Ph. Totté [1]

D. Blankaert [1]

P. Zilimwabagabo [1]

J. Wérenne [1]

Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells

TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibition de l'infectiosité de *Cowdria ruminantium* dans des cellules endothéliales par les interférons alpha et gamma. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

Une corrélation positive entre la résistance de bovins à l'infection par *Cowdria* et la production précoce d'IFN a été démontrée auparavant. Les études *in vitro* rapportées ici ont montré une activité de rBoIFNα2C et de rBoIFNγ contre *Cowdria* dans des cellules endothéliales bovines de la microvasculature cérébrale (BMEC). Le rBoIFNγ est beaucoup plus actif que le rBoIFNα2C. Ces résultats suggèrent un rôle des IFNs dans la résistance contre la maladie. Étonnamment, dans les mêmes conditions le rBoIFNα2C n'a pas d'effet sur la production de *Cowdria* infectieuses dans des cellules endothéliales bovines originaires de l'artère ombilicale (BUEC). Il a déjà été démontré que le HuIFNα n'a pas d'effet sur la multiplication de *Cowdria* dans des cellules endothéliales ombilicales humaines. Nous n'avons pas trouvé de différence dans la capacité de cellules BUE et BME à fixer le rBoIFNα2C. Ceci pourrait indiquer une véritable différence entre les capillaires et les grands vaisseaux sanguins.

Mots clés : Bovin - *Cowdria ruminantium* - Résistance aux maladies - Culture de cellule - Cellule endothéliale bovine - Interféron.

INTRODUCTION

Cytokines are a family of proteins which are synthesized by the cells of vertebrates in response to a wide variety of stimuli. They have pleiotropic effects and act both in an autocrine and paracrine way (3). There are more than 40 different cytokines known so far which interact in a very complex and still obscure manner to orchestrate the body's immune response. The understanding and mastering of the cytokine network would enable us to control immunity and that is one of the great challenges of the future. Of course, there is still much work to do but we are beginning to get a glimpse at this very fine equilibrium between cytokines which makes the immune response beneficial or detrimental for the host. In this perspective, the study of the role played by cytokines in a new model, e.g. *Cowdria ruminantium* infections, is of considerable fundamental interest.

From a more practical point of view, cytokines may help to explain the mechanisms involved in the protective immune response against *Cowdria* and in the pathogenesis of cowdriosis which in turn would be of help in the search for a safe, easy to use and efficient vaccine against this disease. Our group has started an extensive study on the involvement of interferons (IFN α and γ), interleukins (IL-1 and IL-6) and tumor necrosis factor (TNF) in *Cowdria ruminantium* infections. We have shown (11) that cattle that resisted experimental infection with the rickettsia produced significant level of circulating IFN whereas animals that died did not. IFNs were first known for their antiviral activity but have been shown both *in vitro* and *in vivo* to have antirickettsia (2) and antichlamydia (8) properties. In this report we demonstrate for the first time that IFN a and g are capable of inducing, *in vitro* an anticowdria state in the cells treated with subsequent reduction of the yield of infectious *Cowdria* organisms. The possible role of IFNs in the resistance of cattle against *Cowdria* infection is discussed.

MATERIAL AND METHODS

Isolation and culture of cells

Bovine endothelial cells from the brain microvasculature (BMEC) were a kind gift of Dr. G. TARONE (University of Torino, Department of Biology and Medical Chemistry, Italy). Bovine endothelial cells from umbilical cord arteries were a kind gift of Dr. F. JONGEJAN (The Netherlands). These cells were grown in BHK-21 (Glasgow modification) supplemented with 10 % foetal calf serum, penicillin (100 U/ml), streptomycin (100 µg), fungizone and L-Glutamine (2 mM). BMEC and BUEC at passage 20 were used in this study. Primary cultures of human endothelial cells from the umbilical vein (HUVEC) were initiated in our laboratory (for further details see accompanying paper "Bovine and human endothelial cells growth on collagen microspheres and their infection with the rickettsia *Cowdria ruminantium*", p. 153). HUVEC at passage 3 to 7 were used in this study.

Cowdria cultivation *in vitro*

The Senegal stock of *Cowdria* was given to us as sucrose-phosphate-glutamate (SPG) cryopreserved stabilates by Dr. F. JONGEJAN. The cultivation of *Cowdria* was identical for all endothelial cells and was done as previously described for BUE cells (4). Briefly, the cells were

1. Animal Cell Biotechnology, Faculty of Sciences, Interfaculty Section of Agronomy, Université Libre de Bruxelles, Belgique.

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grown to confluency in 80 cm² tissue culture flasks (Nunc). The cells were infected using SPG cryopreserved stabilates of *Cowdria* diluted in complete growth medium (see isolation and culture of cells) supplemented (subsequently reported as "infection medium") with tryptose phosphate broth (Gibco) at 2.9 g/l and hepes buffer (20 mM) (pH 7.0-7.2). The cells were incubated in a 5 % CO_2 - 37 °C incubator (no rocking platform was used). When more than 70 % of the cells were lysed, the culture supernatant was centrifuged for 15 min at 1,500 g; the pellet obtained from 2 ml of supernatant was resuspended in 1 ml SPG buffer before snap freezing in liquid nitrogen. Stabilates of *Cowdria* at passage 9 to 11 (with an incubation period of about 10 days between each passage) were used in this study.

IFNs, IFN assay, anti-IFN antibodies

Recombinant Bovine Interferon alpha C (rBoIFNαC, specific activity 2.10^8 U/mg) was cloned and expressed in *E. coli* (provided by Dr. A. SHAFFERMAN, Israel Institute of Biological Research, Ness-Ziona). Recombinant Bovine Interferon gamma (rBoIFNγ, specific activity 2.5 10^6 U/mg) and neutralizing antibodies to BoIFN α and γ were kindly given to us by Dr. R. STEIGER from CIBA-GEIGY. Recombinant human interferon alpha 2 (rHuIFNα2, specific activity 10^8 IU/mg) was a gift of Dr. C. WEISSMANN from Zürich University. The antiviral activity of all IFNs was estimated before use on Madin Darby bovine kidney cells (MDBK) using the classical test of reduction of the cytopathogenicity of Vesicular Stomatitis Virus (10). The antiviral activity is expressed in laboratory units, no international standards being available as reference for bovine IFNs. One unit of antiviral activity is defined as the reciprocal of the dilution that gives 50 % protection against viral challenge under standard conditions.

Anti-*Cowdria* activity of interferons *in vitro*

We have studied the effect of IFNs on the infectious yield of *Cowdria ruminantium*. It was done by titrating the infectivity of supernatants collected from *Cowdria*-infected cells (in the presence or absence of IFN) using a Tissue Culture Lethal dose 50 % (TCLD50) test we have established (see below). Supernatant freshly collected from an 80 cm² flask, showing 70 % lysis, or more, due to *Cowdria* infection, was used as inoculum. The infected supernatant was centrifuged 5 min at 300 g to remove cell debris and diluted 2-fold in "infection medium" (see *Cowdria* culture *in vitro*). 500 µl of the diluted supernatant was added to confluent BME cells in a 24-well plate at 37 °C-5 % $C0_2$. After 24 hours incubation the medium was renewed. At day five post-infection 500 µl of fresh medium was added to the wells. IFNs were added at different times (see results) and the medium was replaced 24 h after every treatment. Control cells, IFN-treated but not infected, and infected but not treated, were included in each experiment. In order to demonstrate the involvement of BoIFN α and γ, these IFNs were incubated together with their respective neutralising antibodies (20 min at 37 °C) before addition to the cells and compared to IFN alone. The progress of the infection was followed daily by light microscopy. Supernatants were collected in all wells at day 9 post-infection. Each supernatant was centrifuged at 15,000 g for 15 min and the pellet was resuspended in 100 µl SPG before snap freezing in liquid nitrogen. The infectivity of these supernatants was measured using the tissue culture lethal dose 50 % (TCLD50) assay described below.

TCLD50 assay for *Cowdria ruminantium* titration

BME cells were grown to confluency in 96-well plates (Nunc). *Cowdria* samples to be tested and cryopreserved in SPG were thawed at 37°C, serially diluted 2-fold in "infection medium" with a final volume of 100 µl/well and added to the cells. The medium was replaced 24 h after infection and from there on one half of it was replaced every four days. The plates were incubated 15 days in a 37 °C-5 % CO_2 incubator. After incubation the cells were fixed in formaldehyde for 30 min and then stained with crystal violet. The end point (50 % lysis) can be determined using a light microscope or by measuring the absorbance (A_{540}). The TCLD50 of the sample is the reciprocal of the dilution that gives 50 % lysis of the cells after 15 days of incubation. The TCLD50 test measures the infectivity of the samples which depends on the concentration in *Cowdria* elementary bodies. An example of *Cowdria* titration is shown in fig. 1.

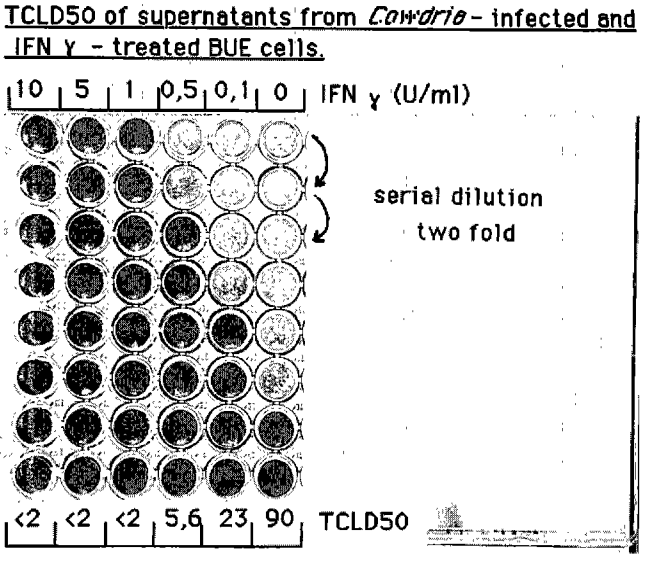

Figure 1 : Illustration of a Cowdria *titration using the TCLD50 method. BUE cells were IFN treated at day 0 and 1. Supernatants were collected at day 9 post-infection.*

2-5A synthetase assay

2-5A synthetase activity was assayed in the cytoplasmic fraction of the cells according to a method described elsewhere (see accompanying paper "Role of Interferons in infectious diseases in the bovine species").

RESULTS

Kinetics of *Cowdria* yield as measured by the TCLD50 method

The development of *Cowdria* (followed by light microscopy) in BME cells in the absence of IFN was similar to observations previously reported with BUE cells (5). Non-fusing colonies (morulae) of *Cowdria* were detected in the cytoplasm of the cells as soon as day four after infection. Cell lysis (with release of infectious organisms) occurred at day five and progressively increased until complete destruction of the monolayer by day 10 - 11 post-infection. The infectious yield of *Cowdria* was not detected by the TCLD50 assay before day 7 post-infection (fig. 2). Thereafter the yield rapidly increased and reached a peak at day 9, which corresponded to more or less 80 % cell lysis, and then slowly decreased (fig. 2).

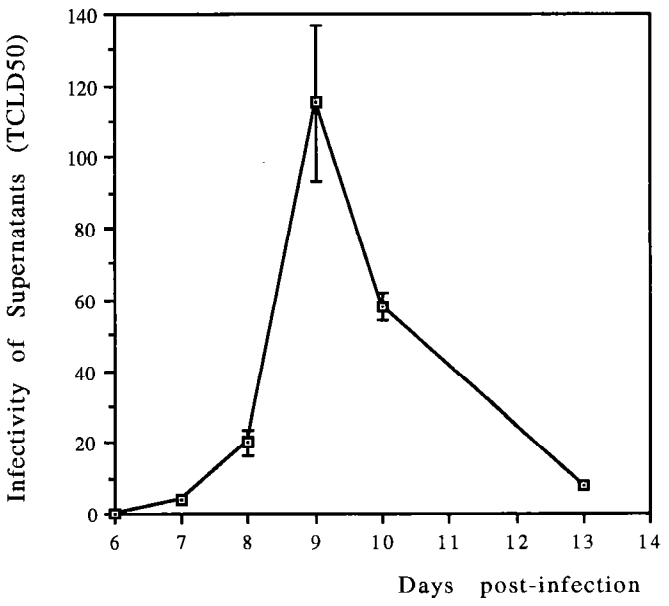

Figure 2 : Kinetics of Cowdria *yield as measured by the TCLD50 assay in the supernatants of infected BME cells. Each point represents the mean value of three different wells in the same experiment, bars indicate standard deviation .*

Effect of IFNs α and IFN γ on the infectious yield of *Cowdria* in endothelial cells

We found that the infectivity of supernatant collected from *Cowdria*-infected BME cells was significantly reduced when rBoIFNαC was added to the medium. The inhibitory effect was dependent on the dose of rBoIFNαC and completely blocked by anti-rBoIFNα antibodies (fig. 3). The number of colonies was significantly reduced in the IFN-treated cells (not shown) but a few morulae were still visible even at the highest IFN concentration and ultimately lead to a complete destruction of the monolayer. rBoIFNαC was not cytotoxic for the cells in the presence or absence of *Cowdria*.

Strangely enough, in the same experimental conditions, rBoIFNα C had no anti-*Cowdria* activity on BUE cells nor did rHuIFNα2 on HUVEC (fig. 3).

rBoIFNγ was found to strongly reduce the infectious yield of *Cowdria* in both BME and BUE cells (fig. 4). The inhibitory effect was completely reversed by anti-rBoIFNγ. In contrast with rBoIFNαC, complete protection of the cells was easily achieved with rBoIFNγ . When the cells received 10 U/ml of rBoIFNγ at day 0 and 1 no colonies were observed in these cells for up to 30 days post-infection (reinfection was not tested). Cytotoxicity of rBoIFNγ for uninfected BME and BUE cells was observed but only when 50 U/ml or more were added to the medium for three consecutive days.

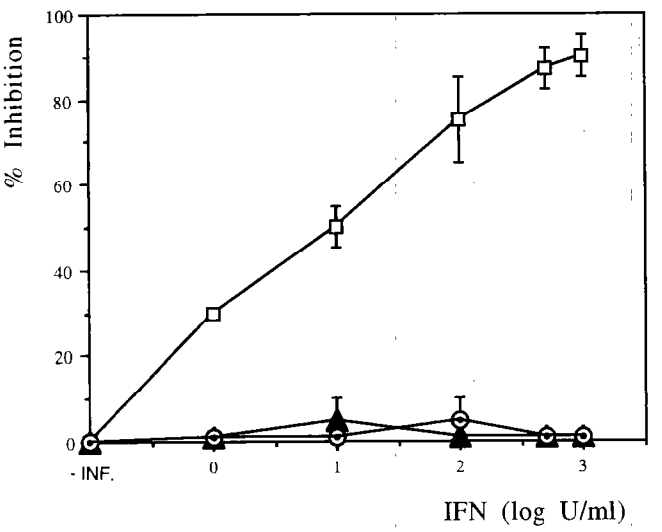

Figure 3 : Inhibitory effect of IFNα on the yield of Cowdria. *BME (□), BUE (O) and HUVEC (Δ) cells were treated with IFN of the homologous species at day 0, 1 and 2. A control with anti-IFN alpha antibodies (•) was included for BME cells. Each point represents the mean value of three different experiments, bars indicate standard deviation.*

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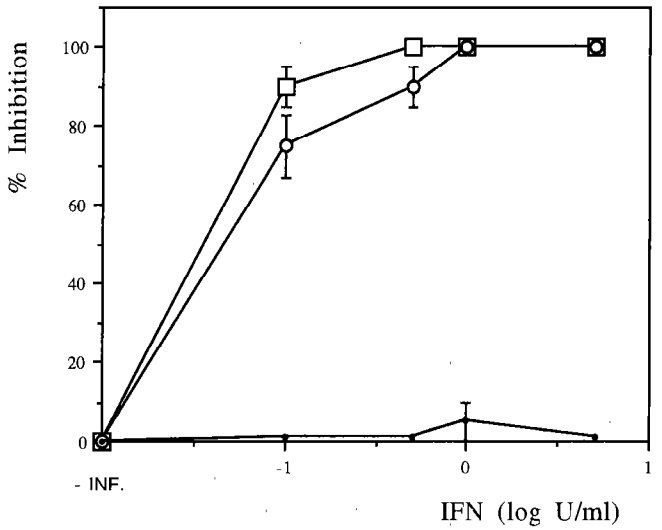

Figure 4 : Inhibitory effect of IFNγ on the yield of Cowdria. *BME (□) and BUE (O) cells were IFN treated at day 0 and day 1. A control with anti-IFN gamma antibodies (•) was included for BME cells. The experiment was run in triplicate, bars indicate standard deviation .*

Comparison of Antiviral, 2-5A synthetase and cowdriacidal activity of rBoIFNαC in BME and BUE cells

In order to investigate the possibility that IFN-induced activities other than the cowdriacidal activity could also differ from one cell type to another, we compared the antiviral and 2-5A synthetase (an enzyme of which synthesis is induced by IFNs, 10) activities of rBoIFNαC in BME and BUE cells. We found (table I) that these cells have the same sensitivity to the antiviral effect of rBoIFNαC and that the small difference observed in the 2-5A synthetase activity can not explain the difference in the activity against *Cowdria*.

TABLE I Antiviral, 2-5 A synthetase and anticowdria activity of rBoIFNα in BME and BUE cells.

Cell type	Antiviral activity (U/ml)	2-5A synthetase activity (a)	% reduction of *Cowdria* yield
BUEC	100 500	219 (± 9) 242 (± 16)	0 0
BMEC	100 500	157 (± 27) 288 (± 16)	75 (± 10) 90 (± 5)

(a) : pmoles ATP polymerized/ µ g protein/h. (standard deviation)

Kinetics of rBoIFN (α and γ) induction of an anticowdria state in BME cells

The inhibitory effect was highest (fig. 5) when both IFNs were present on day one (when *Cowdria* organisms have been removed from the medium) which demonstrates that IFNs act through the host cells and not directly on the free organisms. The anticowdria state induced by rBoIFNαC in BME cells has a very short life time compared to that of rBoIFNγ. Pretreatment of the cells with rBoIFNαC did not affect the yield of *Cowdria*. In contrast, an anticowdria state could be induced in BME cells by addition of rBoIFNγ as soon as two days before infection.

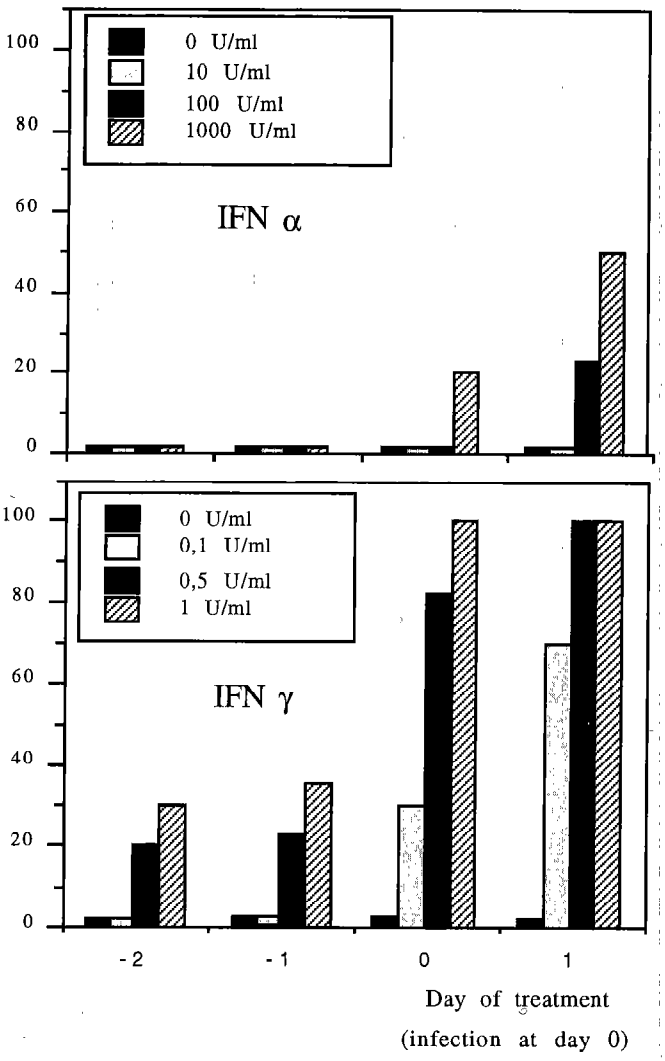

Figure 5 : Kinetics of rBoIFN (α and γ) induction of an anticowdria state in BME cells. IFNs were added to the cells at various times relative to infection (as indicated on the figure). Cowdria *yield (TCLD50) was determined at day 9 after infection (one-step-growth-yield assay). Two repetitions of this experiment yielded similar results.*

When both IFNs anticowdria activities are compared in experimental conditions that give maximal inhibition (e.g. day 1) rBoIFNγ appears 10,000 times more efficient than rBoIFNαC.

DISCUSSION AND CONCLUSION

We have shown that rBoIFNαC has the property to induce *in vitro* an anticowdria state in BME cells. These results, together with previous data showing that IFN was produced in the plasma of animals that resisted an experimental infection with the rickettsiale (11), suggest that IFNα plays a role in the resistance of cattle to cowdriosis. However, rBoIFNαC has no prophylactic effect *in vitro*. Moreover, complete inhibition of *Cowdria* growth in BME cells was never achieved even at high IFN concentration. Therefore, IFNα may be very useful *in vivo* to slow down the infection allowing other mechanisms to take place in order to ensure survival of the infected animals. One possibility is that IFNγ is also produced in response to the infection. We have shown here that rBoIFNγ is a very powerful anticowdria agent *in vitro* but it remains to be demonstrated that it is also produced *in vivo*.

The mechanisms underlaying the IFNs-induced anticowdria activity *in vitro* are not known. The anticowdria activity of rBoIFNαC is undoubtedly dissociated from its antiviral and 2-5A synthetase inducing activities. We have shown that IFNs act on the cells to render them unsuitable for *Cowdria* growth but only electronic microscopy will tell us at which stage of the *Cowdria* replication cycle (fixation, transformation of elementary bodies to reticulate bodies, metabolism of reticulate bodies, etc.) IFNs actually intervene. It has been shown that degradation of tryptophan in the case of *Chlamydia trachomatis* (6) and metabolism of L-arginine in the case of *Ehrlichia risticii* (9) are among the possible pathways involved in the *in vitro* effect of IFNγ. The possible role played by amino acids in our model is under study.

We have found that, in contrast to BMEC, BUE cells were not sensitive to the anticowdria effect of rBoIFNαC. Differences in receptor to BoIFNα are unlikely to be the cause here since in both type of cells rBoIFNαC has similar antiviral and 2-5A synthetase activities. This may reflect a true difference in cell type between capillary and large blood vessels. Endothelial cells isolated from large vessels and capillaries have indeed been shown to differ in the concentration of insulin receptors (1) and in their collagen secretory phenotypes (7). On the other hand, the difference we observed may result from cell isolation and initial culture conditions, in which case, we should not observe the same phenomenom in primary cultures. We know already that HUVEC is insensitive to IFNα- mediated anticowdria activity. We have now undertaken a study in order to compare the anticowdria activity of IFNα in primary cultures of human endothelial cells from the macrovasculature (HUVEC) and the microvasculature (endothelial cells of the human foreskin-HEMEC). In both cell types *Cowdria ruminantium* multiplies efficiently (unpublished data). However, endothelial cells from capillaries and from large blood vessels may very well respond differently to *Cowdria* infection in a way which may be relevant to the immunity and pathogenesis of cowdriosis.

ACKNOWLEDGEMENTS

Special thank to Dr. F. JONGEJAN who provided us with *Cowdria ruminantium* stabilates and useful advice. J. WÉRENNE and Ph. TOTTÉ wish to dedicate this paper to the late Pr. I. SAROV with whom they started a collaboration a few years ago.

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TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

We have shown before that there is a positive correlation between resistance of cattle against *Cowdria* infection and early IFN production. Our *in vitro* studies demonstrated an activity of rBoIFNα2C and rBoIFNγ against *Cowdria* in bovine endothelial cells of brain microvasculature (BMEC). rBoIFNγ is much more active in this respect than rBoIFNα2C. These results suggest a role of IFNs in the resistance against the disease. Strikingly, in the same conditions rBoIFNα2C has no effect on the yield of *Cowdria* from infected bovine endothelial cells of umbilical artery origin (BUEC). Similarly we showed that HuIFNa had no effect on the multiplication of *Cowdria* in human vein umbilical endothelial cells (HUVEC). We found no differences in the capacity of BUE and BME cells to bind rBoIFNα2C. This may reflect a true difference between capillary and large blood vessels.

Key words : Cattle - *Cowdria ruminantium* - Disease resistance - Cell culture - Bovin endothelial cell - Interferon.

TOTTÉ (Ph.), BLANKAERT (D.), ZILIMWABAGABO (P.), WÉRENNE (J.). Inhibición de las infecciones por *Cowdria ruminantium* mediante el interferón alfa y gama en células endoteliales. *Revue Élev. Méd. vét. Pays trop.*, 1993, **46** (1-2) : 189-194

Anteriormente se demostró la existencia de una correlación positiva entre la resistencia del ganado contra la infección por *Cowdria* y la producción temprana de IFN. Nuestros estudios *in vitro* demuestran una actividad de rBoIFNα2C y rBoIFNg contra *Cowdria* en las células endoteliales bovinas de los microcapilares cerebrales (BMEC). El rBoIFNγ es mucho más activo que rBoIFNα2C. Estos resultados sugieren un posible papel del IFNs en la resistencia contra la enfermedad. Sorprendentemente, bajo las mismas condiciones, el rBoIFNα2C no actuó sobre *Cowdria* en las células de endotelio bovino infectado, provenientes de la arteria umbilical (BUEC). Así mismo, se desmostró que el HuIFNα no actúa en la multiplicación de *Cowdria* en las células de endotelio de lavena umbilical humana (HUVEC). No se encontraron diferencias en cuanto a la capacidad de unión de BUE y de BME con rBoIFNα2C, lo cual podría reflejar una diferencia entre los grandes vasos sanguíneos y los capilares.

Palabras claves : Bovino - *Cowdria ruminantium* - Resistancia a la enfermedad - Cultivo de celulas - Célular endotelial bovina - Interferón.