Ultrastructure of Ehrlichia canis

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Received for publication 8 August 1972

The ultrastructure of Ehrlichia canis was examined in both pulmonary mononuclear cells and in monocytes cultured from an infected dog. The cytoplasmic inclusions, or morulae, of E. canis consisted of a membrane-lined vacuole-containing elementary bodies which varied in size and number. The elementary bodies were bound by two trilamellar membranes. The organism shared morphological properties of both the genus Rickettsia and genus Chlamydia.

Ehrlichiosis in the dog is a widespread, tick-borne, infectious disease caused by Ehrlichia canis. The organism was originally described by Donatien and Lestoquard in Algeria in 1935 (6), and was subsequently reported in dogs in other parts of Africa, the Middle East, and the Orient (7). E. canis was first recognized in the Western Hemisphere in 1957 on the Dutch Island of Aruba (4) and later by Ewing and Buckner in dogs in Oklahoma in 1963 (8). The agent has recently been identified as the etiological agent of tropical canine pancytopenia (TCP) which has been reported in Southeast Asia, the Middle East, the Caribbean and the United States (11, 12, 19). The clinical manifestation and pathology of TCP have been described for both the natural and experimental disease by Walker et al. (21) and Hildebrandt et al. (Fed. Proc., p. 754, 1970; Lab. Invest., p. 500, 1970). E. canis is characterized by intracytoplasmic inclusions which it produces in circulating monocytes, lymphocytes, and rarely neutrophils. These inclusions have been referred to as morulae (7). Blood films prepared from acutely ill dogs and stained with any of the Romanovsky stains contain mononuclear cells with single or multiple inclusions which apparently consist of aggregates of smaller elementary bodies (7). Although these inclusions can be demonstrated more readily in impression smears prepared from lung tissue, the true origin of these infected mononuclear cells and the site of multiplication have not been well established.

E. canis has been included in the family Rickettsiaceae (Bergey's Manual, p. 948–950). The ultrastructure of the organism has not been adequately compared to that of other microorganisms within the group. All observations have been made by means of light microscopy which has limitations in disclosing the true structure of the agent. Electron microscopy has been hampered by the fact that the organism has not been grown in any host other than the family Canidae, and attempts in this laboratory to concentrate the organism from dog tissues have been unsuccessful. Early in the course of disease, numerous infected mononuclear cells were observed in the lumina of small blood vessels in the lung. Organisms were also found in what appeared to be sloughing endothelial cells of the pulmonary vessels. The number of infected cells in these areas was of sufficient magnitude that sections for electron microscopy seemed feasible.

Recently Nyindo and co-workers (16) described the propagation of E. canis in monocyte cell cultures derived from dogs acutely affected with TCP. The infected cell cultures afforded an opportunity to further study the ultrastructure of E. canis and to compare organisms in lung tissue of the dog with organisms propagated in vitro.

MATERIALS AND METHODS

The isolant of E. canis used in this study was recovered from a German shepherd dog with typical signs of TCP in Southeast Asia. The organism had been maintained by blood passage in laboratory beagles.

For this study a young adult beagle dog was inoculated with 10 ml of whole blood collected in
ethylenediaminetetraacetic acid from a carrier dog. At 14 days postinoculation when early signs of infection were evident, the dog was killed. The lungs were removed and perfused with glutaraldehyde. Multiple sections of the lung were made and stained with hematoxylin and eosin to determine areas of high concentration of infected mononuclear cells. Opposing surfaces of fixed lung containing numerous infected cells were selected for electron microscope examination.

Monocyte cell cultures derived from the blood of dogs with acute disease were harvested from 2 to 12 days. The monolayers were stained with Giemsa stain, and fractions were concentrated into pellets by centrifugation. The pellets and lung tissues were fixed with 1% glutaraldehyde in 250 nmoles of Colidine buffer at 4 C, postfixed with 1.0% osmium tetroxide, and stained with 2% uranyl acetate solution. Specimens were processed through graded ethanol and xylene and embedded in an epoxy resin. Sections were cut at a thickness of 60 nm on an ultramicrotome (Porter-Blum model MK2), stained with 0.2% lead citrate, and examined in a Siemens Elmiskop IA electron microscope at 80 kv and a Hitachi 11A electron microscope at 50 kv.

RESULTS

In hematoxylin-and-eosin-stained lung tissues, many infected mononuclear cells, the origin of which could not be determined, were observed in the lumina of the pulmonary vessels (Fig. 1). It was not always possible to determine whether the cells containing inclusions were marginated leukocytes or endothelial cells in the process of sloughing. Occasionally, inclusions of *E. canis* were observed in mononuclear cells of septal walls.

In Giemsa-stained preparations of monocyte cultures derived from infected dogs, inclusions of *E. canis* were observed in the cytoplasm (Fig. 2). Infected monocytes contained from 1 to 20 typical inclusions.

Ultrastructurally, inclusions of *E. canis* were detected in endothelial cells of pulmonary vessels and reticuloendothelial cells of the alveolar septal wall. The inclusions consisted of membrane-lined vacuoles containing round to ovoid elementary bodies (Fig. 3, 4). The inclusions in cultured monocytes were indistinguishable from those observed in infected lung tissue (Fig. 5, 6). The majority of the elementary bodies were 0.5 to 0.9 μm in diameter, and the number of bodies in each vacuole ranged from 2 to 40.

Elementary bodies were bound by two distinct tri-layered membranes. The outer membrane, or cell wall, tended to be rippled, whereas the underlying plasma membrane appeared to be fused to the underlying constituents of the particle (Fig. 5). The inner structures of each particle consisted of dense and pale areas. The more dense area con-

![Fig. 1. Pulmonary blood vessel containing numerous mononuclear cells, one of which contains a cytoplasmic inclusion of *E. canis* (arrow). ×1400. Hematoxylin and eosin stain.]
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Fig. 2. Numerous inclusions of E. canis (I) in the cytoplasm of a monocyte in infected cell cultures. Nucleus of monocyte (N). Giemsa stain.

tained granules suggestive of ribosomes. Inclusions frequently contained a few bodies in which membranes were not discernible (Fig. 5). The internal structure of these particles resembled the inner structure of the membrane-bound bodies.

Dumbbell-shaped bodies were evident in some vacuoles. Such forms may represent elementary bodies in various stages of division (Fig. 4, 5). Other forms were observed in which division was almost complete (Fig. 6).

DISCUSSION

The ultrastructure of E. canis is similar to that which has been described for other members of the genus Rickettsia and large particles of the genus Chlamydia (1-3, 5, 13, 14, 15). Although the particles were confined to a membrane-lined vacuole characteristic of the Chlamydia, no definite cycle of development as described for chlamydial agents involving the initial, intermediate, and mature elementary bodies was observed in this study. These distinct developmental forms were not observed in either the infected canine lung tissue or monocyte cell cultures. The number of elementary bodies in each vacuole was variable. This may be due to the manner in which the vacuoles were sectioned; however, if the inclusion originates from a single elementary body which multiples by binary fission, a variation of 2 to 40 elementary bodies in a single vacuole would be expected.

Although variation in size of the particles was observed, this variation could not be associated with a developmental cycle. Since the cell cultures examined in this study were derived from infected dogs, the time at which
FIG. 3. Inclusion of E. canis in a pulmonary endothelial cell. The elementary bodies (EB) are bound by two distinct membranes and are enclosed in a membrane-bound vacuole (VM). Variation in particle size is also evident. Nucleus of endothelial cell (N).

FIG. 4. Inclusion of E. canis in a mononuclear cell of pulmonary septal wall. The membrane of the vacuole (VM), the cell wall (CW), and plasma membrane (PM) of the elementary bodies (EB) are indicated. Nucleus of mononuclear cell (N).
Fig. 5. Inclusion of *E. canis* in a cultured monocyte. Numerous elementary bodies (EB) with distinct plasma membranes (PM) and rippled outer cell walls (CW) are observed. Dumbell-shaped forms indicate early particle division and small particles without distinct membranes are evident. The membrane of the vacuole (VM) and a mitochondria (M) are indicated.

A given cell became infected could not be established for either the cells in culture or those in lung tissue. When techniques are developed allowing a determination of the definite time at which a cell becomes infected, a relationship between particle size and the developmental cycle may become apparent.

The ultrastructure of *E. canis* is similar to
that described for Ehrlichia recovered from horses (9) and tick-borne fever agent (20). Tuomi and von Bonsdorff (20) provided evidence that the tick-borne fever agent may occupy a taxonomic position between the family Rickettsiaceae and the family Chlamydiaceae. The same taxonomic position has been suggested for Cowdria ruminantium, the causative agent of heart water (17, 18). The present study provides evidence that E. canis may occupy a similar position. Further studies on this group of organisms may clarify their true taxonomic position.

LITERATURE CITED

Fig. 6. An inclusion in the cytoplasm of a cultured monocyte. Elementary body (EB) undergoing division is apparent.