



RESEARCH COMMUNICATION

Continuous *in vitro* propagation of *Cowdria ruminantium* (Welgevonden stock) in a canine macrophage-monocyte cell line

E. ZWEYGARTH and ANTOINETTE I. JOSEMANS

Onderstepoort Veterinary Institute, Parasitology Division
Private Bag X05, Onderstepoort, 0110 South Africa

ABSTRACT

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The Welgevonden stock of *Cowdria ruminantium*, aetiologic agent of heartwater, was continuously propagated in DH82 cells, a continuous canine macrophage-monocyte cell line. Cultures of DH82 cells were readily infected provided that the culture medium was supplemented with cycloheximide. Cultures were split at regular 3-day intervals and infection rates ranged between 60% and 95%. Cultures were continuously propagated through more than 125 passages over a period of more than one year.

Keywords: Canine macrophage-monocyte cell line, *Cowdria ruminantium*, cycloheximide, DH82, heartwater, *in vitro* cultivation

INTRODUCTION

Heartwater is an infectious, non-contagious disease of domestic ruminants caused by the rickettsia *Cowdria ruminantium*. It is transmitted by ticks of the genus *Amblyomma* (Lounsbury 1900). The prevalence of the disease throughout sub-Saharan Africa, the islands of Madagascar, La Réunion, Mauritius and São Tomé coincides with the distribution of the vector ticks (Uilenberg 1983). The disease also occurs on three Caribbean islands (Perreau, Morel, Barré & Durand 1980; Birnie, Burridge, Camus & Barré 1984).

Cowdry (1926) was the first to describe, from a histological study, the multiplication of *C. ruminantium* (then known as *Rickettsia ruminantium*) within the endothelial cells of infected animals. It is therefore

not surprising that the first successful *in vitro* propagation of *Cowdria* was achieved using bovine umbilical cord endothelial cells (Bezuidenhout, Paterson & Barnard 1985), although other cell types have also been used. Logan, Whyard, Quintero & Mebus (1987) devised a primary neutrophil culture system suitable for the production of *Cowdria* to be used in serological tests. This system was, however, of short duration and unsuitable for continuous *in vitro* propagation of the organism. Macrophages are also infected by *Cowdria* (Du Plessis 1975) but these cells are unsuitable for the continuous cultivation of the organism, because normal mature macrophages do not proliferate. However, Jongejan & Bekker (1999) tested several cell lines, which were in use for the cultivation of other ehrlichial species (P388D1; MDH-SP; DH82) and were able to grow *Cowdria* in monocyte-macrophage cell lines from mice and dogs. Infection rates remained low, however, and no persistent infections were established.

We report here the first continuous propagation of the Welgevonden stock of *C. ruminantium* in DH82 cells

through more than 125 passages over a period of more than one year.

MATERIALS AND METHODS

Stock of *C. ruminantium*

The Welgevonden stock was isolated by injecting into a mouse a tick homogenate prepared from a single tick. The tick, a male *Amblyomma hebraeum*, was collected on the farm Welgevonden in the Northern Province of South Africa (Du Plessis 1985).

Cell cultures

DH82 cells originally derived from a dog suffering from malignant histiocytosis (Wellman, Krakowka, Jacobs & Kociba 1988) were grown in Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) (Sigma, St Louis, MO, USA; D 0547) containing 15 mM HEPES and 1.2 g/l sodium bicarbonate. It was further supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. This medium was used for infected and uninfected cell cultures.

Infection of DH82 cells

The propagation of *C. ruminantium* in endothelial cells was carried out as described in detail elsewhere (Zweygarth, Vogel, Josemans & Horn 1997). In brief, endothelial cell cultures heavily infected with *C. ruminantium* were harvested by scraping off the cell monolayer into the medium. The cell suspension was centrifuged (800 x g for 10 min at room temperature) and 2.5 ml of the supernatant, containing mainly elementary bodies, was distributed into culture flasks containing DH82 cells. Attempts were made to initiate two different cultures, one using the medium described above and one using the same medium supplemented with 1 µg/ml cycloheximide. Cycloheximide is a specific inhibitor of protein synthesis in eucaryotic cells (Ennis & Lubin 1964) and has been used to promote growth of chlamydial organisms in cell cultures (Hobson, Johnson & Byng 1977). The cultures were incubated at 37 °C. In each case the initial medium was removed after 24 h and replaced with 5 ml of the same medium.

Monitoring of cultures

C. ruminantium infections were detected by light microscopy. Small samples of adherent cells were removed from the cultures, using a sterile 21 gauge needle with a bent tip, and smears were prepared. These were air-dried, methanol-fixed, and quick-stained (RapiDiff; Clinical Sciences Diagnostics, Booyens, South Africa). Occasionally cytospin preparations of infected cells floating in the culture supernatant were also monitored.

RESULTS

Infection rates with *C. ruminantium* in DH82 cell cultures in the absence of cycloheximide were 3.6% in adherent cells, and less than 1% in cells floating in the supernatant, and because of these low infection rates the cultures were terminated after two passages. The experiments were repeated twice with similar results (data not shown).

In contrast, the cultures of DH82 cells which contained cycloheximide were readily infected by the Welgevonden stock of *C. ruminantium*, with approximately 35% of adherent cells and 70% of detached cells showing infection before the first subculture. Cultures were split at regular intervals of 3 days at a ratio of 2. Occasionally many of the adherent cells were found to have detached from the surface of the flask and were subsequently lost when the medium was changed 24 h after passage. In such cases the cultures were passaged onto fresh host cells without being split. Infection rates were high, i.e. usually between 60% and 95%.

Continuous cultures of *Cowdria* could be maintained only when cycloheximide was added to the medium, and this continued to be true even after 116 passages. If cycloheximide was omitted from cultures having an infection rate close to 100% the rate began to decline after 3 days and was close to 0% after only four passages.

DISCUSSION

Cowdria ruminantium is particularly closely related to those *Ehrlichia* species in the phylogenetic clade known as Genogroup III (Allsopp, Visser, Du Plessis, Vogel & Allsopp 1997) with somewhat more distant relatives in Genogroups II and I. Several of these species have been successfully propagated in DH82 cells: from Genogroup III *Ehrlichia canis* (Dawson, Rikihisa, Ewing & Fishbein 1991) and *Ehrlichia chaffeensis* (Dawson, Anderson, Fishbein, Sanchez, Goldsmith, Wilson & Duntley 1991) and from Genogroup I *Ehrlichia risticii* (Van Heeckeren, Rikihisa, Park & Fertel 1993) and *Neorickettsia helminthoeca* (Rikihisa, Stills & Zimmerman 1991). The results of our study demonstrate that *C. ruminantium* too can readily be propagated in DH82 cells, a permanent canine macrophage-monocyte cell line, but only if cycloheximide is present in the culture medium. Split ratios of two and subculture intervals of 3 days were regularly observed, although in rare instances a split ratio of one had to be used.

Jongejan & Bekker (1999) failed to establish persistent infections of *Cowdria* in the DH82 cell line, and we speculate that this was because they did not incorporate cycloheximide in the medium. It should be noted that Bezuidenhout (1987) and Yunker, Byrom & Semu (1988) found that the presence of cyclohex-

imide in the culture did not improve the propagation of *Cowdria* in bovine endothelial cells. This might have been due to the fact bovine endothelial cells usually show contact inhibition after confluency, with a concomitant reduction in their rate of metabolism. DH82 cells do not show this characteristic, and we propose that the crucial factor in our experiments was the suppression of protein synthesis in the DH82 cells by cycloheximide (Ennis & Lubin 1964) thus reducing their high metabolic turnover. It has been shown that *Chlamydia psittaci* and L cells compete for the amino acid isoleucine (Hatch 1975) and it is likely that competition between *Cowdria* and DH82 cells for certain amino acids would have been suppressed by cycloheximide. Such competition could have been the factor limiting the propagation of *Cowdria* in the absence of cycloheximide.

In conclusion, it has been shown that the Welgevonden stock of *C. ruminantium* can be propagated in DH82 cells, provided suitable culture conditions prevail.

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