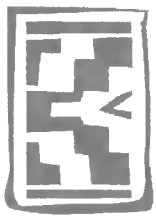

Electron microscopy of *Cowdria*-infected macrophages suggests that in the absence of binary fission a mosaic of organisms develops from an amorphous electron dense matrix

J.L. DU PLESSIS*

Protozoology Division, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110 South Africa

ABSTRACT

DU PLESSIS, J.L. 1999. Electron microscopy of *Cowdria*-infected macrophages suggests that in the absence of binary fission a mosaic of organisms develops from an amorphous electron dense matrix. *Onderstepoort Journal of Veterinary Research*, 66:39–46

Electron microscopy of mouse peritoneal macrophages infected with the Kümm stock of *Cowdria ruminantium* suggests that in the final stage of intracellular growth, a mosaic of organisms develops from an amorphous matrix of varying electron density by a process in which double unit membranes portion off the *Cowdria* particles. This stage is preceded by inclusions consisting of a network of aggregated electron dense granules and these in turn by homogeneous dense bodies. The study failed to show how these dense bodies develop from internalized *Cowdria* particles introduced in the infective inoculum. The replication of the heartwater agent in macrophages differs from that in vascular endothelial cells in two important respects. First, at no stage during the course of development in macrophages is binary fission in evidence and second, in the absence of a limiting membrane the inclusions and colonies of organisms throughout the cycle of development in macrophages are in intimate contact with the host cell cytoplasm.

Keywords: Amorphous electron dense bodies, binary fission, *Cowdria*-infected macrophages

INTRODUCTION

Ultrastructural studies on *Cowdria ruminantium* have been conducted on parasitized cells of the midgut epithelium (Kocan, Bezuidenhout & Hart 1987a; Kocan, Morzaria, Voigt, Kiarie & Irvin 1987b; Hart, Kocan, Bezuidenhout & Prozesky 1991) and the salivary glands (Kocan *et al.* 1987a) of the tick vector, as well as in tissue culture (Prozesky, Bezuidenhout & Paterson 1986; Prozesky 1987; Prozesky, Hart & Brett 1993; Prozesky & Du Plessis 1987; Jongejan, Zandbergen, Van de Wiel, De Groot & Uilenberg 1991) and the tissue cells of the vertebrate host (Pienaar 1970; Du Plessis 1975; Prozesky & Du Plessis 1985; Rikihisa & Logan 1986; Prozesky 1987; Logan, Whyard, Quintero & Mebus 1987). In the case of the vertebrate host cells, attention was focused on colonies of the heartwater agent in vascular endothelial cells, except for one study on parasitized macrophages (Du Plessis 1975) and another on the neutrophils of an infected goat (Logan *et al.* 1987).

Because the findings on infected macrophages have so far not been confirmed (Hart *et al.* 1991) and since there is mounting evidence that macrophages play an important role in the pathogenesis of (Du Plessis 1970; 1982) and the immune response against *C. ruminantium* (Du Plessis, Berche & Van Gas 1991; Totté 1998), a further study has been carried out on the peritoneal macrophages of mice infected with one of the murinotropic stocks of *C. ruminantium*.

* Present address: 156 First Avenue, Kleinmond 7195, South Africa

Accepted for publication 22 January 1999—Editor

MATERIALS AND METHODS

Six-week-old specified-pathogen-free (SPF) NMRI mice were inoculated intraperitoneally with 0,2 mℓ (400 LD_{50} doses) of a mouse spleen homogenate infected with the Kümm stock of *C. ruminantium* (Du Plessis & Kümm 1971). The homogenate was prepared from the spleens of five SPF NMRI mice inoculated 10 d earlier with deep-frozen stock antigen. At the appearance of the first clinical signs, the mice were killed, their spleens removed and homogenized in 10 mℓ buffered lactose peptone, centrifuged at 1 000 r.p.m. for 5 min and the supernatant deep-frozen in liquid nitrogen in suitable quantities. A sample was withdrawn and an infectivity titre of $10^{4,3}$ determined in mice as previously described (Du Plessis 1982).

At 6, 12, 24, 36, 72 and 96 h post-infection (p.i.), the peritoneal cells of two to four mice were collected in 2 mℓ cell culture medium per mouse and pooled in conical centrifuge tubes. An equal volume of 2% glutaraldehyde in a 0,2 M sodium cacodylate buffer with 0,5% sucrose was added immediately. Without any delay the cell pellets were sedimented by centrifugation at ± 3 000 r.p.m., and the supernatant was discarded. The cells were resuspended in cold fresh 2% glutaraldehyde and fixed in the cold for 2 d. They were pelleted again by centrifugation, post-fixed in 2% osmium tetroxide in 0,2 M sodium cacodylate buffer and processed for transmission electron microscopy according to the method of Kocan, Venable & Brock (1978). Ultra-thin sections were collected on 200–300 mesh copper grids and stained with uranyl acetate and lead citrate (Venable & Coggeshall 1978).

Control uninfected peritoneal cells were collected on similar days from mice inoculated with non-infected spleen homogenate and processed in a similar manner.

RESULTS

The developmental cycle of *C. ruminantium* as suggested below assumes credibility if the ultrastructural findings are presented in retrospect, i.e. commencing with a clearly distinguishable colony of *Cowdria* particles. In the cytoplasm of peritoneal macrophages collected 96 h p.i. (Fig. 1), colonies were detected with fully developed organisms, each with a double unit membrane and 0,4 to 0,43 µm in size. There were also colonies with clearly distinguishable single organisms with only partially developed double membranes (Fig. 5), in association with closely packed as yet not fully individualized organisms with indistinct double membranes in groups (Fig. 5, arrows).

Immediately prior to these stages, colonies consist of densely packed organisms vaguely outlined by double membranes in the process of development (Fig. 2, 6 and 7). These colonies were seen in cells collected 72 and 96 h p.i. Although prominent endoplasmic reticulum may cursorily suggest that these colonies are surrounded by a membrane (Fig. 2 and 6), closer observation confirms that they are in intimate contact with the cell cytoplasm.

At 72 (Fig. 3, 4 and 12) and 48 h p.i. (Fig. 11), inclusions were detected which closely resemble and presumably precede those just described. Islands of finely granular electron dense material are either interspersed with loosely arranged material with a similar granularity (Fig. 3 and 4), or the electron dense granules are distributed homogeneously throughout the inclusion (Fig. 12). The granularity of these inclusions is indistinguishable from that of readily identifiable organisms described above. At this stage there is no evidence of double membrane development, so that individual organisms are not even vaguely discernible. There are once again no limiting membranes between the inclusions and the cytoplasm of the host cells.

Still earlier developmental stages observed in cells collected 36 h p.i. (Fig. 13) are represented by inclusions consisting of closely packed almost homogeneous electron dense material of a similar granularity. These inclusions were also detected in cells collected 48 h p.i. (Fig. 14).

The earliest developmental stages, suspected to be those of the heartwater agent and obviously distinct from typical fully developed individual organisms phagocytosed as such, were detected in cells collected 24 h p.i. (Fig. 8 and 9). Extremely dense, homogeneous bodies with ill-defined borders and devoid of a limiting membrane, were seen in the cytoplasm of macrophages. These bodies were usually surrounded by a translucent zone free from endoplasmic reticulum.

In cells collected 6 and 12 h p.i. (Fig. 10), single organisms indistinguishable from typical fully developed particles were observed. A double membrane between the cytoplasm of the organism and that of the host cell, often observed at this early stage, appeared to have originated rather from the organism than from the host cell cytoplasm. Exhaustive search failed to reveal any organisms in the process of binary fission. Furthermore, no inclusions that could possibly be considered as a stage between newly introduced recognisable organisms and the dense body stage described above, were detected.

The intracytoplasmic inclusions observed in cells collected from control mice inoculated with uninfected homogenized mouse spleen (Fig. 15–20), showed a superficial resemblance to some of the structures described in the cells of infected mice. Structures that may be confused with homogeneous dense bodies

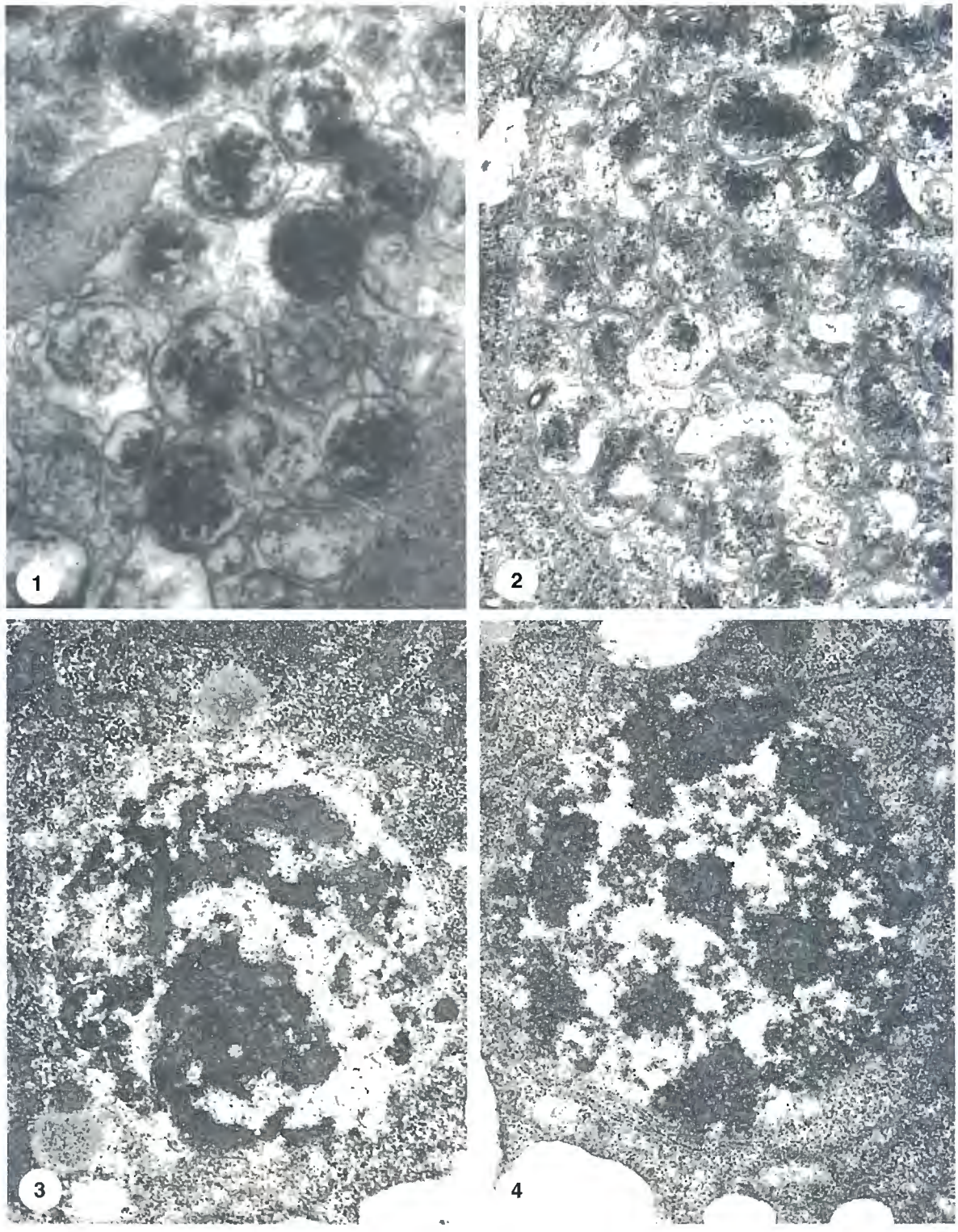

FIG. 1–4 Peritoneal macrophages from mice infected with *C. ruminantium*

FIG. 1 Part of a colony of fully developed organisms in a macrophage collected 96 h p.i. x 39 500

FIG. 2 Part of a colony in final stage of development Double unit membranes closing around individual organisms. 72 h p.i. x 40 000

FIG. 3–4 Inclusions 2,7 and 3,3 µm in size, respectively, 72 h p.i., consisting of islands of electron dense granular material. x 28 000 and 26 000, respectively

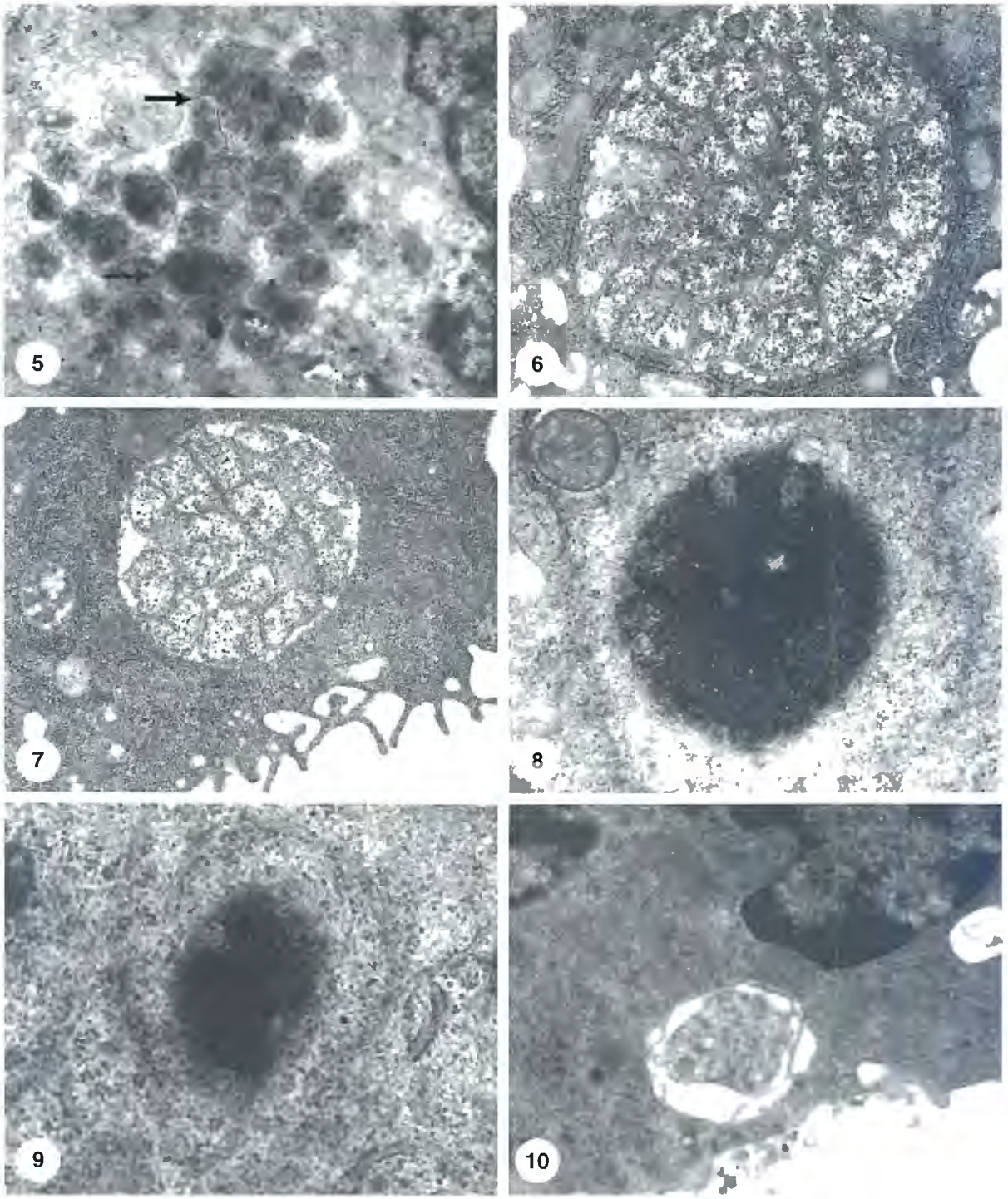

FIG. 5–10 Infected cells

FIG. 5 Almost fully developed colony, 96 h p.i., with parts of colony (arrows) not yet fully divided. x 16 000

FIG. 6 Colony in final stage of development, 72 h p.i. Double unit membranes become visible. Note prominent endoplasmic reticulum around colony and absence of limiting membrane. x 21 000

FIG. 7 Small colony, 96 h p.i. x 15 000

FIG. 8 Homogeneous, dense body, 1,4 µm in diameter, 24 h p.i. x 30 800

FIG. 9 Homogeneous, dense body, 0,8 µm in diameter, 24 h p.i. x 18 000

FIG. 10 Single organism, 6 h p.i. Note separation of unit membrane from cytoplasm of organism. x 15 000

detected in cells from infected mice 24 h p.i., were observed in control cells collected 12 (Fig. 15) and 48 h (Fig. 16) after inoculation. These bodies were smaller, had a different structure, did not merge with the host cell cytoplasm and were invariably separated from it by a membrane of host cell origin (arrow). Inclusions that may resemble the developmental stage in infected cells preceeding colonies with clearly distinguishable organisms, were detectable in cells collected from control mice 24 (Fig. 17, 18 and 19) and 48 h (Fig. 20) after inoculation and were likewise separated from the host cell cytoplasm by membranes (arrows). Dense homogeneous bodies (Fig. 17, arrow) and bodies with peripheral electron dense material (Fig. 18), were readily distinguishable from superficially similar *Cowdria* developmental stages in cells from infected mice.

An inclusion observed in a control cell 48 h after inoculation (Fig. 20) bears superficial resemblance to part of a colony in the final process of development (Fig. 2), which forms an important link in the developmental chain. Whereas individual organisms become visible and there is no sign of a membrane limiting the colony in the case of the cell from an infected mouse (Fig. 2), the contents of the inclusion, surrounded by a membrane in the control cell (Fig. 20), has no definite pattern.

Developmental stages of *C. ruminantium* can therefore be differentiated from the intracytoplasmic inclusions described in the control cells, which in all probability represent phagosomes (Fig. 15 and 16), phagocytosed cells (Fig. 18) or cell debris (Fig. 17, 19 and 20).

DISCUSSION

In this time sequence study, the final stages of the intracellular development of *C. ruminantium* in macrophages suggest that an amorphous body of granular electron dense material develops into a colony of clearly distinguishable particles. In the key image of the portion of a cell collected 72 h p.i. (Fig. 2), islets of finely granular material are portioned off as organisms by developing membranes, resulting in a mosaic of closely packed *Cowdria* particles (Fig. 6 and 7), which become detached one from another in the final stage of development (Fig. 1). At no stage during this latter part of the cycle is binary fission in evidence. In this respect the development of *Cowdria* in macrophages differs from that in midgut epithelial cells (Kocan *et al.* 1987a; Hart *et al.* 1991) and salivary glands (Kocan *et al.* 1987a) of the tick vector, capillary endothelial cells of the vertebrate host (Pienaar 1970) and in endothelial cell tissue culture (Prozesky *et al.* 1986; Jongejan *et al.* 1991). In all these cases binary fission is a regular feature.

The organisms in the final stages of development in macrophages are uniform in size, shape and internal structure and cannot be differentiated into elementary, intermediate and reticular bodies as is the case in other mammalian, tick vector and cultured cells cited above.

The inclusions suspected to be developing colonies of *C. ruminantium* detected in cells collected 24–48 h p.i., are less convincing from a morphological point of view. It is, however, inconceivable that in the total absence of binary fission a developing colony, such as the key inclusion depicted in Fig. 2, can develop from a single phagocytosed organism (Fig. 10) without intermediate stages such as e.g. those described in this study. The only structures, absent in control cells, detected in time sequence, that could possibly represent intermediate stages, are inclusions without surrounding membranes, containing evenly distributed, more or less dense granular material from which the mosaic of *Cowdria* particles, rounded off by double membrane development, eventually evolve.

The findings in this study confirm most of the observations made in an earlier study (Du Plessis 1975). The criticism that these findings have not been confirmed (Hart *et al.* 1991), can be refuted by stating that no one has published findings on macrophages of *Cowdria*-infected ruminants or mice unequivocally contradicting the observations described here and elsewhere (Du Plessis 1975). In view of indications that macrophages play an important role in the pathogenesis (Du Plessis 1970; 1982) and the immune response (Du Plessis 1982; Du Plessis *et al.* 1991; Totté 1998) in heartwater, it is perhaps unfortunate that the great majority of electron microscopic studies have so far been confined to mammalian host vascular endothelial, tick vector cells and endothelial cell cultures.

Despite exhaustive search in cells collected 6–24 h p.i., no further clarity on the fate of *Cowdria* after entry into macrophages could be obtained. One would expect some morphological evidence of the parasite intermediate between newly phagocytosed recognisable organisms and the dense bodies presumed to be associated with *C. ruminantium* detected 24 h p.i. In the absence of any evidence of binary fission, it would therefore appear that the organism goes through an eclipse phase during which *Cowdria* building material becomes integrated into the cytoplasm of the macrophage in a process not readily detectable by electron microscopy. If these observations are valid, are we in the realm of viruses at this stage of the development of *Cowdria*?

Indications in this and an earlier (Du Plessis 1975) study suggest that not only the morphology (no clear differentiation into elementary, intermediate and reticulate forms and no limiting membrane surrounding inclusions and colonies in macrophages) but also the mode of replication of *C. ruminantium* in macro-

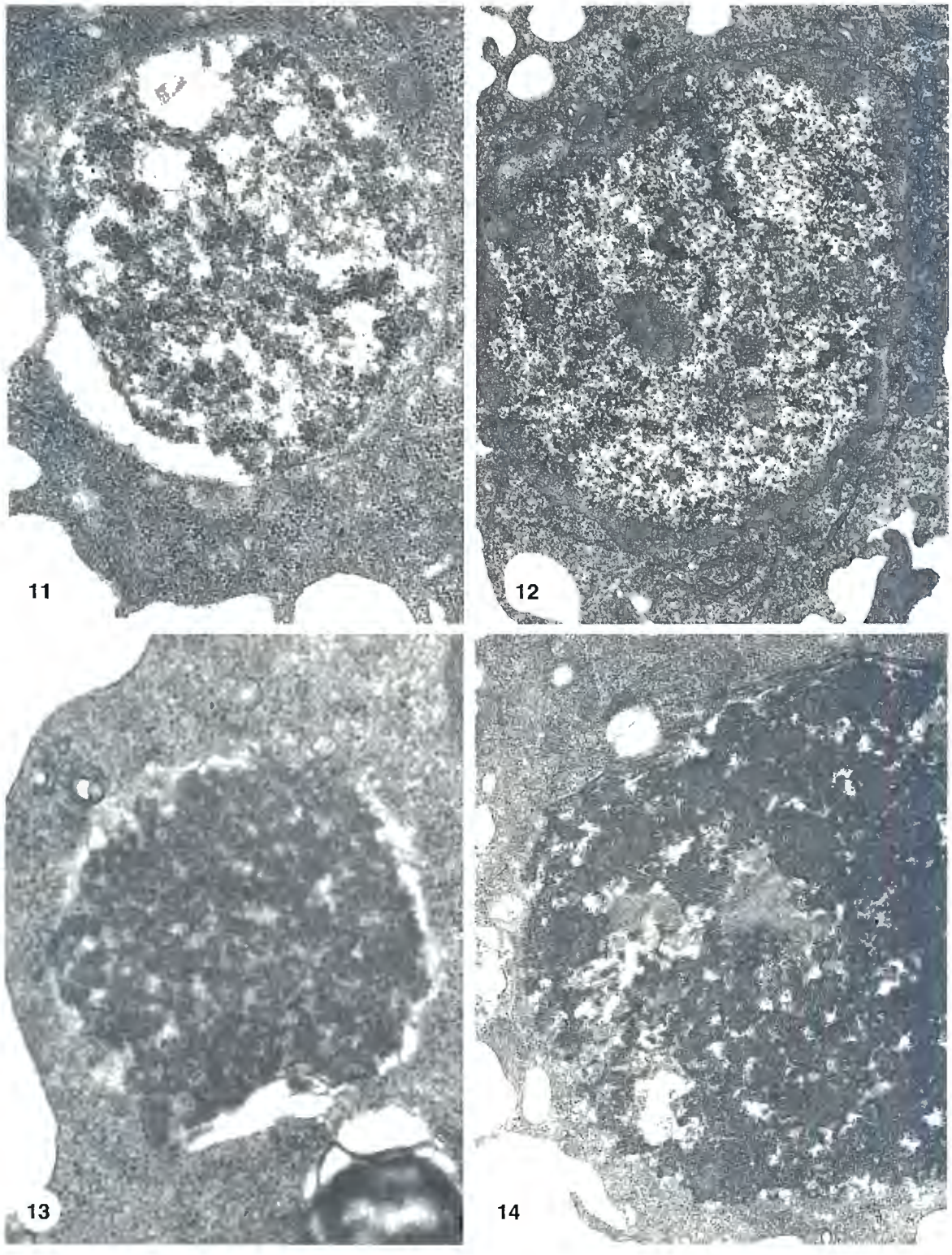

FIG. 11–14 Infected cells

FIG. 11 Inclusion, 2,3 µm in diameter in macrophage collected 48 h p.i. x 32 000

FIG. 12 Inclusion with more evenly distributed granular material, 72 h p.i. Note prominent endoplasmic reticulum. x 15 000

FIG. 13 Inclusion, 1,6 µm in diameter, with more closely packed electron dense granular material, 36 h p.i. x 40 000

FIG. 14 Inclusion 5,2 µm in diameter, 48 h p.i. x 16 000

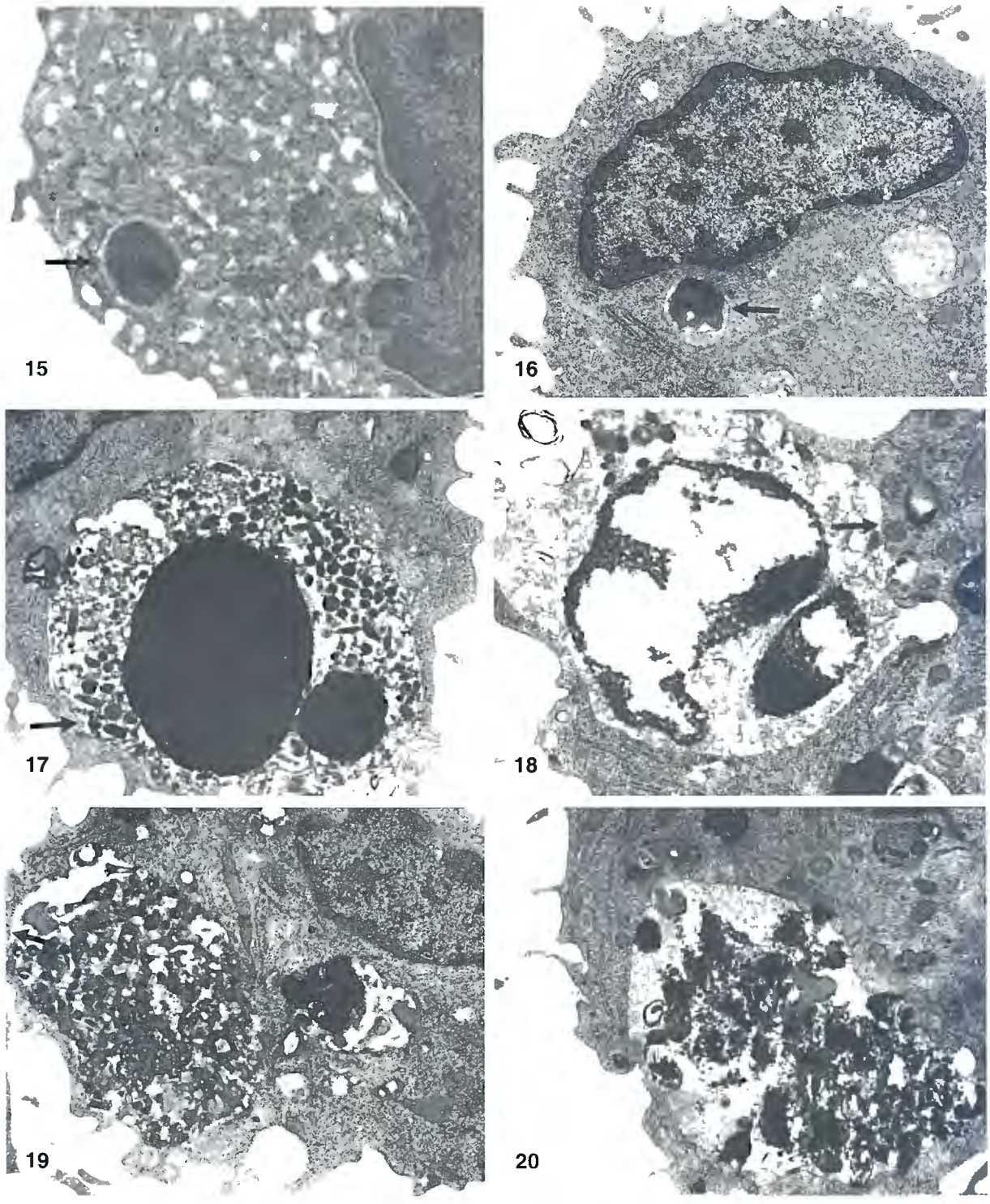

FIG. 15–20 Control peritoneal macrophages from mice inoculated with non-infected tissue homogenate

FIG. 15 Phagosome (arrow), 12 h after inoculation. x 20 000

FIG. 16 Phagosome (arrow) with clearly distinguishable limiting membrane, 48 h after inoculation. x 11 800

FIG. 17–20 Macrophages with phagocytosed cell (Fig. 18) and cell debris in cells collected 24 (Fig. 17–19) and 48 h (Fig. 20) after inoculation. Note the limiting membranes (arrows)

cells, both *in vivo* and *in vitro*. The interesting and important question arises whether these differences could possibly be associated with the observation that the infectivity to mice of cultures of *C. ruminantium* in endothelial cells decreases after storage in liquid nitrogen, whereas deep-frozen mouse spleen homogenates infected with the same stock, containing organisms that originate from both macrophages and endothelial cells, retain their infectivity (J.L. du Plessis, unpublished observation 1993). Furthermore, if this question is valid, is it advisable that molecular biological studies, particularly those aimed at identifying immunogenic antigens, are exclusively conducted on tissue culture-derived *Cowdria*?

The Kümm stock differs from most other stocks of *C. ruminantium* in that it appears to have a greater affinity for macrophages (J.L. du Plessis, unpublished observation 1992) and that only limited growth of this stock has as yet been achieved in cultured DH82 but not in endothelial cells (E. Zweygarth, personal communication 1998). It must therefore be emphasized that the above observations have been made in this particular stock and cannot be extrapolated to other stocks without some reservation and further proof. The author believes, however, that the above developmental cycle in macrophages occurs in all stocks of *C. ruminantium* and most probably also in *Ehrlichia*.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the advice and assistance by Drs Katherine Kocan, Jaco van der Lugt and Leon Prozesky, the able technical assistance by Mr J.F. Putterill and Mrs Louise Limper and gratefully recalls the help and encouragement given by the late Alet Hart. A special word of thanks to Mrs M.M. Viljoen for the final preparation on computer of the manuscript long after my retirement.

REFERENCES

DU PLESSIS, J.L. 1970. Pathogenesis of heartwater. I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort Journal of Veterinary Research*, 37:89–96.

DU PLESSIS, J.L. 1975. Electron microscopy of *Cowdria ruminantium* infected reticulo-endothelial cells of the mammalian host. *Onderstepoort Journal of Veterinary Research*, 42:1–14.

DU PLESSIS, J.L. 1982. Mice infected with a *Cowdria ruminantium*-like agent as a model in the study of heartwater. D.V.Sc. thesis, Pretoria: University of Pretoria.

DU PLESSIS, J.L. & KÜMM, N.A.L. 1971. The passage of *Cowdria ruminantium* in mice. *Journal of the South African Veterinary Medical Association*, 42:217–221.

DU PLESSIS, J.L., BERCHE, P. & VAN GAS, L. 1991. T cell-mediated immunity to *Cowdria ruminantium* in mice: The protective role of Lyt-2$^+$ T cells. *Onderstepoort Journal of Veterinary Research*, 58:171–179.

HART, A., KOCAN, K.M., BEZUIDENHOUT, J.D. & PROZESKY, L. 1991. Ultrastructural morphology of *Cowdria ruminantium* in midgut epithelial cells of adult *Amblyomma hebraeum* female ticks. *Onderstepoort Journal of Veterinary Research*, 58:187–193.

JONGEJAN, F., ZANDBERGEN, T.A., VAN DE WIEL, P.A., DE GROOT, M. & UILENBERG, G. 1991. The tick-borne rickettsia *Cowdria ruminantium* has a *Chlamydia*-like developmental cycle. *Onderstepoort Journal of Veterinary Research*, 58: 227–237.

KOCAN, K.M., VENABLE, J.H. & BROCK, W.E. 1978. Ultrastructure of anaplasmal inclusions (Pawhuska isolate) and their appendages in intact and hemolyzed erythrocytes and in complement fixation antigen. *American Journal of Veterinary Research*, 39:1123–1130.

KOCAN, K.M., BEZUIDENHOUT, J.D., HART, A. 1987a. Ultrastructural features of *Cowdria ruminantium* in midgut epithelial cells and salivary glands of nymphal *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research*, 54: 87–93.

KOCAN, K.M., MORZARIA, S.P., VOIGT, W.P., KIARIE, J. & IRVIN, A.D. 1987b. Demonstration of colonies of *Cowdria ruminantium* in midgut epithelial cells of *Amblyomma variegatum*. *American Journal of Veterinary Research*, 48:356–360.

LOGAN, L.L., WHYARD, T.C., QUINTERO, J.C. & MEBUS, C.A. 1987. The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort Journal of Veterinary Research*, 54:197–204.

PIENAAR, J.G. 1970. Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry 1926) in the endothelial cells of the vertebrate host. *Onderstepoort Journal of Veterinary Research*, 37:67–78.

PROZESKY, L. & DU PLESSIS, J.L. 1985. The pathology of heartwater. I. A study of mice infected with the Welgevonden strain of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 52:71–79.

PROZESKY, L. BEZUIDENHOUT, J.D. & PATERSON, C.L. 1986. An *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research*, 53:153–159.

PROZESKY, L. 1987. Heartwater. The morphology of *Cowdria ruminantium* and its staining characteristics in the vertebrate host and *in vitro*. *Onderstepoort Journal of Veterinary Research*, 52:71–79.

PROZESKY, L. & DU PLESSIS, J.L. 1987. Heartwater. The development and life cycle of *Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells. *Onderstepoort Journal of Veterinary Research*, 54:193–196.

PROZESKY, L., HART, A. & BRETT, M.S. 1993. An *in vitro* study of the life cycle of *Cowdria ruminantium*. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, 46:247

RIKIHISA, Y. & LOGAN, L.L. 1986. Unusual peptidoglycan-like substance in *Cowdria ruminantium* in the endothelial cells of the choroid plexus of the goat. *Proceedings of the XIth International Congress on Electron microscopy*, 1986, Kyoto, Japan, 3345–3346.

TOTTÉ, P. 1998. Protective immune mechanisms to *Cowdria ruminantium*: The present state of knowledge (in press).

VENABLE, J.H. & COGGESHALL, R. 1978. A simplified lead citrate stain for use in electron microscopy. *Journal of Cellular Biology*, 25:407–408.