Bovine and human endothelial cell growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium*: prospects for cells and vaccine production

INTRODUCTION

Cowdriosis (or heartwater) is one of the main causes of economical losses for cattle breeding in Subsaharan Africa. As the disease appeared recently in the Caribbean, the American mainland is at risk also.

Methods of vaccination against heartwater exist (e.g. deliberate infection of the animals with virulent sheep blood “vaccine” followed by tetracycline treatment at the time of hyperthermia), but their application is risky and laborious (4). The method is not applicable on a large scale either.

Other means of control are therefore needed. Before one could use an active recombinant vaccine on a large scale, there is still room for a more classical approach. The recent attenuation of one strain of *Cowdria ruminantium* by long term passage in BUEC cells (2) paved the way to this direction. Despite trials with different strains of *Cowdria ruminantium*, up to now success in attenuation has been limited. Generalization of this approach requires the possibility to attenuate different isolates of the rickettsia. As a contribution to this goal, we have undertaken a series of trials using different kinds of endothelial cells as host for the rickettsia multiplication, with the hope of extending the cell systems available to grow *Cowdria ruminantium*.

Another essential aspect in vaccine production is the development of an appropriate method for large scale cell production. Indeed the classical vaccine approach requires production of attenuated variants of the rickettsia in a convenient mass cell culture system.

The present investigation had also this prospect as a goal. As a first step towards mass cell-culture of endothelial cells in a bioreactor, we have studied adhesion of cells and vaccine production on microspheres. A second trial was carried out in order to extend the cell systems available to grow *Cowdria ruminantium*.

MATERIAL AND METHODS

Isolation and culture of endothelial cells

Bovine endothelial cells from the brain microvasculature (BMC) were received from Dr. G. TARONE (University of Torino, Department of Biology and Medical Chemistry, Torino, Italy). Bovine endothelial cells from umbilical cord arteries were obtained from Dr. F. JONGEJAN (Utrecht University, Faculty of Veterinary Medicine, Utrecht, The Netherlands).

Human endothelial cells from the microvasculature (HEMEC) were received from Dr VAN HINSBERG (TNO, Leiden, The Netherlands). Primary cultures from human endothelial cells from the umbilical vein (HUVEC) were initiated in our laboratory as well as bovine endothelial cells from the aorta (BAEC), using essentially the same basic method. HUVEC cells were isolated from the umbilical cord by collagenase (Boehringer Mannheim, 153
Germany) digestion according to the method of Gimbrone essentially as follows. Cells pooled from three to six umbilical cords were cultured on gelatin-coated 100 mm plastic tissue culture dishes (Nunc, Denmark) in medium 199 (Gibco) supplemented with 20% foetal calf serum (Gibco), 100 mg/ml bovine brain extract, 100 mg/ml porcine heparin (Sigma), 100 Units/ml penicillin and 100 mg/ml streptomycin (M199 complete medium). Seven to eight passages could be obtained from this primary culture. For the BAEC cells, a similar method but adapted for the handling of bovine aorta was applied. In both cases, a good yield of cells was routinely obtained.

Medium BHK 21 was used for the maintenance of the cells in culture (details are described in an accompanying paper, "Inhibition of Cowdria ruminantium infectious yield by interferons alpha and gamma in endothelial cells").

For the culture on porous VERAX collagen unweighted microspheres, we adapted the standard method of the producer to the needs of endothelial cells.

### Culture of the cells on collagen microspheres

The porous collagen microsphere is the basic element of the unique fluidized-bed culture system manufactured by VERAX. It is made of collagen derived from native bovine collagen using a proprietary process. Both weighted (with a density of about 1.6 for fluidized-bed technology) and unweighted (for batch procedure) forms of microspheres are available (5). The seeding conditions were as follows: 3x10^6 of endothelial cells were incubated in conical plastic flasks containing 50 ml of medium supplemented with non-essential amino-acids, penicillin and streptomycin with 10% foetal calf serum, together with 5 ml of standard VERAX collagen microsphere suspension. To allow fixation the culture was operated first for 24 h without agitation; further incubation was performed with shaking to allow oxygenation.

### RESULTS AND DISCUSSION

#### Multiplication of Cowdria ruminantium in bovine and human endothelial cells

In all the endothelial cells used in the present study, *Cowdria ruminantium* (Senegal), was shown to replicate efficiently, including in the human cells whether of umbilical vein or microvasculature origin (not shown). In the different systems, the same kind of developmental pathway leading to the maturation of the rickettsia seems to occur. The morphology and development of *Cowdria* observed both in HUVEC and in BMC is similar (photo 1). Colonies of *Cowdria* were visible in the cytoplasm of the cells which finally lysed. However, the *Cowdria* morulae appeared to be less numerous per cell in the HUVEC compared to infected BMC.

#### Adhesion and growth of endothelial cells on porous collagen microspheres

Using unweighted porous collagen microspheres in non-agitated batch culture, a high proportion (more than 70%) of freshly trypsinized endothelial cells adhere quickly in the carriers. After a latency of about one day, in these non-agitated procedures, cell multiplication occurs first in an exponential way and then continues at

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**Photo 1**: Diff-Quick staining of *Cowdria ruminantium* in vitro in BMEC (a) and HUVEC (b), 10 days post-infection (X 735).

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a slower rate for a long period at a more or less linear fashion before reaching a plateau. The situation observed with the BAEC are presented in figure 1 as an example.

\[ \begin{align*}
&CELEL \quad 1,00E^{+07} \\
&LSEL \quad 1,00E^{+06} \\
&GSEL \quad 1,00E^{+05} \\
&0 \quad 2 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \\
\end{align*} \]

**Figure 1 : BAEC growth on VERAX unweighted porous collagen microspheres.**

**CONCLUSION**

Altogether, our results offer interesting prospects for the future development of a vaccine against cowdriosis and raise important questions concerning the possible relevance of the infection observed in human endothelial cells for human health.

The possibility to grow one isolate of *Cowdria ruminantium* in endothelial cells both of bovine and human origin that we demonstrated here, together with our previous observation that interferons and other cytokines play a role in the natural resistance against *Cowdria* (6), open new avenues to the search of attenuated variants not only for the Senegal isolate studied but for other isolates also. Systematic trials of the pathogenicity of the *Cowdria* variant obtained in cells of the heterologous species should be made.

We have also approached the question of scaling-up the production of endothelial cells in a bioreactor, and as a first step, the adhesion of BAEC on collagen microspheres is already encouraging. The differences noticed between the properties of bovine and human primary cells, indicate that specific conditions should be applied in each case and our results also indicate a need for further investigations in order to find less fragile cells for growing *Cowdria*. We are presently screening a number of different cell lines for the properties we are looking for.

The question of the relevance of the infection for human health is of particular importance in Africa since immunodeficiency conditions may affect the resistance of individuals. In AIDS related conditions, many opportunistic infections may develop that were not observed in a previous situation. In a recent survey, sera from humans exposed to vector ticks were found to be free of antibodies directed against *Cowdria ruminantium* (3). However, this does not rule out a possible immunologically silent infection already described (1), and other methods of screening should also be used in the future to adress this important question.

**REFERENCES**

1. DU PLESSIS (J.L.), LUDEMANN (C.J.F.), VAN STRYP (F.). The value of the indirect fluorescent antibody test to determine the infection rate of *Cowdria ruminantium* in cattle. Onderstepoort Newsletter , 1991, 9 (3) : 15-16.


We successfully cultivated the rickettsia *Cowdria ruminantium*, in bovine endothelial cell lines (Bovine Umbilical Endothelial Cells/BUEC and Bovine microvascular Cells/BMC) and also in primary endothelial cells of bovine origin (Bovine Aorta Endothelial cells/BAEC) and more surprisingly in cells of human origin - Human Umbilical Vein Endothelial Cells/HUVEC - and Human Endothelial Cells from the Microvasculature/HEMEC. This first evidence of the pathogenicity of this bovine rickettsia in the human cell system generates new interest as regards its possible relevance for human health. It provides also further possibilities for the attenuation of *Cowdria ruminantium* isolates, and therefore brings new prospects for vaccine preparation. In vaccine production, mass cell culture is essential. Our results indicate that endothelial cells attach efficiently on collagen microspheres. As BAEC cells grow well on them in a batch mode, and if the process could be optimized for the different cell types (using appropriate adhesion and growth factors) our observations offer interesting prospects for the future development of a *Cowdria ruminantium* vaccine production in the fluidized-bed reactor VERAX System one, which provides easy control of growth conditions.

Key words: Heartwater - Rickettsia - *Cowdria ruminantium* - Cell growth - Bovine endothelial cell - Human endothelial cell - Collagen - Vaccine.

La rickettsia *Cowdria ruminantium* se cultivó, con éxito, en células de endotelio bovino (células de endotelio umbilical bovino/BUEC y células microvasculares bovinas/BMC), así como en células de endotelio primario de origen bovino (células aórtica endotelio BAEC) y células de origen humano (células humanas de endotelio de la vena umbilical/HUVEC y células de endotelio microvascular humano/HEMEC). Esta primera evidencia de la patogenicidad de la rickettsia bovina sobre el sistema celular humano, presenta un nuevo interés en cuanto a su posible importancia para la salud humana. También provee otras posibilidades para la attenuación de los ailamientos de *Cowdria ruminantium* y nuevas perspectivas para la preparación de vacunas. El cultivo cellular en masa es esencial para la producción de vacunas. Nuestros resultados indican que las células endoteliales atacan en forma eficiente en las microesferas de colágeno. En vista de que las células BAEC crecen correctamente en grupos en esta materia y siempre y cuando el proceso se mejore para los diferentes tipos celulares (mediante el uso apropiado de factores de crecimiento y de adhesión), nuestras observaciones representan una perspectiva interesante para el desarrollo futuro de la producción de la vacuna contra *Cowdria ruminantium*, en una capa fluida del agente reactor del Sistema uno VERAX, el cual presenta facilidades en las condiciones de crecimiento.

Palabras claves: Cowdriosis - Rickettsia - *Cowdria ruminantium* - Cultivo de célula - Célula endotelial bovina - Célula endotelial humana - Colágeno - Vacuna.