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THE PRESENT STATE OF *COWDRIA RUMINANTIUM* CULTIVATION IN CELL LINES

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ABSTRACT

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Attempts were made to grow 4 isolates of *Cowdria ruminantium* in cell lines. Three of these isolates, viz. Ball 3, Welgevonden and Kwanyanga, could be cultivated in a calf endothelial cell line, but experiments with the Kümm isolate have so far failed.

The successful *in vitro* cultivation of 2 isolates (Welgevonden and Kwanyanga), which are also pathogenic for mice, has great potential for future studies and these aspects are discussed in this review.

INTRODUCTION

Apart from a recent report on the successful *in vitro* cultivation of *Cowdria ruminantium* (Bezuidenhout, Paterson & Barnard, 1985) all previous attempts to cultivate the organism in cell cultures have met with little or no success (Uilenberg, 1983).

Unfortunately few details of these experiments with mammalian cells (B. J. Erasmus, personal communication, 1985) or insect cell lines in the form of *Aedes albopictus* (J. L. du Plessis, unpublished results, 1972) are available or published. This has resulted into a situation where there has been almost no scientific basis for future researchers to build upon. It was therefore also not possible to plan new strategies through a process of elimination of previous techniques.

The most significant contribution in the work by Bezuidenhout *et al.* (1985) is the demonstration that various factors, such as the inoculum, cell line, medium, antibiotics and the techniques used, all affect the growth of the organism. This information forms the basis for the development of better and more efficient techniques which, it is hoped, will lead to the mass production of organisms in culture.

MATERIALS AND METHODS APPLIED

Isolates of C. ruminantium

Up to the present, attempts have been made at the Veterinary Research Institute, Onderstepoort, to cultivate 4 different isolates of *C. ruminantium* in cell cultures. They are: the Ball 3 isolate (Haig, 1952), the Welgevonden isolate (Du Plessis, 1985), the Kwanyanga isolate (Mackenzie & Van Rooyen, 1981) and the Kümm isolate (Du Plessis & Kümm, 1971).

Cell lines

In most cases a calf endothelial cell line (E5) was used (Bezuidenhout *et al.*, 1985). Other cell lines such as Vero, lamb foetal kidney (LFK) and mouse L-cells were also used in some instances (Table 1).

Medium

Eagle's medium (Glasgow modification), containing 10 % locally prepared bovine serum, sodium benzylpenicillin (200 IU/mℓ), streptomycin sulphate (200 μg/mℓ) and amphotericin B (2,5 μg/mℓ) was used throughout these investigations. After the addition of the above, the pH of the medium is usually 6,5.

Inoculum

In the case of the Ball 3 isolate cultures were inoculated with infected choroid plexus (B. J. Erasmus, unpublished data, 1969), tick suspension (Bezuidenhout *et al.*, 1985) or 2 mℓ of fresh blood in heparin per 80 cm² plastic flask. Suspensions of liver and spleen, prepared from moribund mice according to the method of Du Plessis (1982), were used as inoculum in the case of all other isolates. These suspensions were diluted (0,5 mℓ suspension in 4,5 mℓ medium) and inoculated onto culture flasks (80 cm²).

Special techniques applied prior to and after inoculation of cultures

(1) *Irradiation:* To facilitate initial growth of organisms most cultures were irradiated at 45 GY or 90 GY in a Cesium source 1–4 days prior to inoculation (Bezuidenhout *et al.*, 1985). Once growth of the organism was established, as determined by animal inoculation studies or microscopy, culture material was passaged in many instances onto non-irradiated cells. Attempts were also made to grow the organism from original inocula on non-irradiated cells.

(2) *Cycloheximide*[1] *in culture medium:* in certain instances medium containing 0,1–0,5 % cycloheximide was added to non-irradiated cultures 1 day prior to the inoculation of cultures. The same medium was also used to replace old medium at weekly intervals. Cycloheximide, a specific inhibitor of protein synthesis in eucaryotic cells (Ennis & Lubin, 1964) has been used to promote growth of chlamydial agents in cell cultures (Hobson, Johnson & Byng, 1977; Spears & Storz, 1979).

Techniques applied to facilitate better contact between cells and inoculum

Three techniques have been applied viz.:

(1) Infected tick suspension was clarified by centrifugation at low speed (2 000 × g for 5 min), were then centrifuged at 30 000 × g for 20 min after which the supernatant was discarded. Non-irradiated cells were then added to the sediment and centrifuged at 4 500 × g for 20 min (Bezuidenhout *et al.*, 1985).

(2) Inoculated cultures in 80 cm² plastic flasks containing 6 mℓ of medium were secured in swing-out microtiter plate holders and centrifuged at a speed of 250 × g for 20 min.

(3) Prior to inoculation Polybrene (hexadimethrine bromide)[2] was added at a concentration of 8 μg/mℓ to irradiated or non-irradiated cultures and incubated for 30 min at 37 °C. The medium in the flasks was then discarded and the cultures inoculated with tick suspension (Ball 3) or mouse material (Welgevonden isolate). Polybrene is a polycation frequently used to facilitate infection of cells by retroviruses (Payne, York, De Villiers, Verwoerd, Quèrat, Barban, Sauze & Vigne, 1986).

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[1] Sigma Chemical Co., St. Louis, MO. 63178 U.S.A.

General handling of cultures

Cultures were propagated at 37 °C in a variety of plastic and glass containers. After inoculation and centrifugation cultures were incubated for 1 h at 37 °C after which more medium was added. About half of the culture medium was usually replaced 1–2 days after inoculation and thereafter at weekly intervals. Before the transfer of culture material from 1 passage to the next, cells were released either by activated trypsin versene (ATV) or by means of a cell scraper.

Testing for the presence of C. ruminantium *in cultures*

(1) *Microscopic examinations*. In the case of plastic culture flasks most of the cells were released either by trypsinization or by means of a cell scraper. Areas of cells were left behind for microscopic examination. The medium with suspended cells was poured off and used for further passages or for animal inoculation. The flasks were rinsed twice with serum free medium or phosphate buffered salt solution (PBS). Cells were fixed for 2–3 min in methanol before staining with Giemsa (5 % solution for 55 min or 25 % solution for 20 min). The flasks were then left to dry and pieces of plastic containing cells were cut out by means of a Dremel tool[3] fitted with a fine rotating saw blade. The pieces of plastic were secured onto glass microscope slides by means of a transparent contact adhesive. In cultures which were not subjected to centrifugation cells were grown on glass cover slips. Cells from larger glass containers such as Roux flasks, or Roller bottles (500–2 000 mℓ) were carefully scraped off, centrifuged at 400 × g in a Cyto-spin centrifuge on glass slides which were then stained for microscopic examination. Some slides were stained with Gimenèz (Gimenèz, 1964) or used for direct or indirect immunofluorescence studies (Bezuidenhout *et al.*, 1985).

(2) *Animal inoculation*. The infectivity of cultures was tested on various days after inoculation and also after certain passages by intravenous (i.v.) injection into sheep and/or mice. Cultures inoculated with the Kümm isolate were also injected intraperitoneally (i.p.) into mice. In the case of the Ball 3 isolate some of the reacting sheep were treated intramuscularly with oxytetracycline (20 mg/kg) and then challenged i.v. at different intervals with 5 mℓ infective blood (Ball 3). Culture material for animal inoculation was prepared by the release of 20 cm² of culture surface per 1 mℓ of medium. Sheep were injected with 1 mℓ and mice with 0,2 mℓ of this material intravenously. Animals were monitored for heartwater reactions as previously described (Bezuidenhout *et al.*, 1985).

Results and Discussion

The results of investigations conducted are summarized in Tables 1, 2, 3 and 4.

So far 3 of the 4 isolates tested have been successfully cultured. The only exception is the Kümm isolate which did not multiply in bovine endothelial or mouse L cells.

Irradiation of cells and centrifugation of the inoculum onto cells consistently enhanced the initial infection of cultures. Once the organisms have established themselves in culture irradiation and centrifugation do not seem to be all that important. Cultures treated with cycloheximide or polybrene were also infective but very few colonies were present in these cultures.

In all the isolates where success was obtained a calf endothelial cell was used. Vero and lamb foetal kidney cells did not support the growth of the Ball 3 isolate. Mouse L-cells also did not become infected with the Welgevonden isolate. Eagle's medium with 10 % bovine serum and antibiotics, described earlier gave good results.

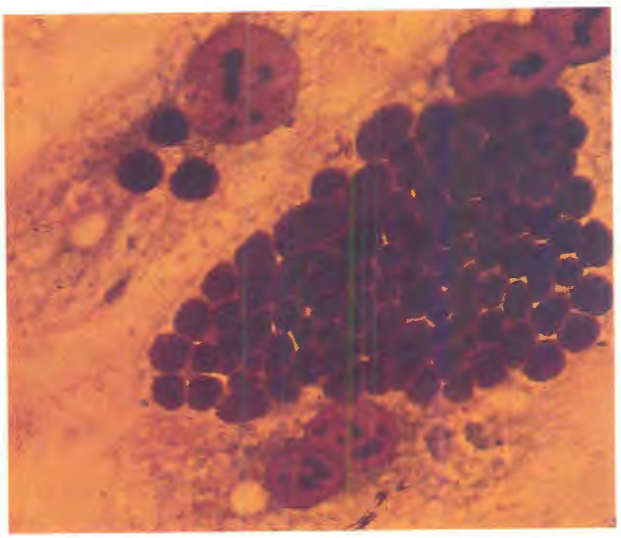

FIG. 1 A Giemsa-stained preperation showing colonies of *C. ruminantium* (Welgevonden isolate) in a bovine endothelium cell line (E5), 14 days post infection. × 400

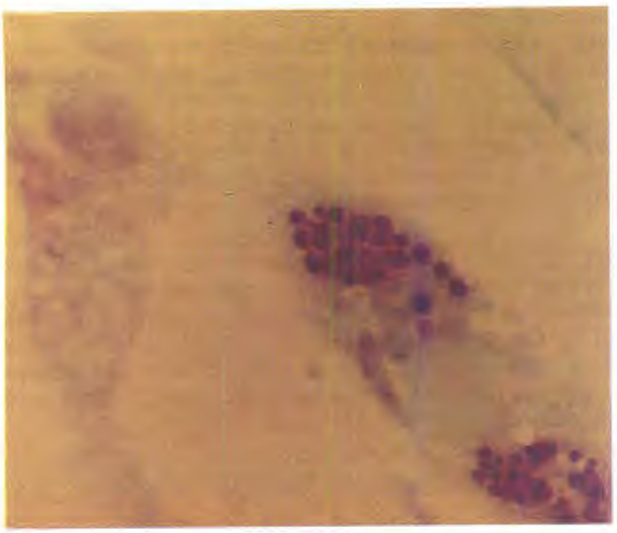

FIG. 2 Colonies of *C. ruminantium* (Kwanyanga isolate) in E5 cultures, 19 days post infection, staining red with Gimenèz. × 400

In the case of the Ball 3 isolate tick suspensions were the inocula most widely used. Infected choroid plexus of a reacting sheep once gave positive results (B. J. Erasmus, personal communication, 1986, cited in Bezuidenhout *et al.*, 1985). Cultures inoculated with fresh blood from a reacting sheep were infective 18 and 25 days after inoculation. Microscopic examination of these cultures also revealed typical colonies of the organism. However, growth of the organism could not be established by inoculation with the same blood after it had been frozen and kept at −80 °C for 31 days.

Homogenates from mouse liver and spleen were found to be suitable inocula in the case of the Welgevonden and Kwanyanga isolates. No other inocula were tested in these cases.

The presence of organisms in cultures can be demonstrated with relative ease in Giemsa- and also Gimenèz-stained preparations (Fig. 1 & 2). Undisturbed cells gave better results than those that were detached and centrifuged in a cyto-spin. Giemsa staining gave the most

[2] Serva, Feinbiochemica, Heidelberg, Germany

[3] Dremel, MFG, Racine, Wisconsin 53406, U.S.A.

TABLE 1 Cultivation of the Ball 3 isolate in cell lines

Cell line used	Inoculum	Techniques to:		Results			Reference
		Retard division of cells	Improve contact cells and inoculum	Micro-scopy	Animal inoculation		
					Sheep	Mice	
Calf endothelium (E5)	Choroid plexus (sheep)	None	None	—	+	*	B. J. Erasmus unpublished—1969
	Tick stabilate	None	None	—	—	*	Bezuidenhout, Paterson & Barnard, 1985
	Tick stabilate	Irradiation (45 GY)	None	+	+	*	Bezuidenhout, Paterson & Barnard, 1985
	Tick stabilate	Irradiation (90 GY)	None	+	+	*	Bezuidenhout, Paterson & Barnard, 1985
	Tick stabilate	None	Centrifugation in tube	+	+	*	Bezuidenhout, Paterson & Barnard, 1985
	Tick stabilate	Irradiation (90 GY)	Centrifugation in flasks	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
		Irradiation (90 GY)	None	+	+	*	
	Tick stabilate	Cycloheximide (0,1 & 0,5%)	None	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
	Tick stabilate	Irradiation (90 GY)	Polybrene	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
	Tissue culture	Irradiation (90 GY)	Centrifugation in flasks	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
		None	None	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
	Blood (fresh)	Irradiation (90 GY)	Centrifugation in flasks	+	+	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
	Blood (frozen)	Irradiation (90 GY)	Centrifugation in flasks	—	—	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
Vero	Tick stabilate	Irradiation	Centrifugation in flasks	—	—	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986
LFK	Tick stabilate	Irradiation	Centrifugation in flasks	—	—	*	Bezuidenhout, J. D., Brett, S. & Olivier, J. A. Unpublished, 1986

* Not attempted so far

TABLE 2 Cultivation of the Welgevonden isolate in cell lines

Cell line used	Inoculum	Techniques to:		Results			Reference
		Retard division of cells	Improve contact cells and inoculum	Micro-scopy	Animal inoculation		
					Sheep	Mice	
Calf endothelium (E5)	Mouse liver and spleen	Irradiation (90 GY)	Centrifugation in flasks	+	+	+	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
		None	Centrifugation in flasks	+	*	*	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
		Irradiation (90 GY)	None	+	*	+	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
	Tissue culture	None	None	+	+	+	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
		Irradiation (90 GY)	Centrifugation in flasks	+	*	+	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
Mouse fibro-blasts (L cells)	Mouse liver and spleen	Irradiation	None	—	*	—	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986
		Irradiation (90 GY)	Centrifugation in flasks	—	*	—	Bezuidenhout, J. D., Du Plessis, J. L. & Brett, S. Unpublished, 1986

* Not attempted so far

TABLE 3 Cultivation of the Kwanyanga isolate in cell lines

Cell line used	Inoculum	Techniques to:		Results			Reference
		Retard division of cells	Improve contact cells and inoculum	Micro-scopy	Animal inoculation		
					Sheep	Mice	
Calf endothelium (E5)	Mouse liver and spleen	Irradiation (90 GY)	Centrifugation in flasks	+	*	+	Bezuidenhout, J. D., MacKenzie, P. K. I., Brett, S. & Olivier, J. A. Unpublished, 1986
	Tissue culture	None	None	+	*	*	Bezuidenhout, J. D., MacKenzie, P. K. I., Brett, S. & Olivier, J. A. Unpublished, 1986

* Not attempted so far

TABLE 4 Cultivation of the Kümm isolate in cell lines

Cell line used	Inoculum	Techniques to:		Results			Reference
		Retard division of cells	Improve contact cells and inoculum	Micro-scopy	Animal inoculation		
					Sheep	Mice	
Calf endothelium (E5)	Mouse tissue	Irradiation (90 GY)	Centrifugation in flasks	—	—	—	Bezuidenhout, J. D., Du Plessis, J. L., & Brett, S. unpublished, 1986
Mouse fibroblasts (L cells)		None	None	—	—	—	Bezuidenhout, J. D., Du Plessis, J. L., & Brett, S. unpublished, 1986
Aedes albopictus[1]		None	None	S	S	*	J. L. du Plessis, unpublished, 1972

[1] In this case Hank's medium was used
S Inconclusive results
* Not attempted

reliable results. Although Gimenez staining was also suitable, optimal counter-staining was not always obtained.

Cowdria colonies can first be seen in cultures as early as Day 11 after infection (Ball 3). They become more apparent as time passes and 21 days after inoculation of irradiated cells numerous dark staining colonies are clearly visible. Thereafter, they seem to disintegrate, releasing numerous organisms. In the case of the Welgevonden isolate, colonies were seen 12 days after inoculation with mouse tissues. However, when these cultures were homogenized with a syringe and needle (18G) and transferred onto new cultures, numerous colonies were observed 3 days later. In further trials these results were not always reproduceable.

Generally speaking more colonies were seen in cultures initially infected with the Welgevonden and Kwanyanga isolates than in the case of the Ball 3 isolate. Although in certain cultures a very high percentage of cells were infected (>50 %), the majority only had a few colonies and these had a patchy distribution. In culture the organism exhibited the typical morphology of the organism in its mammalian hosts. This was also confirmed in electron microscopic studies of culture material (Prozesky, Bezuidenhout & Paterson, 1986).

Culture material is highly infective for susceptible animals. After treatment and recovery from infection, sheep were immune for at least 6 months. The Ball 3 isolate was still highly pathogenic for sheep after 8 passages and the Welgevonden for sheep and mice after 5 passages, the limit of testing undertaken to date.

FUTURE STUDIES

Reasons for the variation in the infectivity rates of cells in cultures that were treated in the same way should be sought. It is of the utmost importance that the inoculum and culture techniques be standardized in the greatest detail. Cloning and characterization of the E5 cell line should be done as this may lead to the identification of a more suitable isolate of cells for the cultivation of the organism.

The optimum time after inoculation for passaging and harvesting has not been determined. This aspect should be looked into as it could lead to higher yields.

The successful growth of isolates pathogenic for mice is seen as a valuable tool to study the immunogenicity of inactivated organisms. The mouse would also be a cheap model to evaluate the possibility of attenuating the organism in culture systems. It is also an ideal system for freeze-drying experiments. This opportunity should be exploited to the full.

Purification of the organism from cultures should receive more attention in order to obtain antigen for serological tests, monoclonal antibodies and, possibly, for the isolation of *Cowdria* DNA which could be used in studies for the development of a DNA-recombinant vaccine.

The possibilities of using infected cultures as an alternative antigen for vaccination should be studied. This may mean that alternatives for irradiation and centrifugation will have to be found.

Tissue cultures may also prove to be a useful system for the screening of a new chemotherapeutics with potential for use in the treatment of affected animals.

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