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CULTIVATION OF COWDRIA RUMINANTIUM IN BOVINE VASCULAR ENDOTHELIAL CELLS

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The causal agent of heartwater disease of ruminants, Cowdria ruminantium, was cultured for the first time in 4 cell lines derived from bovine vascular endothelium, specifically aorta, pulmonary artery and fetal bovine heart. The organism had previously been grown only in bovine umbilical cells (Bezuidenhout et al. 1985). Two Zimbabwean isolates of C. ruminantium (Crystal Springs, Palm River) as well as one from South Africa (Welgevonden), were repeatedly isolated from animal sources in one or more of the vascular cell line tested. Repeated serial passage of the Crystal Springs and Welgevonden isolates was obtained in 3 of the 4 lines. The organism was infective for sheep after in vitro growth and extracellular elementary bodies or from infected cell cultures fluoresced brightly through the 1:1250 dilution of immune bovine serum. This fluorescence was absent from higher dilutions of serum and cell controls.

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CULTIVATION OF *COWDRIA RUMINANTIUM* IN BOVINE VASCULAR ENDOTHELIAL CELLS

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The first successful propagation of *Cowdria ruminantium in vitro* was reported by Bezuidenhout *et al* in 1985, who infected a cell line, E5, derived from bovine umbilical endothelium. Prior to this, all cell culture systems tested allowed little more than limited maintenance of infectivity of the organism (reviewed by Uilenberg 1983; Bezuidenhout *et al* 1985; and Bezuidenhout 1987). Recently, a primary neutrophil culture system for *Cowdria* was devised by Logan *et al* (1987) which, while also of short duration, did permit intracellular multiplication of the rickettsia with production of antigen in amounts adequate for serological tests. In this paper, we report results of efforts to cultivate the heartwater organism in 11 cell lines other than the E5 line used by Bezuidenhout *et al* (1985) and Bezuidenhout (1987).

Materials and methods

Cells and cell-lines. Two cell lines from bovine aorta and one from pulmonary artery were derived by the methods in use at ILRAD for preparation of endothelial cell primary cultures (H. Hirumi personal communication). Briefly, these vessels were aseptically removed from the animal and transferred to Hank's basic salt solution (HBSS), where the external layers of the vessels were removed. The internal layers were cut into small squares and placed, lumen side down, on drops of collagenase (1 mg/ml) (Worthington Biochemical Corp., Freehold, NJ, USA) in a petri dish. After incubation at 37°C for 10-15 minutes, detaching cells were separated from the vessel pieces by pipette-dispersion in 0.5 mg/ml collagenase. Cells were pelleted by low speed centrifugation (1000/rpm/5 min) and dispersed in medium M199 in HBSS (Gibco Ltd., Paisley, Scotland, UK, Cat. No. 071-1200) or Eagle's minimal essential medium (Glasgow modification GMEM, Gibco, Cat. No. 072-02100) that contained antibiotics but lacked other additives. Following a second low speed centrifugation, the cells were resuspended in complete medium and distributed among wells of 24-well plastic tissue culture cluster. Each well was brought to 1 or 2 ml with complete medium, and clusters were sealed and incubated at 37°C for seven days. Following this, 1 or 2 ml of medium were added to each well. When monolayers were confluent in at least 2 wells, cells were passaged into 25ml plastic flasks (Costar, Cambridge MA, USA; Cat. No. 3055) following brief enzymatic (trypsin-versene) treatment.

Two additional primary cell cultures were similarly derived from ovine aorta and pulmonary artery endothelium and tested at early passage levels.

Five cell lines obtained from the American Type Culture Collection (ATCC; Hay *et al* 1985), were tested for their ability to support growth of *C.ruminantium*. These were cow pulmonary artery endothelium [CPAE cells (ATCC CRL-209)], CPA-47 cells (ATCC CRL-1733), foetal bovine heart endothelium [FBHE cells (ATCC CRL-1395)], ovine choroid plexus [SCP cells (ATCC CRL-1700)], and mouse fibroblasts [(McCoy cells (ATCC CRL-1696)]. Also tested was an embryonic tick cell line from *Dermacentor variabilis* (RML/C; Yunker *et al* 1987).

Culture media. Medium used for preparation of primary endothelial cell cultures was M199 or GMEM, to which was added 10% tryptose phosphate broth (Oxoid Ltd., Basingstoke, UK), 20% foetal bovine serum (FBS, Flow Labs, Herts., UK), L-glutamine (292 ug/ml) (Gibco) and the antibiotics penicillin-G (100 units/ml) and streptomycin sulphate (100 ug/ml) (Caps, Harare, Zimbabwe). Endothelial cell growth supplement (ECGS, 300 ug/ml, Sigma, St Louis, MO, USA) was also added in the initial culture and early passages.

For infection of cells, the medium used in most instances was that of Bezuidenhout (1987). This consisted of GMEM with the above additives (except that 10% adult bovine serum was substituted for FBS and ECGS was omitted) and 2.5ug/ml amphotericin B (Fungizone, Squibb, Isando, Tvl., SA) was added. In later experiments, streptomycin sulphate was deleted from the medium and FBS in concentrations as low as 3% was used in place of adult bovine serum.

Inocula. A number of infectious stocks of *C.ruminantium* were used to inoculate cell cultures. Included were five from Zimbabwe and four from other parts of Africa. The former were Mbizi, Lemco, Palm River and Crystal S

prings, from the Southern lowveld and Nyatsanga, from an area in the marginal highveld of north-central Zimbabwe. All had been isolated from *Amblyomma hebraeum* ticks by feeding them on susceptible Merino sheep. Non-indigenous stocks included: the Ball-3 vaccine strain (Bezuidenhout 1981) and Welgenvonden (De Plessis 1985) from South Africa, Kiswani (Kocan *et al* 1987) from Kenya, and Um Banein, an isolate from Sudan. These were exposed to the cells in various infectious preparations, principally blood or blood fractions, organ or tissue homogenates, or tick suspensions. Homogenates were obtained by grinding 2.0g of tissue with 20ml of tissue culture medium at pH 6.0-6.5. Particulate matter was allowed to settle and the supernatant was further diluted in five volumes of fresh medium. Tick suspensions were prepared by grinding ticks with sterile sand in a small amount of the same tissue culture medium. The suspension was further diluted, approximately 1:10, with medium and particulate matter removed by low speed centrifugation. Monolayers, in 25ml flasks from which growth medium had been decanted, were inoculated with 1.0ml of supernatant and incubated on a rocking platform at 37°C. Both tissue and tick suspensions were decanted from the flask after one hour of absorption and 4-5ml of fresh medium was added. Medium was changed as needed in the next two or three days. In order to retard

cell growth, the cells had been irradiated at 45 or 90 GY (Bezuidenhout *et al* 1985) by means of a cobalt-60 source 3-5 days prior to inoculation. Alternatively, they were treated with cyclohexamide (1ug/ml) (Sigma) (Bezuidenhout 1987), which was removed 24 hours before inoculation, iododeoxyuridine (25ug/ml) (Sigma) (Wentworth and Alexander 1974) which was removed just before inoculation, or they were inoculated without prior treatment.

Culture sampling and passage. Cells were observed for cytopathic effect (CPE) daily and for cytoplasmic inclusions typical of *C. ruminantium* at intervals beginning as early as 3-5 days after inoculation. Flasks were tilted to expose part of the monolayer, from which a small sample of cells was scraped by means of a sterile, 21 g. needle with a bent tip (S. Brett. personal communication). The sample was transferred to a microscope slide and teased out slightly with the needle to dissociate cells. The smear was allowed to dry and was fixed in methanol and stained with Giemsa's stain or leukostat (Fischer Scientific, Orangeburg, NY. USA). Some culture samples were also used in immunofluorescence studies and others were tested for infectivity by intravenous (i.v.) inoculation of susceptible animals (bovines, sheep, goats or Swiss White mice).

Infected cultures were prepared for passage by dislodging the monolayer into the supernatant medium with a plastic cell scraper. The mixture was drawn into a 5.0ml syringe fitted with a 21 g. needle and expressed forcefully (but without creating froth) into the flask three times. The suspension was divided between two new cultures that had been decanted of medium, and flasks were incubated on a slowly tilting rocker platform for 2-24 hours at 37°C. Four or five ml of fresh growth medium was used to replace the inoculum after incubation. If cultures were negative for *Cowdria* by microscopic examination after 7 or 14 days, medium was again replaced. If positive, the cultures were passaged, harvested for other studies, or reincubated without adding fresh medium to allow further development of infection.

Indirect immunofluorescence antibody (IFA) test to demonstrate *Cowdria ruminantium* in infected cell cultures.

Ten circular wells were marked on microscope slides with a diamond stylus. Five wells in one row were streaked with BA987 cells from a culture infected with *C.ruminantium*. The other row of five wells received uninfected BA987 cells. The smears were air-dried, fixed in methanol overnight at 4°C, dried again and stored at - 60°C until used. Prior to staining, the slides were thawed to room temperatures, and rinsed three times with 0.01M (PBS, pH 7.2). Preimmune and immune serum from bovines infected with *C.ruminantium* (blood stabilate or infected culture material) was used as the test serum. The serum was diluted 2-fold, starting with a 1:40 dilution and ending with a 1:10240 dilution. The slides were incubated for 45 minutes at 37°C, and washed three times for 10 minutes with 0.01M phosphate buffered saline, pH 7.4 (PBS). Antibovine fluorescein isothiocyanate (FITC)-labelled immunoglobulin (Kirkegaard Perry Labs., Inc., Gaithersburg, MD, USA) diluted 1:60 in 0.04% Evans blue/PBS, was added. The slides were incubated for a further 45 minutes at 37°C, then washed three times for 10 minutes in PBS. Non-specific fluorescence controls were prepared by substituting PBS for either serum or conjugate; for autofluorescence controls both the serum and conjugate were omitted. Slides were rinsed briefly in distilled water, air-dried and mounted in buffered glycerol, and observed with a Zeiss or Leitz microscope fitted for epifluorescent illumination.

Indirect immunofluorescence assay for Factor VIII-related antigen.

The marker specific for endothelial cells, Factor VIII antigen (Pyan 1986) was assayed in four bovine endothelial cell lines: BA886 (passage 24), BA987 (passage 23), BPA987 (passage 64) and E5 (passage 128). The established fibroblast line, VERO (ATCC CCL-81; passage unknown), was included as a control. Cells were grown to confluence in 8-well tissue culture chambers (LabTek 4808; Miles Scientific, Napierville, IL, USA), seeded at 1x105 cells per ml. The growth medium was discarded and cells were washed three times with PBS; they were fixed in cold methanol and air-dried. Before assaying, the slides were rinsed three times with PBS and incubated in duplicate with rabbit antiserum to human Factor VIII (Behring Diagnostics, La Jolla, CA, USA) and a control rabbit serum; each serum had been diluted 1:50. Additional controls were included in which either serum or conjugate was substituted with PBS and one in which both serum and conjugate were omitted. The slides were incubated for 30 minutes at 37°C then washed three times with PBS for 10 minutes. Fluorescein isothiocyanate labelled sheep anti-rabbit immunoglobulin (Kirkegaard Perry) diluted 1:5 was added. The slides were incubated for another 30 minutes at 37°C, washed thrice with PBS, rinsed gently in distilled water, air-dried and mounted in p-phynylenediamine (Sigma) and observed for fluorescence.

Results

Infection of cultured cells. Ninety-nine infectious samples prepared as 11 different inocula were exposed to 672 cultures of various cell-lines. Isolations *in vitro* resulted from 65 of these samples (Table 1).

Three cell lines, BA886, BA987 and BPA987 (all originating from bovine arterial endothelium), were repeatedly found capable of supporting growth of the organism. A fourth line, FBHE (foetal bovine heart endothelium), supported multiplication of the organism on five occasions. The remaining seven cell lines were refractory to infection under the conditions imposed.

The highest rate of infection of cultured cells was obtained when the Welgevonden stock of *C.ruminantium* was passaged consistently as infectious culture material to BA987 cells. The Welgevonden strain was also isolated in culture on two occasions from whole heparinised blood of an infected sheep and on three occasions from freshly prepared organ homogenate (pooled liver, spleen and kidney) of sick mice that had been injected intravenously with the organism.

To date, one of the Welgevonden isolates has been continuously cultured in BA987 cells for 133 days, during which it was passaged 10 times. The cultures are highly productive of *C.ruminantium* colonies, with the majority of cells becoming infected, often by 8-12 days following inoculation.

The cell line, BPA987, derived from bovine pulmonary artery, which was highly susceptible to infection with

failures resulted from loss of the cultures due to bacteria contamination or toxicity of the inoculum to cells, espe-

Table 1: Infection of cell lines with ***Cowdria ruminantium***

INOCULUM (Stock)	No. samples tested	CELL-LINE (No. cultures positive/No. inoculated) BA886	BA987	BPA987	FBHE
Mouse organs (Welgevonden)	3	6/8	6/10	1/6	2/4
Goat organs (Palm River)	4	0/10	6/10	0/3	0/
Whole blood (Welgevonden)	2	1/6	0/5	4/4	0/4
Plasma (Crystal Springs)	2	5/12	4/11	0/2	1/3
Cell Culture (Welgevonden)	51	12/27	58/75	23/54	
(Crystal Springs)	2	13/19	8/17	0/4	2/3
(Palm River)	1	0/1	0/12	0/4	0/3

BA = bovine aortic endothelial cells; BPA = bovine pulmonary artery cells; FBHE = foetal bovine heart endothelial cells

C.ruminantium in early passages, later became refractory. On or about the 19th passage, the cells lost their endotheloid morphology and became fibroblastic. Thereafter it no longer supported growth of the rickettsia.

Two Zimbabwean stocks of *C.ruminantium*, Crystal Springs and Palm River, were isolated in culture. The former was isolated in BA886, BA987 and FBHE cells from two preparations, infectious plasma and a pellet resulting from high speed centrifugation of the plasma. Irradiated and non-irradiated BA886 and BA987 cells were equally susceptible to infection, and the single isolate in FBHE cells was obtained in non-irradiated cells. The Palm River isolates were obtained from three inocula, brain, kidney and liver homogenates, which were prepared from a goat that died of heartwater. All of six positive cultures were of BA987 cells, one of which was not irradiated prior to inoculation. A third homogenate, from spleen of the same goat, proved toxic to the cells.

After its initial growth in BA886, BA987 and FBHE cells the Crystal Springs isolate was successfully passaged, on days 11 and 14 to both irradiated and non-irradiated cells of all three of these lines. This stock is currently in its third passage. It grows relatively rapidly, and to moderately high infection levels in both lines of arterial origin, but more slowly and to a lower infection level in the foetal heart endothelial cells.

Upon isolation in BA987 cells, the Palm River stock multiplied slowly over a period of three weeks, but only to low levels of infection. At that time, cultures were passaged to seven new cultures of BA987 cells. Results of this passage are pending.

Infectious inocula failing to infect cell cultures included tick stabilates (2), scrapings of arterial lumen (1), organ homogenates (1), whole blood (7), plasma (3), leucocytes (7), neutrophils (6), and macrophages (5). Represented among these negative results were all stocks of *C.ruminantium* tested (see Materials and Methods). Some of the cially in the case of tick stabilates or whole blood. The reasons for other negative results are not known.

Initial infection of cultures was detected typically as early as 14 days after inoculation if the inocula originated from infected animals. When infectious cell culture material was used as inoculum, infections could be seen much earlier (4 days).

Cytopathic effect was often readily observed in *Cowdria* infected cultures. Grossly, this could be seen as clear streaks in the monolayer where infected cells had detached, or were beginning to detach. Microscopically, affected cells were rounded and loosely attached or floating.

Welgevonden-infected cell culture material (both pooled cells and supernatant medium or supernatant medium alone) was infectious for susceptible Merino sheep. Inoculation of 2ml of this material intravenously, produced typical febrile reactions and nervous symptoms. When characteristic organisms were demonstrated in microvessels on brain biopsy, the animals were treated with oxytetracycline (8 mg/kg). After recovery, these sheep were resistant to challenge with virulent *C.ruminantium* (Mbizi stock).

Immunofluorescence of *Cowdria ruminantium* in culture. In smears from infected cultures, extracellular elementary bodies of *C.ruminantium*, which had been released from colonies within cells, fluoresced brightly through the 1:2560 dilution of immune bovine sera. Intracellular organisms and colonies fluoresced only faintly. Cell nuclei exhibited very pale, light green autofluorescence. Some degree of non-specific background staining was seen with immune serum and uninfected cells, as well as with preimmune serum and infected cells at a 1:40 dilution. This non-specific staining was absent from higher dilutions.

Factor VIII immunoreactivity. Both BA886 and BA987 cell lines exhibited bright granular fluorescence in

the presence of antiserum to Factor VIII antigen. This fluorescence was absent in the controls. Neither Vero cells nor controls in which test serum and conjugate were omitted showed fluorescence in any test. Some degree of non-specific fluorescence was observed in E5 and BPA987 cells exposed to normal rabbit serum, even when the serum was absorbed to delete non-specific reactivity. In these instances, however, the staining was uniformly pale green and markedly different from that seen with Factor VIII antiserum.

Discussion

The methods employed in this study for cultivation of *C.ruminantium* in bovine arterial and heart endothelial cells are essentially those outlined by Bezuidenhout *et al* (1985) and Bezuidenhout (1987) in E5 (bovine umbilical) cells and the present findings confirm their usefulness. These workers employed techniques that retarded growth of host cells (irradiation, cyclohexamide) and facilitated contact between cells and inoculum (centrifugation, polybrene). Later, they employed a slowly rocking platform, which allowed a reduced volume of infectious inoculum to flow repeatedly over the cell monolayer (J.D. Bezuidenhout and S. Brett personal communication). This replaced centrifugation as a means of insuring pathogen-cell contact. Most of our positive cultures had been irradiated prior to inoculation. Cyclohexamide- and iododeoxyuridine-treated cells failed to propagate the organism. However, the two Zimbabwean stocks of *C.ruminantium* were isolated in non-irradiated cells.

The conditions that obtain during absorption are apparently critical. In addition to the requirement for close contact of organism and cell, an acid environment may be necessary to ensure infection. We have had best results when culture media was gassed with $C0_2$ before inoculation. Once infection is established, the shifting extracellular pH does not seem to affect the development and maturation of intracellular colonies. It remains to be seen how it affects the infectivity of mature organisms (elementary bodies) as they are released into the culture media.

The nature and amount of bovine serum added to the culture medium is apparently not critical. Bezuidenhout *et al* (1985) and Bezuidenhout (1987) routinely used 10% adult bovine serum, but we have found that the organism can be isolated initially and propagated continuously *in vitro* in the presence of only 3.0% FBS. This reduced amount is sometimes necessary to slow the growth of bovine endothelial cells.

One of the cell lines initially found susceptible to infection with *C.ruminantium*, BPA987, underwent a change in morphology from endotheloid to fibroblastic on or about the 19th passage. At this time it also became refractory to infection. Thus, we attempted to determine whether or not susceptibility to the organism was related to the endothelial nature of the cell. Four bovine cell lines were tested for the presence of Factor VIII antigen, a specific marker for endothelial cells. Two of the lines, BA886 and BA987, at low passage levels with endotheloid morphology, and susceptible to infection with the rickettsia, exhibited marked fluorescence with Factor VIII antisera. A third line, BPA987, at a high passage level of fibroblastic morphology and no longer susceptible to infection, failed to exhibit specific fluorescence. However, E5 cells, which are also fibroblastic and at a high passage level, but susceptible to infection, were also negative in our tests for this antigen. This would indicate that, while bovine cells of known endothelial nature are definitely susceptible to infection with *C.ruminantium*, at least one cell line that is probably not endothelial is also susceptible.

Obviously much more work is needed on the basic relationships between this rickettsia and its host cell, inasmuch as the organism cannot yet be routinely isolated or passaged with the success rate seen for many other cell-dependent pathogens. Nevertheless, the existing *Cowdria* culture system will undoubtedly find many uses. Some of these uses have been listed by Bezuidenhout (1987) and Bigalke (1987). We have been separating and stockpiling culture supernatants in preparation for the establishment of a *Cowdria* DNA library, which will be applicable to the development of vaccines and nuclear probes through recombinant technology. In addition, we have begun to isolate and characterise *Cowdria* proteins from infected cultures and culture supernatants. These proteins will be tested for immunogenicity, specificity and sensitivity. An understanding of the relevance of these antigens, which are readily obtainable from culture, to immunity in heartwater could conceivably follow.

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