferating membrane limited cytoplasmic processes. Magnification, $\times 92,000$.

FIG. 6. Fine structure of 277F specimens in extracellular space between basement membrane (bm) and underlying cells of coxal organ of *O. moubata*. Note ribosomes and fibrillar reticulum in cytoplasmic ground substance of these organisms. Magnification, $\times 59,000$.

FIG. 7. Cross and longitudinal sections of 277F specimens in neural sheath of central nerve mass of *O. moubata*. Organisms are delimited by a trilayered unit membrane with a velvety glycocalyx-like coat on exterior surface and contain ribosomes throughout their cytoplasm. Magnification, $\times 111,000$.

FIG. 8. Prominent fibrillar reticulum and ribosomes in cytoplasm of 277F organisms located in extracellular space subjacent to basement membrane of coxal organ of *O. moubata*. Magnification, $\times 81,000$.

FIG. 9. Spherical body of 277F organism possessing a distinctly trilaminar unit membrane with a velvety coat on its exterior surface. The cytoplasm consists of a fine reticulum with patches of amorphous material and ribosomes. Ribosomes are also present in helical portion of organism. Magnification, $\times 111,000$.

(3, 4). As with *S. citri*, the 277F agent possessed neither a cell wall nor axial filaments. How these organisms maintain their structural helical symmetry remains to be determined.

The presence of spherical bodies or blebs protruding from the surface of the 277F agent as extensions of the plasma membrane constituted another important similarity in the gross morphology of this organism with *S. citri* (3, 4). Pickens et al. (10) suggested that granules associated with the 277F agent may be infectious and capable of producing spirochetes. The presence of slender cytoplasmic processes radiating from large spheroidal bodies suggests that these spheres function in the budding of new helices.

Several subcellular structures of the 277F agent, such as the plasma membrane, ribosomes, fibrillar reticulum of probable genetic material, and a thin glycocalyx-like coat on the cell surface, agreed closely in morphology and size with the same structures of *S. citri* (3, 4).

Similarity is also evident between the 277F agent and the suckling mouse cataract agent (SMCA) isolated from a pool of four *H. leporispalustris* ticks removed from a dead cottontail rabbit (*Sylvilagus putorius*) in Georgia (2). This agent, first thought to be a virus, was later found to be a member of the order *Mycoplasmatales* (17; R. F. Zeigel and H. F. Clark, J. Cell. Biol. 43:1632). Like the 277F agent, SMCA does not possess a cell wall, and its fine structure is characterized by a dense-staining, granular, cortical cytoplasm, with the central region containing a fine ribbon-like reticulum of probable genomic material.

Based on the ability of these organisms to multiply prolifically in both ticks and insects (9, 11, 15, 16), their association with arthropods is doubtless more extensive than heretofore realized.

ADDENDUM

Upon completion of our study, J. G. Tully and associates called our attention to a manuscript (submitted for publication to *Nature*) describing the suckling mouse cataract agent (SMCA) as a helical, wall-free prokaryote (*Spiroplasma*) pathogenic for vertebrates. These authors also suggest that the 277F agent may be similar to SMCA and other spiroplasmas.

REPRINT REQUESTS

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