Onderstepoort J. vet. Res., 58, 227–237 (1991)

THE TICK-BORNE RICKETTSIA *COWDRIA RUMINANTIUM* HAS A *CHLAMYDIA*-LIKE DEVELOPMENTAL CYCLE

FRANS JONGEJAN[1], THIJS A. ZANDBERGEN[2], PAUL A. VAN DE WIEL[3], MIA DE GROOT[1] and GERRIT UILENBERG[4]

ABSTRACT

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

The development of the tick-borne rickettsial pathogen *Cowdria ruminantium* (S stock) was studied in bovine umbilical endothelial (BUE) cell cultures and in goat choroid plexus, by light- and electron microscopy. *Cowdria* divided by binary fission within intracytoplasmic vacuoles resulting in large colonies of reticulate bodies. After three to four days in culture, reticulate bodies developed into smaller intermediate bodies characterized by an electron-dense core. Shortly before disruption of the host cells, intermediate bodies condensed further into electron-dense elementary bodies, which were released into the culture medium. Elementary bodies invade other endothelial cells thus initiating a new infectious cycle which lasts between 5 and 6 days.

In the infected goat choroid plexus similar reticulate and intermediate bodies were identified within vacuoles of capillary endothelial cells. However, extracellular elementary bodies were not detected.

Another stock of *Cowdria* (W) showed an identical developmental cycle as that of the S stock. The W isolate was also pathogenic for mice, making it possible to test the infectivity of reticulate and elementary bodies in these animals. Reticulate bodies appeared to be less infective than elementary bodies.

The developmental cycle of *Cowdria* resembles the cycle known to occur in *Chlamydia*. Moreover, *Cowdria* has other similarities with *Chlamydia*. It has a Gram-negative envelope, it does not store iodine-stainable carbohydrates and may lack peptidoglycan as does *Chlamydia*. It is concluded, that *Cowdria* and *Chlamydia* are to a certain extent related, confirming a recent report that both organisms have certain antigenic determinants in common. Since *Cowdria* is also related to *Ehrlichia* it may well be that *Cowdria* takes an intermediate position between *Chlamydia* and *Ehrlichia*. The phylogenetic relationship between *Cowdria* and *Chlamydia* and also with *Ehrlichia* should be further elucidated by molecular analysis using 16S ribosomal DNA sequences.

INTRODUCTION

The rickettsia *Cowdria ruminantium* is an obligatory intracellular pathogen, which causes the disease heartwater. The agent is exclusively transmitted by *Amblyomma* ticks to wild and domestic ruminants in Africa and the Caribbean region (Uilenberg, 1983). It has been known for some time that *Cowdria* develops within membrane-bound vacuoles in the cytoplasm of endothelial cells of its ruminant host, (Pienaar, 1970) and in gut epithelial cells of its vector tick, *Amblyomma* (Cowdry, 1925; Kocan, Morzaria, Voigt, Kiarie & Irvin, 1987). Because of similarities in ultrastructure between *Cowdria* and *Chlamydia,* several authors have commented upon a possible phylogenetic relationship between these organisms (Kocan *et al.*, 1987; Pienaar, 1970; Rake, Alexander & Hamre, 1945; Uilenberg, 1983). Moreover, recognition of *Cowdria* by a monoclonal antibody directed against the major outer membrane of *Chlamydia trachomatis,* has provided evidence that both organisms are to a certain extent antigenically related (Jongejan, Bax, Meddens & Quint, 1991).

The lack of a suitable method for *in vitro* cultivating *Cowdria* has for a long time restricted research on *Cowdria*. For instance, the persistence of *Cowdria* for up to 13 days in primary goat kidney cell cultures has been the unimpressive record until 1985 (Jongejan, Van Winkelhoff & Uilenberg, 1980). A breakthrough was the cultivation of *Cowdria* in an irradiated bovine endothelial cell line (Bezuidenhout, Paterson & Barnard, 1985). In these endothelial cells the occurrence of two distinct forms of *Cowdria,* elementary bodies and reticulate bodies, has been reported (Prozesky, Bezuidenhout & Paterson, 1986; Prozesky & Du Plessis, 1987). However, studies on the sequential morphology of *Cowdria* have not been reported as yet.

We have simplified the cultivation of *Cowdria* by using cultures of non-irradiated primary bovine endothelial cell cultures isolated from umbilical cord arteries. This enabled us to study the development of *Cowdria in vitro* and to compare its ultrastructure *in vitro* with the ultrastructure of *Cowdria* inclusion bodies found in choroid plexus of heartwater-infected goats. In this paper we describe the developmental cycle of *Cowdria* in endothelial cell cultures using electron- and light microscopy. The data on *Cowdria* are compared with the developmental cycle known to occur in *Chlamydia*.

MATERIALS AND METHODS

Cowdria ruminantium infection

Two isolated of *Cowdria* were used: one from Senegal, designated "S" (Jongejan, Uilenberg, Franssen, Gueye & Nieuwenhuijs, 1988) and the mouse-pathogenic South African isolate Welgevonden, designated "W" (Du Plessis, 1985). The isolates were stored in liquid nitrogen as infected blood stabilates described previously (Uilenberg, 1983). Goats were infected by intravenous inoculation of thawed blood stabilate and were monitored by daily rectal temperature records and clinical inspection. Heparinized blood was collected from infected animals when their rectal temperature reached 41 °C and the blood was used to initiate infection of cultures of endothelial cells. After blood sampling, the animals were treated immediately with oxytetracycline at a dosage of 20 mg/kg to prevent their death from the otherwise lethal infection. *Cowdria* infec-

[1] Department of Tropical Veterinary Medicine and Protozoology, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands

[2] Department of Experimental Zoology, Research Group for Comparative Endocrinology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

[3] Research Institute of Toxicology, Section of Immunotoxicology, University of Utrecht, P.O. Box 80.176, 3508 TD Utrecht, The Netherlands

[4] Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux, 10 rue Pierre Curie, 94704 Maisons-Alfort, France

Received 22 August 1991—Editor

tion was confirmed serologically by using an indirect fluorescent antibody assay based on infected endothelial cells as antigen (Martinez, Swinkels, Camus & Jongejan, 1990).

Two goats infected with *Cowdria* "S" were left untreated. Brain squash smears were prepared to confirm heartwater, by staining with Diff-Quik (Merz & Dada AG, Switzerland) and examining for *Cowdria* inclusion bodies. Choroid plexus material was removed as soon as possible after death and processed for electron-microscopy.

Endothelial cell cultures

Bovine umbilical endothelial (BUE) cells were isolated from umbilical cord arteries as described previously (Van de Wiel, Pieters, Van der Pijl & Bloksma, 1989) with the following modifications. Umbilical cord arteries were rinsed with PBS, filled with collagenase (1 mg/mℓ) (Boehringer, Mannheim) in PBS without Ca^{2+} and Mg^{2+} and incubated for 40 min at 37 °C. Detached endothelial cells were collected, centrifuged 10 min at 200 g and resuspended in RPMI 1640 medium (Gibco/BRL) supplemented with penicillin (100 IU/mℓ), streptomycin (100 µg/mℓ), amphotericin B (1,25 µg/mℓ), HEPES buffer (20 mM) (pH 7,0–7,2), L-glutamine (2 mM) and 10 % newborn or fetal calf serum (Gibco/BRL). The cells were seeded in plastic flasks (Costar) coated with fibronectin (50 µg/cm^2) and incubated at 37 °C with 5 % CO_2 and humidified atmosphere until confluency and subcultured after trypsinization.

Cultivation of *Cowdria*

Confluent monolayers of endothelial cells were inoculated with infected goat blood using a modification of the methods of Bezuidenhout (1987). Two parts of heparinized blood were diluted with one part sucrose-phosphate-glutamate (SPG) buffer (Bovarnick, Miller & Snyder, 1950). Three mℓ of diluted blood was used to inoculate a 25 cm^2 tissue culture flask containing a monolayer of BUE cells. Flasks were incubated at 37 °C on a rocking platform (10 cycles/min) for 2 to 24 h. Thereafter, cultures were rinsed with Hank's balanced salt solution and *Cowdria* growth medium was added. This consisted of Glasgow minimal essential medium (GMEM) supplemented with tryptose phosphate broth (TPB) (Difco Lab., Detroit, USA) at 2,9 g/ℓ, penicillin (100 IU/mℓ), streptomycin (100 µg/mℓ), amphotericin B (1,25 µg/mℓ), HEPES buffer (20 mM) (pH 7,0–7,2), L-glutamine (2 mM) and 10 % newborn calf serum (Gibco/BRL). Cultures were further incubated on the rocking platform at 37 °C. Samples of BUE cells were scraped from the bottom of the culture flask, smeared onto a glass slide and examined for *Cowdria* inclusions after staining with Diff-Quik (Merz & Dada AG, Switzerland)

Infection of BUE cells with reticulate bodies (RBs) and elementary bodies (EBs) was scored as follows; RBs: (1+), scanty intracellular colonies less than 1 per cent of BUE cells infected; (2+), approximately 10 % of the cells infected; (3+), virtually all cells infected. Score for EBs: (1+), scanty extracellular particles; (2+), present in large numbers coinciding with moderate cytophatic effect; (3+) heavily infected culture supernatant coinciding with destruction of most BUE cells.

Cultures containing extracellular elementary bodies (score of 3+) were used to passage *Cowdria* onto other BUE cell cultures. Alternatively, culture supernatant (EB score of +3) was centrifuged for 10 min at 10 000 *g*, the pellet resuspended in SPG buffer and preserved in liquid nitrogen. When required, stabilates were rapidly thawed and used to initiate *Cowdria* infection of monolayers of endothelial cells. BUE cultures infected with the "S" stock of *Cowdria* were fixed and processed for light and electron-microscopy on day 2, 4, 5 and 6 post inoculation.

Moreover, some infected BUE cultures were stained according to Gram and also with iodine to detect possible carbohydrate storage.

Infectivity for mice

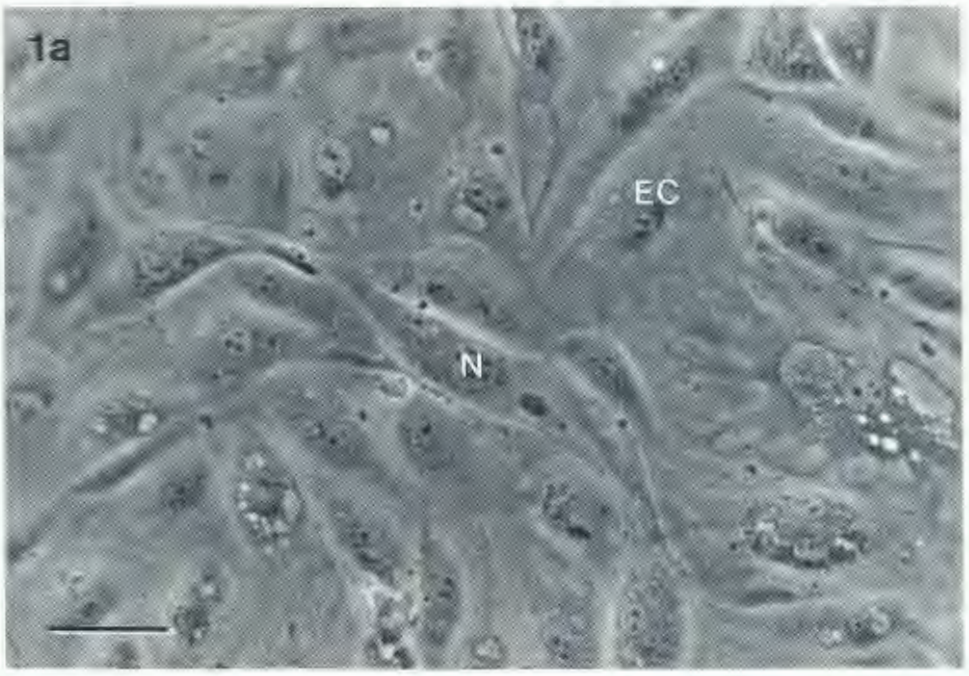
BUE cultures infected with the W isolate of *Cowdria*, known to be lethal for mice, were used to study the infectivity of intracellular and extracellular *Cowdria* stages for mice. For this purpose, six 25 cm^2 culture flasks containing monolayers of BUE cells were inoculated with 2 mℓ of a BUE culture supernatant containing 3+ EBs of *Cowdria* (W). This supernatant caused death in six mice after inoculation of 0,3 mℓ intravenously. Additional groups of six mice each were inoculated intravenously with the contents of one culture flask (0,3 mℓ per mouse) collected at intervals after 24 h incubation. A control group of 6 mice was inoculated with a suspension of non-infected BUE cells.

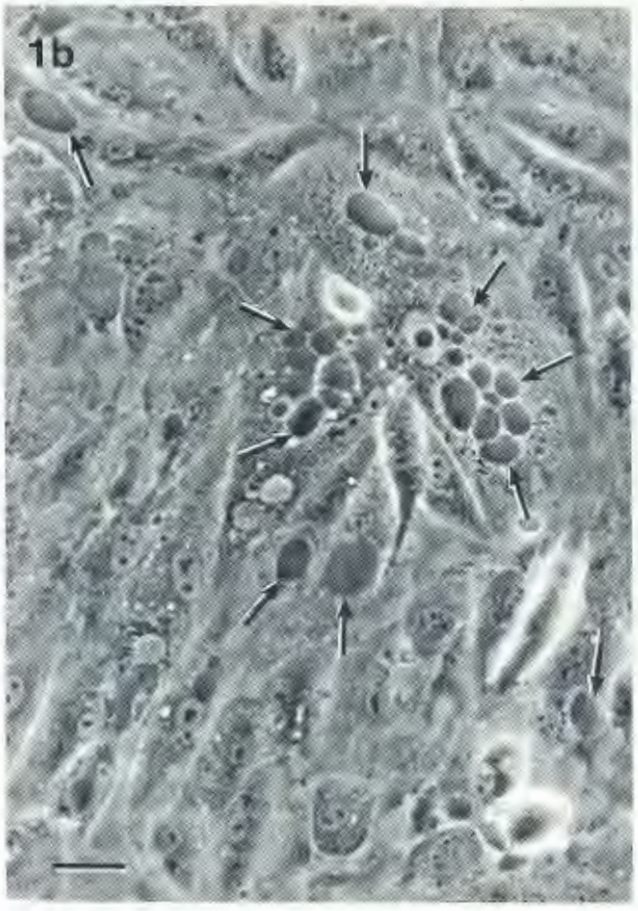
Electron microscopy

Cowdria-infected BUE cell cultures were fixed in situ for 60 min at 4 °C in 1,25 % formaldehyde, 2,5 % glutaraldehyde, 0,03 % $CaCl_2$ and 0,03 % picric acid in 0,05 % cacodylate buffer of pH 7,4 (Ito & Rikihisa, 1981). Thereafter the cells were collected with a cell scraper, centrifuged for 10 min at 3 000 *g* and the pellet was embedded in 3 % agar. Subsequently, the agar embedded samples were washed in 0,2 M cacodylate buffer, post-fixed for 60 min with 2 % osmium-tetroxide in 0,1 % cacodylate buffer (pH 7,4) at 4 °C, washed again, dehydrated in graded concentrations of ethanol and embedded in Epon. For light microscopical studies, 1 µm sections were stained with a mixture of Azur II and methylene blue in borax buffer and mounted with Depex (Serva, Germany). For electron microscopy, sections of 60–90 nm were cut with a diamond knife on an ultramicrotome. Contrast was improved with uranylacetate and lead citrate and examined in a Zeiss EM-10A transmission electron microscope at 60 kV. Excised caprine choroid plexus material was processed for electron microscopy in the same way, except that the samples were not embedded in agar.

RESULTS

Bovine endothelial cells of passage 5 to 25 established from umbilical cord arteries supported sustained intracellular growth of *C. ruminantium* (Fig. 1). *Cowdria* growth was observed in 19 different umbilical cord isolates, provided Glasgow MEM medium was supplemented with tryptose phosphate broth (TPB) at 2,9 g/ℓ. In GMEM (or RPMI 1640) without TPB the organisms degenerated within 24 h. Small intra-cytoplasmic bodies and small rickettsial colonies [S stock were demonstrated as early as 2 days post inoculation (p.i.)] (Fig. 2b). The inclusion morphology of *Cowdria* was characterized by a number of discrete colonies (or morula's) per cell (Fig. 1b and 2b). From day 5 p.i. onwards clear cytopathic effects of *Cowdria* infection on BUE cells were visible (Fig. 1c), which coincided with the release of large numbers of rickettsial bodies (Fig. 2c).





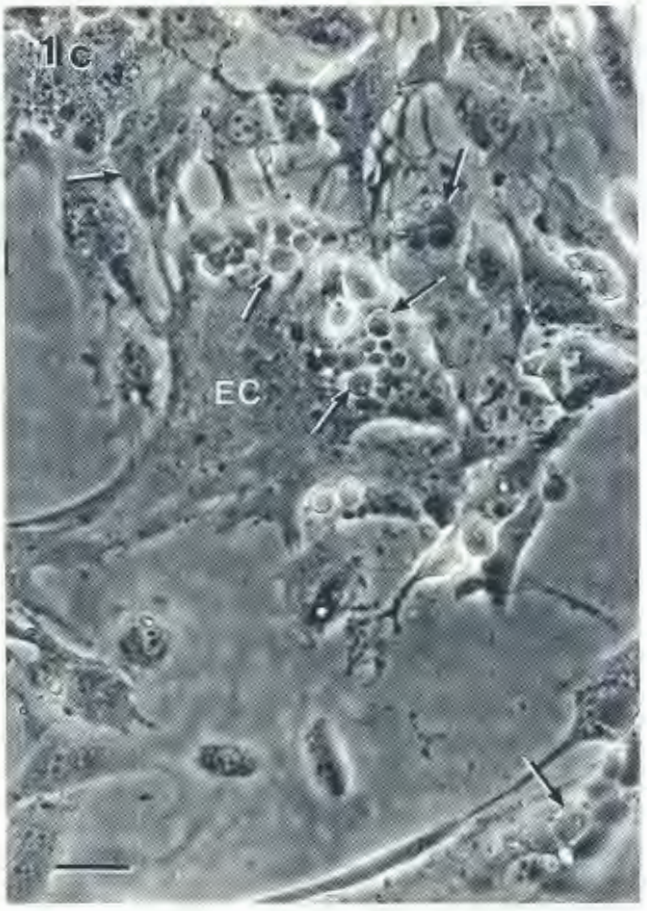


FIG. 1 Bovine endothelial cells isolated from an umbilical cord artery. (a) Monolayer of non-infected bovine umbilical endothelial cells (BUE isolate No. 9, passage No. 7). EC, endothelial cell; N, nucleus. (b) BUE monolayer 4 days after infection with *C. ruminantium* (S stock). The cells contain a number of discrete colonies (arrows) of *Cowdria* per cell. (c) Cytopathic effect of *Cowdria* infection on BUE cells 5 days p.i. Some cells are ruptured, other are still heavily infected. Bars represent 10 μm.

Electron microscopical analysis of infected cultures revealed three developmental stages. The first stage, detected between day 2 and day 4 p.i. within intracytoplasmic vacuoles of BUE cells, were the reticulate bodies (RBs) (Fig. 3a), of approximately 1,2–2,8 μm, named after their fine reticulated network of filaments interspersed with numerous ribosomes (Fig. 4, insert). The second stage detected on day 4 and day 5 within the same intracytoplasmic vacuoles were called intermediate bodies with a centrally located, electron-dense nucleoid containing partially condensed DNA and a somewhat smaller size (0,9–2,2 μm), leaving more extra-rickettsial space within the vacuole (Fig. 4). Further internal

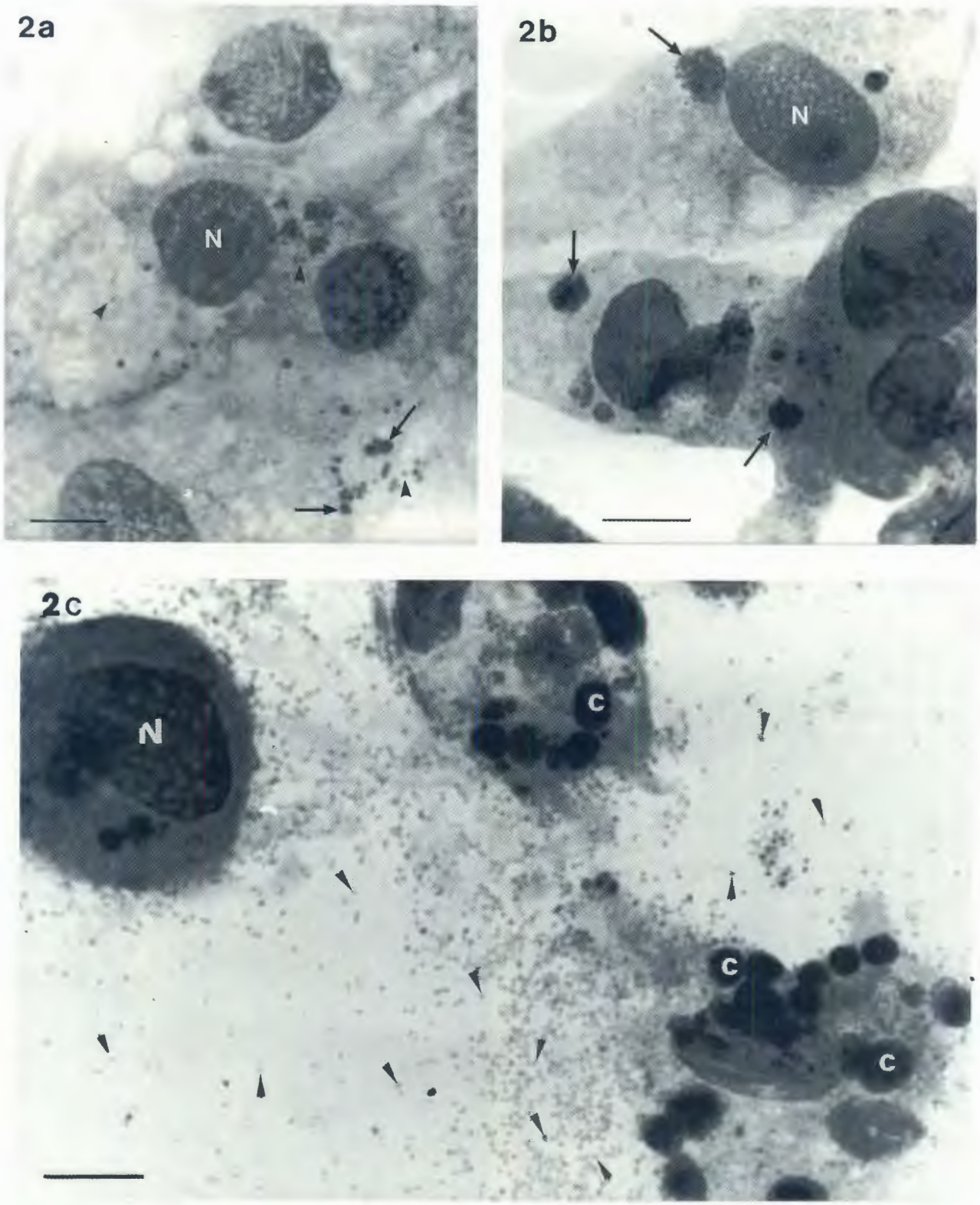


FIG. 2 Development of *C. ruminantium* (S stock), in cultures of BUE cells. (a) Intra-cytoplasmic bodies (arrow heads) and small rickettsial colonies (arrows) can be detected as early as 2 days p.i. (b) The same culture as shown in (a) at 4 days p.i.; large colonies are present (arrows). (c) On day 5 p.i. many extracellular elementary bodies (EBs) (arrowheads) of *Cowdria* have been released after destruction of the host cells. Other cells remain heavily infected. C, colony; N, nucleus. Bars represent 10 μm.

reorganization resulted in the formation of the small electron-dense elementary bodies (EBs) (0,6–1,2 μm), which are the third and final stage (Fig. 3). Large numbers of elementary bodies were detected after disruption of the host cell on day 5 or day 6 p.i. (Fig. 2c). The deduced *Cowdria* developmental cycle of 5 to 6 days is shown in Fig. 5.

Electron microscopy of *C. ruminantium* (S stock) infection in capillary endothelial cells from the choroid plexus of a goat which had died of heartwater, revealed the presence of both reticulate bodies as well as intermediate bodies (Fig. 6). Both stages divided by binary fission (Fig. 6a). However, extracellular elementary bodies were not observed in any of the thin sections examined.

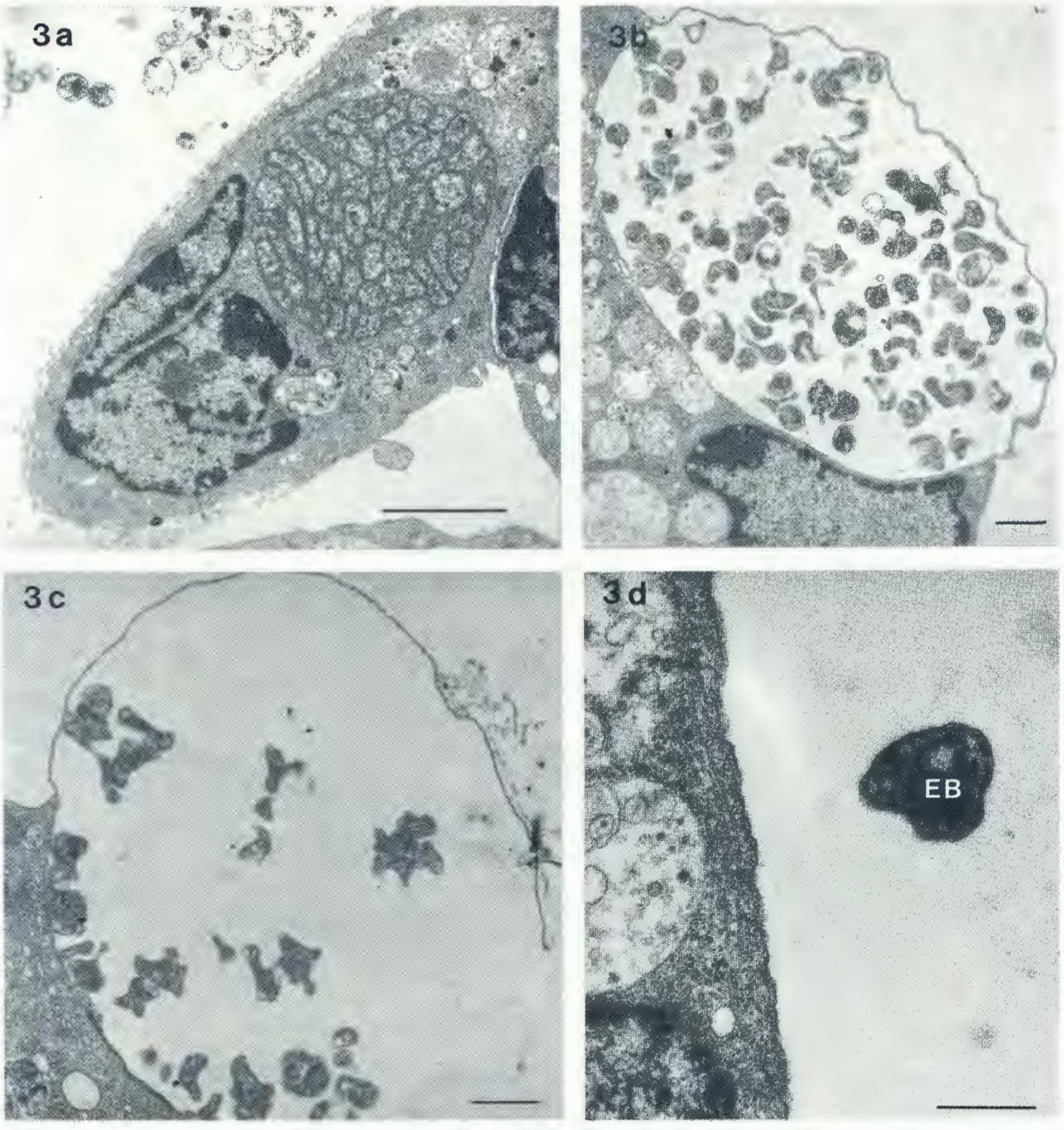


FIG. 3 Electron-micrographs of developmental stages of *Cowdria* (S) in cultures of BUE cells. (a) BUE cell containing one intracytoplasmic vacuole with closely packed reticulate bodies (4 days p.i.). Bar 5 μm. (b) Infected BUE cell with protruding vacuole containing electron dense bodies with much extrarickettsial space on day 5 p.i. The membrane of the vacuole and plasma membrane of the BUE cell appear to have fused. Bar 2 μm. (c) Ruptured vacuole releasing elementary bodies on day 6 p.i. Bar 2 μm. (d) An extracellular elementary body. EB, elementary body. Bar represents 1 μm.

The infectivity of cultures infected by *Cowdria* (W stock) were tested in mice. There was a prolongation in the number of days to death of mice inoculated with cultures incubated for 24 h to 72 h p.i. (Table 1), indicating reduced infectivity of the reticulate bodies.

Finally, *Cowdria* cells stained Gram-negative and no iodinestainable carbohydrates were detected. Several differentiating characters between *Cowdria* and *Chlamydia* are listed in Table 2.

DISCUSSION

Cultivation of *Cowdria*

Bovine umbilical endothelial cells are a convenient source for cell culture, since bovine placentas are widely available. Although in early experiments cultures were irradiated prior to inoculation with *Cowdria* (Bezuidenhout, 1987), this irradiation appeared not to be essential (Martinez *et al.*, 1990). Glasgow minimal essential medium should contain tryptose phosphate broth; otherwise infected cultures degenerated within 24 h. Nineteen different isolates of BUE cells have so far been prepared and they all supported growth of the S and W stocks of *Cowdria*. Also other *Cowdria* isolates from Zambia (Jongejan *et al.*, 1988) and the Sudan (Jongejan Morzaria, Sheriff & Abdalla, 1984), South Africa, Zimbabwe and Mozambique (unpublished) were successfully grown in these cells. It is likely that the majority of stocks can be cultivated using this *in vitro* system, perhaps with the exception of the Kümm

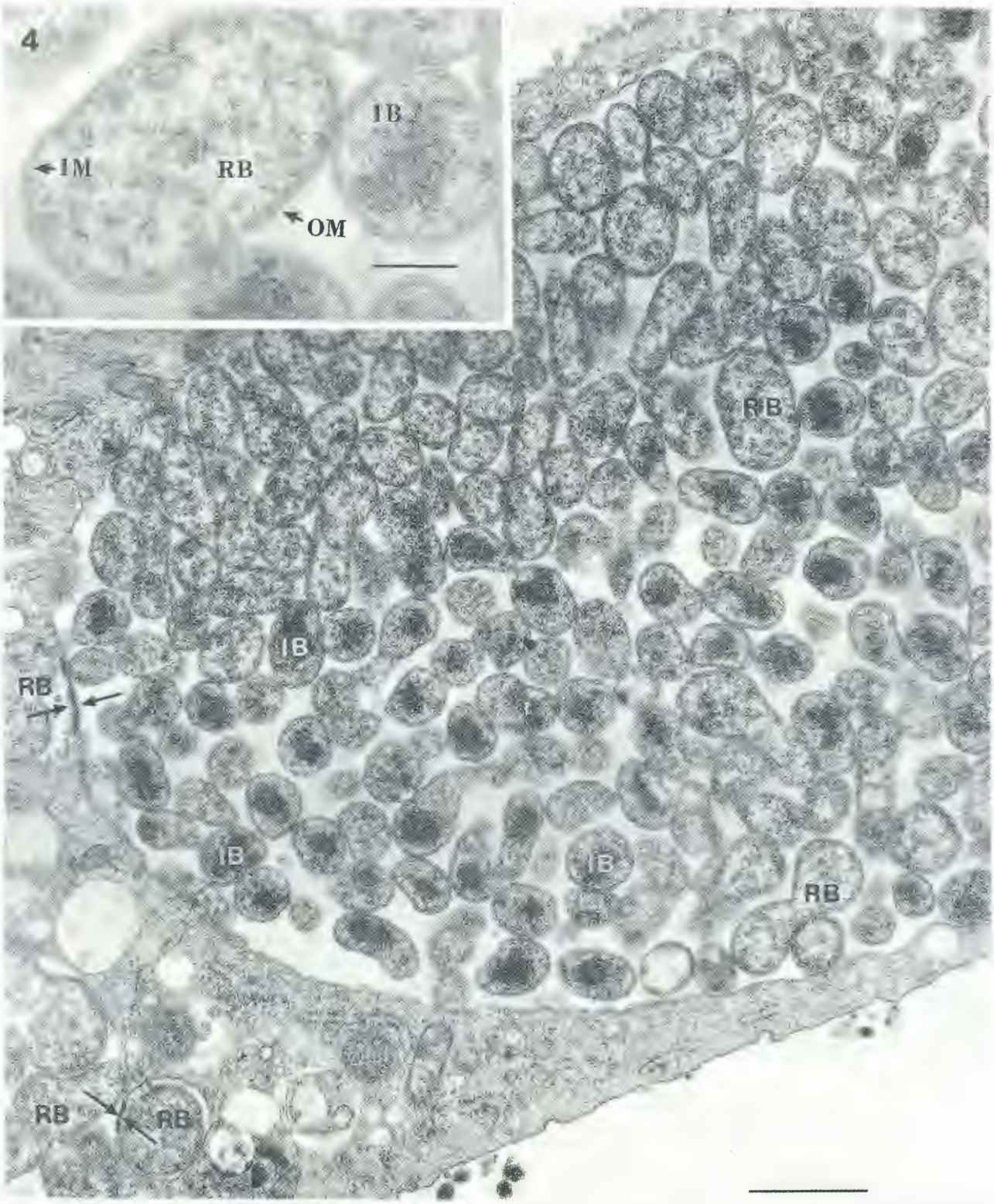


FIG. 4 Electron-micrograph of a BUE cell showing a vacuole with a large number of reticulate bodies (RB) and intermediate bodies (IB) of *Cowdria*. Both stages divide by binary fission. Note the non-synchronized development within the same vacuole. In addition, three small vacuoles containing reticulate bodies can also be seen. Electron-dense structures are present between adjacent infected vacuoles (arrows). Bar represents 2 μm. Insert. magnifiction of RB and one IB with partially condensed DNA. Bar 0,5 μm.

stock due to its tropism for macrophages (Du Plessis & Kümm, 1971). Other vascular endothelial cell cultures (aorta, pulmonary artery) were also shown to support the growth of several Zimbabwean *Cowdria* isolates (Yunker Byrom & Semu, 1988; Byrom, Yunker, Donovan & Smith, 1991).

Despite the fact that *Cowdria* exhibits tropism for endothelial cells, infection of human umbilical vein endothelial cells (Van de Wiel *et al.*, 1989) failed (F. Jongejan, unpublished observations), indicating that *Cowdria* is unlikely to be pathogenic for humans although no human sera have yet been examined in endemic areas for specific antibodies to *Cowdria*.

Relationship between *Cowdria* and *Chlamydia*

Using the BUE *in vitro* system, we have identified three developmental stages of *Cowdria* and named these forms in analogy with *Chlamydia*: "reticulate bodies", "intermediate bodies" and "elementary

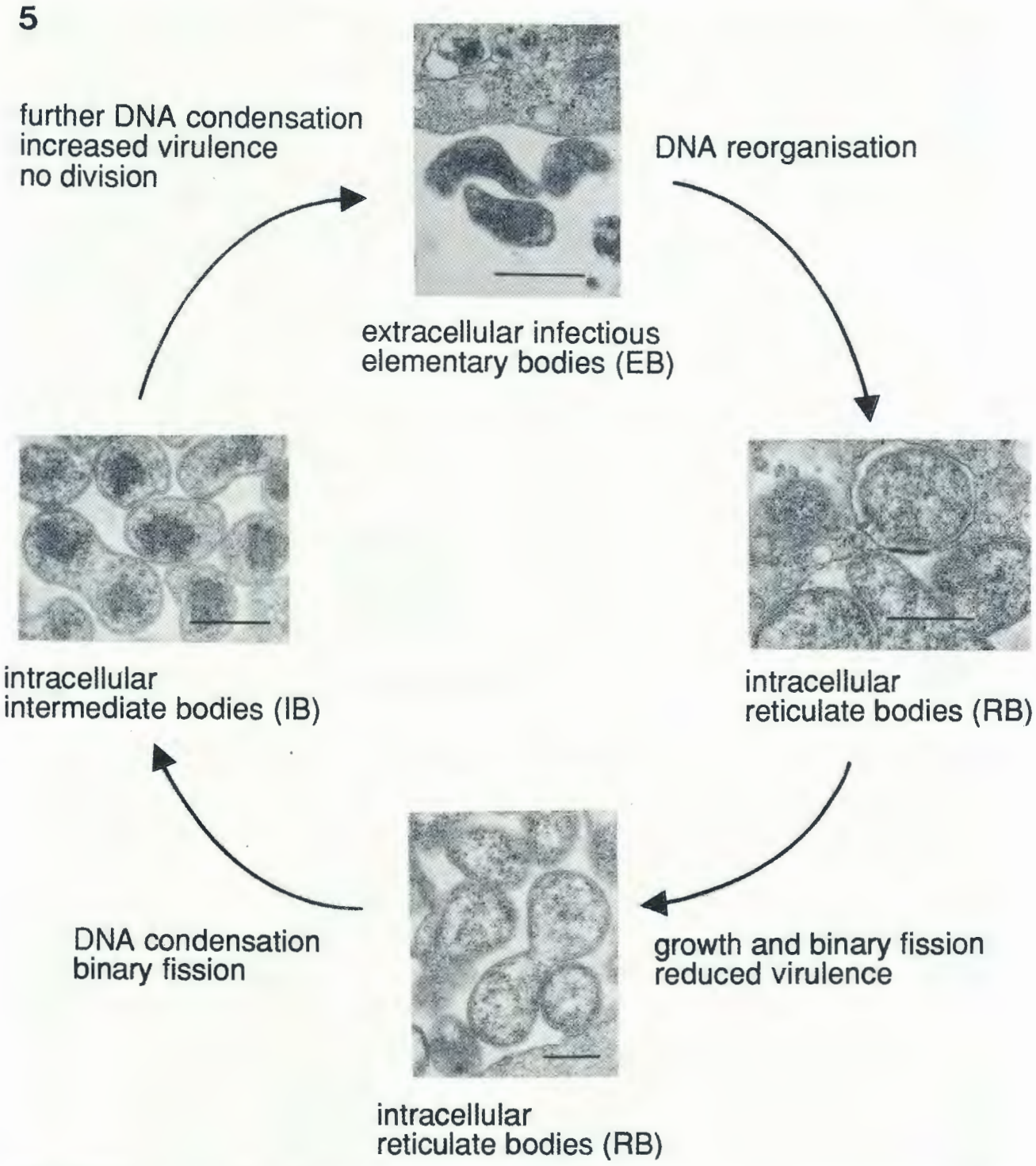


FIG. 5 Deduced sequence of *Cowdria* stages in the course of one developmental cycle of 5 to 6 days. Selected from infected BUE/9 cultures. Bars 1 μm.

bodies". The intermediate bodies in *Cowdria* correspond to the so-called condensing forms in *Chlamydia* (Storz & Spears, 1977). Although this latter name may more accurately reflect the function and morphology of this developmental stage, we did not use it for *Cowdria,* since no dispersing forms as a transition form between elementary bodies and reticulate bodies were detected in *Cowdria*. Such transition forms may possibly be detected in cultures incubated less than 24 h p.i.; this period was not investigated in this study.

The observations made by light and electron-microscopy demonstrate that the appearance of the extracellular elementary bodies coincided with the lysis of the endothelial host cells (Fig. 1c and 2c). This implies that elementary bodies are produced to survive extracellularly and to infect other host cells. Reticulate and intermediate bodies were shown to multiply by binary fission within BUE cells, whereas the elementary bodies were never observed to divide (Fig. 5). This is in agreement with *Chlamydia* developmental stages, which also multiply only intracellularly.

In *Chlamydia,* the non-dividing elementary bodies carry the infection from one cell to the other, where they initiate the next developmental cycle, whereas

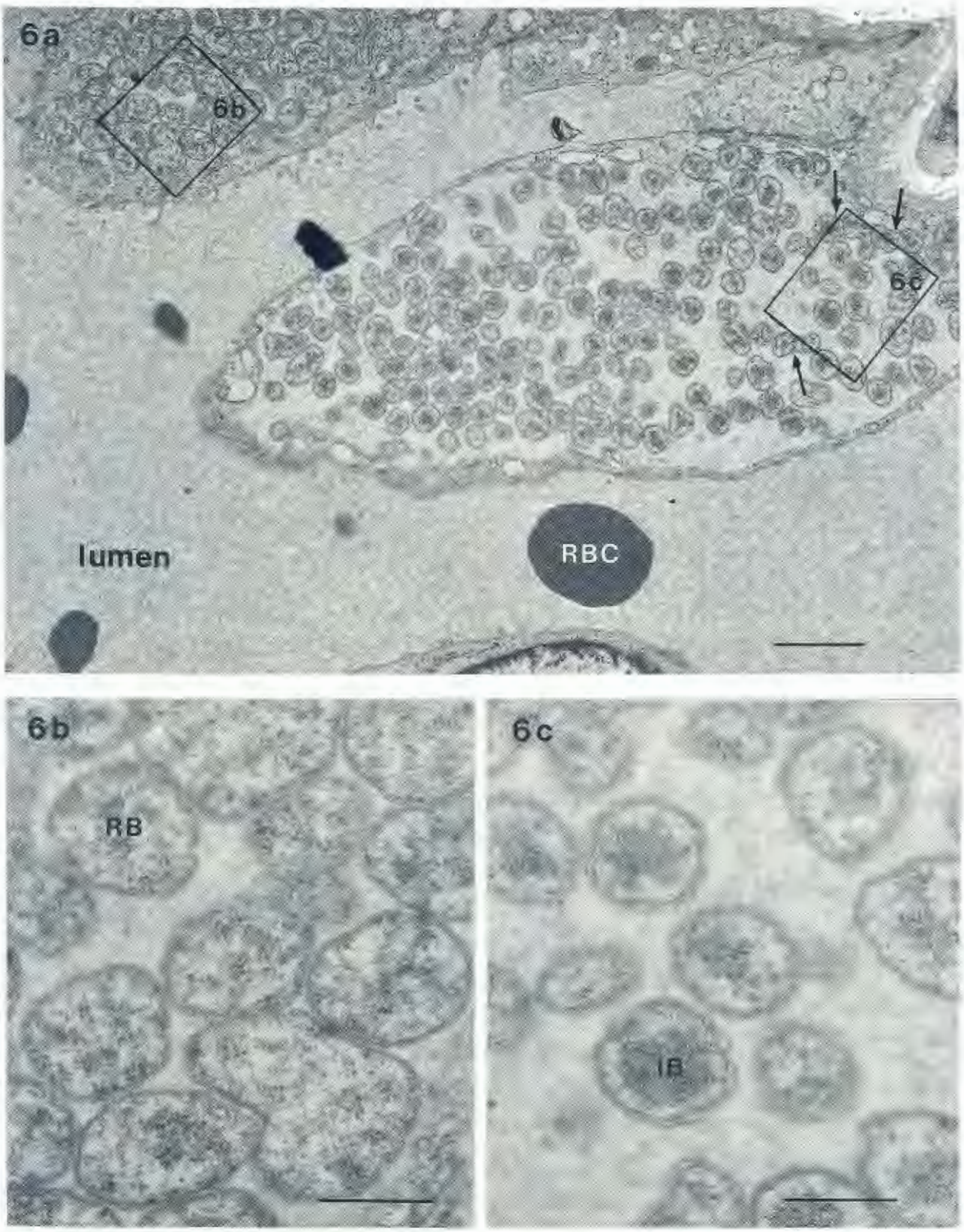


FIG. 6 Electron-micrographs of *Cowdria ruminantium* (S) infection in capillary endothelial cells from the choroid plexus of a goat which had died of heartwater. (a) Two infected cells with their vacuoles protruding into the lumen of the capillary are shown. Bar 5 μm. (b) A small vacuole containing closely packed reticulate bodies. Bar 1 μm. (c) A larger vacuole containing intermediate bodies which leave a relatively large extrarickettsial space. Bar 1 μm. Note synchronized development within the vacuoles. RBC, red blood cell; RB, reticulate body; IB, intermediate body. Arrows point towards binary fission of intermediate bodies.

reticulate bodies are the vegetative, intracellular non-infectious forms. It is most likely that elementary bodies of *Cowdria* have the same function. We have, in analogy with *Chlamydia*, attempted to evaluate infectivity of different developing stages of *Cowdria*. Infected cultures abundant in elementary bodies were more infective for mice than cultures which contained reticulate bodies (Table 1). Further

TABLE 1 Infectivity of *C. ruminantium*-infected bovine umbilical endothelial cell cultures for groups of 6 Balb/C mice

Culture[1]			Mortality in days (P.I.)												
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days[2]	RB[3]	EB[3]														
0	3+	3+	–	–	–	–	–	–	–	–	6	–	–	–	–	–
1	–	–	–	–	–	–	–	–	–	–	–	–	3	2	1	–
2	–	–	–	–	–	–	–	–	–	–	–	1	1	1	3	–
3	–	–	–	–	–	–	–	–	–	–	–	–	1	3	2	–
4	1+	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–
5	2+	1+	–	–	–	–	–	–	–	1	5	–	–	–	–	–
6	2+	1+	–	–	–	–	–	–	–	1	2	3	–	–	–	–

[1] Non-infected BUE cells did not cause any reaction when inoculated intravenously into a control group of 6 Balb/C mice

[2] Days post inoculation

[3] RB and EB rating are on a scale of – = no RB/EB and 1+ to 3+ as defined in Materials and Methods

TABLE 2 Some differentiating characters of *Cowdria* and *Chlamydia*

Character	*Cowdria*	*C. psittaci*	*C. trachomatis*
Duration developmental cycle	5–6 days	2–3 days	2–3 days
Peptidoglycan	Uncertain	–	–
Inclusion morphology[a]	Variable, dense	Variable, dense	Oval, vacuolar
Brownian movement[a]	–	–	+
Host cell tropism	Endothelial cells and phagocytes[b]	Epithelial cells and phagocytes	Epithelial cells and phagocytes
Iodine-stainable carbohydrate	–	–	+
Penicillin sensitive	–	+	+
Sulfadiazine sensitive	+	–	+
Toxic death in mice[c]	–	+	+
Host range	Ruminants, mice, birds[d] reptiles[d]	Birds, mammals	Humans, mice
Transmission	Tick-borne	Air-borne	Sexual

[a] Within intracytoplasmic vacuoles

[b] Mainly polymorphonuclear leucocytes

[c] Lipopolysaccharide-related

[d] Infective but not pathogenic to guinea fowl and leopard tortoise

– = Absent

+ = Present

studies on this aspect of the developmental cycle, with quantified inocula of *Cowdria* in TC LD_{50} assays on BUE monolayers, are in progress.

Several other important differential characters between *Cowdria* and *Chlamydia* spp. have been summarized in Table 2 and will be discussed here in further detail.

Chlamydial cell envelopes differ from those of typical Gram-negative bacteria in that they do not contain peptidoglycan. Electron-micrographs of *C. trachomatis* show that there is no peptidoglycan layer between its inner and outer membranes (Caldwell, Kromhout & Schachter, 1981). In addition, muramic acid, which is the major component of this layer has never been demonstrated by biochemical methods (Barbour, Amano, Hackstadt, Perry & Caldwell, 1982). Electron micrographs of *Cowdria* show that there is no clearly defined periplasmic space between the inner and outer membranes of *Cowdria,* which indicates that *Cowdria* may also lack peptidoglycan (Fig. 4). On the other hand, there has been one report on possible presence of peptidoglycan in *Cowdria* bodies from goat choroid plexus (Rikihisa & Logan, 1986). However, such rickettsial bodies which might contain peptidoglycan were often observed in our studies on the infected choroid plexus. We consider them to be degenerated forms, because of their discontinuous inner membranes.

The morphology of *Cowdria* within its intracellular inclusions is strikingly similar to the appearance of *Chlamydia psittaci.* Both organisms are tightly packed in their intracytoplasmic vesicles, wherein no Brownian motion can be observed using phase contract microscopy. In contast, *C. trachomatis* shows very active rapid movement in their vacuoles, which appears to depend on the low population density of the chlamydial bodies within one inclusion (Matsumoto, 1988). Moreover, inclusions which contain *C. trachomatis* usually fuse, culminating in a single large inclusion per infected host cell. In contrast, cells infected with *C. psittaci* contain multiple inclusions per cell approximately equal in number to the infectious particles previously internalized by the cells (Hodinka *et al.,* 1988). *Cowdria* and also *C. psittaci* may have a common mechanism that prevents the fusion of inclusions as occurs in *C. trachomatis.* In this respect the electron-dense structures between adjacent inclusion bodies of *Cowdria* (Fig. 4), are of special interest, since these may be involved in the prevention of vacuolar fusion.

Yet another similarity with *C. psittaci* is the absence of glycogen storage as demonstrated by negative iodine staining. However, susceptibility to sulphadiazine (Neitz, 1940) is shared between *Cowdria* and *C. trachomatis,* but not with *C. psittaci* (Table 2). *Cowdria* is not susceptible to penicillin and differs in this respect from *Chlamydia,* where penicillin-binding proteins have been demonstrated (Barbour *et al.,* 1982).

Cowdria is exclusively transmitted by *Amblyomma* ticks, whereas chlamydial organisms do not require an arthropod vector and can either be sexually transmitted *(C. trachomatis)* or transmitted via inhalation *(C. psittaci)* (Table 2). One might speculate that *Cowdria* has developed from symbionts within ticks to its vertebrate hosts without losing its invertebrate-dependency due to close association of ticks with those vertebrates. Although *Chlamydia* are considered invertebrate-independent, there is

one interesting report on the experimental transmission of *C. psittaci* by the soft tick *Ornithodorus coriaceus,* causing epizootic bovine abortion in cattle (McKercher, Wada, Ault & Theis, 1980), indicating that transmission by a vector remains possible. There are many examples of chlamydiae that develop in invertebrates other than ticks, for instance in spiders (Osaki, 1973), scorpions (Morel, 1976), isopods (Shay, Bettica, Vernon & Witkus, 1985) and crabs (Sparks, Morado & Hawkes, 1985). These chlamydial organism are usually not transmitted to vertebrates because of only incidental contact with vertebrate hosts. Moulder (1988) considered these invertebrate-dependent agents chlamydiae *sensu lato,* and his phylogenetic tree for *Chlamydia* includes invertebrate-dependent *Chlamydia*-like organisms and could very well also accommodate *Cowdria.*

Relationship between *Cowdria* and *Ehrlichia*

At present *Cowdria* is placed in the tribe Ehrlichieae, order Rickettsiales, together with the genera *Ehrlichia* and *Neorickettsia* (Ristic & Huxsoll, 1984). A taxonomic position near to that of *Ehrlichia* seems logical, as both organisms are tick-borne agents, which develop within intracytoplasmic vacuoles of reticulo-endothelial cells. The close relationship between *Cowdria* and *Ehrlichia* is supported by the finding that *Cowdria* also multiplies *in vitro* within granulocytes, the same host cells for several *Ehrlichia* species (Logan, Whyard, Quintero & Mebus, 1987). Moreover, *Cowdria* has certain antigenic determinants in common with *Ehrlichia phagocytophila* as demonstrated by cross-reactive antibodies (Logan, Holland, Mebus & Ristic, 1986; Jongejan, Wassink, Thielemans, Perié & Uilenberg, 1989).

Although *Chlamydia*-like developmental stages have been reported in *Ehrlichia,* no sequential data on their morphology have been documented. For instance, stages which suggest a developmental cycle have been reported in cultured monocytes infected with *E. canis* (Nyindo, Ristic, Huxsoll & Smith, 1971) and also in human endothelial cell cultures infected with *E. sennetsu* (Kelly, Lee & Lewis, 1985). However, electron microscopic studies carried out on *E. phagocytophila* (Woldehiwet & Scott, 1982) and *E. equi* (Sells, Hildebrandt, Lewis, Nyindo & Ristic, 1976) did not reveal a cycle of development. Therefore, similarities between *Cowdria* and *Chlamydia* reported herein, do not necessarily apply to other members of the tribe Ehrlichieae.

The taxonomic relationship between *Cowdria* and *Chlamydia* and also with *Ehrlichia* should further be elucidated by studies on the nucleotide similarities by 16S ribosomal DNA sequence analysis (Lane, Pace, Olsen, Stahl, Sogin & Pace, 1986; Weisburg Dobson, Samuel, Dasch, Mallavia, Baca, Mandelco Sechrest, Weiss & Woese, 1989; Wersburg, Barns, Pelletier & Lane, 1991), to permit construction of a system of nomenclature consistent with evolutionary relations.

POSTSCRIPT

Meanwhile the complete 16S ribosomal DNA of *Cowdria ruminantium* (Senegal isolate) has been amplified by PCR (polymerase chain reaction) and the complete nucleotide sequence determined. Phylogenetic analysis of this sequence showed that *C. ruminantium* is closest related to several species of *Ehrlichia.* Although intracellular growth of *Cowdria* resembles that of *Chlamydia,* no close phylogenetic relationship with *Chlamydia* was found (A. H. M. van Vliet, F. Jongejan & B. A. M. van der Zeijst, unpublished results).

ACKNOWLEDGEMENTS

This work was supported by grants from the European Community (Directorate General XII) under contract No. TSD-A-249 and No. TS2-0115-C. The expert advice of Mrs Susan Brett on the cultivation of *Cowdria* is very much appreciated. Dr J. L. du Plessis is thanked for making the Welgevonden isolate of *Cowdria* available. Prof. Dr. Ben van der Zeijst and Dr Henk Herwig are thanked for their critical comments on the manuscript.

REFERENCES

BARBOUR, A. G., AMANO, K.-I., HACKSTADT, T., PERRY, L. & CALDWELL, H. D., 1982. *Chlamydia tachomatis* has prenicillin-binding proteins but not detectable muramic acid. *Journal of Bacteriology*, 151, 420–428.

BEZUIDENHOUT, J. D., 1987. The present status of *Cowdria ruminantium* cultivation in cell lines. *Onderstepoort Journal of Veterinary Research,* 54, 205–210.

BEZUIDENHOUT, J. D., PATERSON, C. L. & BARNARD, B. J. H., 1985. *In vitro* cultivation of *Cowdria ruminantium. Onderstepoort Journal of Veterinary Research,* 52, 113–120.

BOVARNICK, M. R., MILLER, J. C. & SNYDER, J. C., 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *Journal of Bacteriology,* 59, 509–522.

BYROM, B., YUNKER, C. E., DONOVAN, P. L. & SMITH, G. E., 1991. *In vitro* isolation of *Cowdria ruminantium* from plasma of infected ruminants. *Veterinary Microbiology,* 26, 263–268.

CALDWELL, H. D., KROMHOUT, J. & SCHACHTER, J., 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis. Infection and Immunity,* 31, 1161–1176.

COWDRY, E. V., 1925. Studies on the etiology of heartwater. II. Observations of a rickettsia, *Rickettsia ruminantium* in the tissues of ticks transmitting the disease. *Journal of Experimental Medicine,* 42, 253–274.

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

DU PLESSIS, J. L., 1985. A method for determining the *Cowdria ruminantium* infection rate of *Amblyomma hebraeum*: Effects in mice infected with tick homogenates. *Onderstepoort Journal of Veterinary Research*, 52, 55–61.

GARRET, A. J., HARRISON, M. J. & MANIRE, G. P., 1974. A search for the bacterial mucopeptide component muramic acid in *Chlamydia. Journal of General Microbiology*, 80, 315–318.

HODINKA *et al.,* 1988. Ultrastructural study of endocytosis of *Chlamydia trachomatis* by McCoy cells. *Infection and Immunity,* 56, 1456–1463.

ITO, S. & RIKIHISA, Y., 1981. Techniques for electron microscopy of rickettsiae. *In:* Rickettsiae and rickettsial diseases (W. Burgdorfer & R. L. Anacker, eds.). Academic Press, New York. pp. 213–227.

JONGEJAN, F., VAN WINKELHOFF, A. J. & UILENBERG, G., 1980. *Cowdria ruminantium* (Rickettsiales) in primary goat kidney cell cultures. *Research in Veterinary Science,* 29, 392–393.

JONGEJAN, F., MORZARIA, S. P., SHARIFF, O. A. & ABDALLA, H. M., 1984. Isolation and transmission of *Cowdria ruminantium* in Blue Nile Province, Sudan. *Veterinary Research Communications,* 8, 141–145.

JONGEJAN, F., UILENBERG, G., FRANSSEN, F. F. J., GUEYE, A. & NIEUWENHUIJS, J., 1988. Antigenic differences betweeen stocks of *Cowdria ruminantium. Research in Veterinary Science,* 44, 186–189.

JONGEJAN, F., WASSINK, L. A., THIELEMANS, M. J. C., PERIÉ, N. M. & UILENBERG, G., 1989. Serotypes in *Cowdria ruminantium* and their relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. *Veterinary Microbiology,* 21, 31–40.

JONGEJAN, F., BAX, R., MEDDENS, M. J. M. & QUINT, W. G. V., 1991. *Cowdria ruminantium* is recognized by a monoclonal antibody directed against the major outer membrane protein of *Chlamydia trachomatis. Veterinary Microbiology,* 27, 115–123.

KELLY, D. J., LEE, M. & LEWIS, G. E., 1985. A light and electron microscopic examination of *Ehrlichia sennetsu* in cultured human endothelial cells. *Japanese Journal of Medical Science and Biology,* 38, 155–168.

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

LANE, D. J., PACE, B., OLSEN, G. J., STAHL, D. A., SOGIN, M. L. & PACE, N. R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proceedings National Academy of Science,* USA, 82, 6955–6959.

LOGAN, L. L., HOLLAND, C. J., MEBUS, C. A. & RISTIC, M., 1986. Serological relationship between *Cowdria ruminantium* and certain *Ehrlichia* species. *Veterinary Record,* 119, 458–459.

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

MARTINEZ, D., SWINKELS, J., CAMUS, E. & JONGEJAN, F., 1990. Comparaison de trois antigènes pour le sérodiagnostique de la cowdriose. *Revue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux,* 43, 159–166.

MATSUMOTO, A., 1988. Structural characteristics of Chlamydial bodies. *In:* A. L. Barron (ed.), Microbiology of *Chlamydia.* CRC Press, Inc., Florida, USA. pp. 21–45.

MCKERCHER, D. G., WADA, E. M., AULT, S. K. & THEIS, J. H., 1980. Preliminary studies on transmission of *Chlamydia* to cattle by ticks *(Ornithodorus coriaceus). American Journal of Veterinary Research,* 41, 922–924.

MOREL, G., 1976. Studies on *Parachlamydia buthi* g. n. sp. n., an intracellular pathogen of the scorpion *Buthus occitanus. Journal of Invertebrate Pathology,* 28, 167–175.

MOULDER, J. W. 1988. Characteristics of Chlamydiae. *In:* A. L. Barron (ed.), Microbiology of *Chlamydia.* CRC Press, Inc., Florida, USA. pp. 4–19.

NEITZ, W. O., 1940. Uleron in the treatment of heartwater. *Journal of the South African Veterinary Medical Association,* 11, 15.

NYINDO, M. B. A. RISTIC, M., HUXSOLL, D. L. & SMITH, A. R., 1971. Tropical Canine Pancytopenia: *in vitro* cultivation of the causative agent, *Ehrlichia canis. American Journal of Veterinary Research,* 32, 1651–1658.

OSAKI, H., 1973. Electron microscopic observations of chlamydia-like organism in hepatopancreas cells of the spider, *Coelotes luctuosus. Acta Arachnologia,* 25, 23–29.

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

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

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.

RAKE, G., ALEXANDER, R. & HAMRE, D. M., 1945. The relationship of the agent of heartwater fever, *Rickettsia ruminantium. Science,* 102, 424–425.

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 XIth International Congres on Electron Microscopy, Kyoto,* pp. 3345–3346.

RISTIC, M. & HUXSOLL, D. L., 1984. The genus *Cowdria,* Moshkovski 1947. *In:* Bergey's manual of Systematic Bacteriology. Vol. 1. (Eds. N. R. Krieg & J. S. Holt). Williams & Wilkins, Baltimore, USA. pp. 709–710.

SELLS, D. M., HILDEBRANDT, P. K., LEWIS, G. E., NYINDO, M. B. A. & RISTIC, M., 1976. Ultrastructural observations on *Ehrlichia equi* organisms in equine granulocytes. *Infection and Immunity,* 13, 273–280.

SHAY, M. T., BETTICA, A., VERNON, G. M. & WITKUS, E. R., 1985. *Chlamydia isopodii* sp. n., an obligate intracellular parasite of *Porcellio scaber. Experimental Cell Biology,* 53, 115–119.

SPARKS, A. K., MORADO, S. F. & HAWKES, J. W., 1985. A systemic microbial disease in the Dungeness crab, *Cancer magister,* caused by a *Chlamydia*-like organism. *Journal of Invertebrate Pathology,* 45, 204–217.

STORZ, J. & SPEARS, P., 1977. Chlamydiales: Properties, cycle of development and effect on eukaryotic host cells. *Current Topics in Microbiology and Immunology,* 76, 168–214.

UILENBERG, G., 1983. Heartwater (*Cowdria ruminantium* infection): current status. *Advances in Veterinary Science and Comparative Medicine,* 27, 427–480.

VAN DE WIEL, P. A., PIETERS, R. H. H., VAN DER PIJL, A. & BLOKSMA, N., 1989. Synergic action between tumor necrosis factor and endotoxins or poly A:U on cultured bovine endothelial cells. *Cancer Immunology and Immunotherapy,* 29, 23–28.

WEISBURG, W. G. , DOBSON, M. E., SAMUEL, J. E., DASCH, G. A., MALLAVIA, L. P., BACA, O., MANDELCO, L., SECHREST, J. E., WEISS, E. & WOESE, C. R., 1989. Phylogenetic diversity of rickettsiae. *Journal of Bacteriology,* 171, 4202–4206.

WEISBURG, W. G., BARNS, S. M., PELLETIER, D. A. & LANE, D. J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173, 697–703.

WOLDEHIWET, Z. & SCOTT, G. R., 1982. Stages in the development of *Cytoecetes phagocytophila,* the causative agent of tick-borne fever. *Journal of Comparative Pathology,* 92, 469–474.

YUNKER, C. E., BYROM, B. & SEMU, S., 1988. Cultivation of *Cowdria ruminantium* in bovine vascular endothelial cells. *The Kenya Veterinarian,* 12, 12–16.