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Original article

In vitro effect of classical swine fever virus on a porcine aortic endothelial cell line

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Abstract – The effect of classical swine fever (CSF) virus on some phenotypic and functional features of an established porcine aortic endothelial cell (AOC) line was investigated. AOC cells show most of the characteristics of primary endothelial cells, avoiding the alterations and senescence that these cells undergo after a few passages in culture. AOC cells were susceptible to CSF virus infection to a high degree, reaching 90% of CSF virus positive cells after 24 h of infection; however as with other porcine susceptible cells, no cytopathic effect could be observed. In these conditions none of the surface molecules studied, including SLA-II MHC antigens, adhesion or co-stimulatory molecules, were altered by virus infection after 24 or 48 h. Functionally CSF virus infection induced a decrease in the pro-coagulant activity of the AOC cells, determined by the increase in the clot formation time shown by the lysates of these cells. This contrasts with the increase observed in the expression of mRNA corresponding to IL-1α and IL-6, two proinflammatory and pro-coagulant cytokines, in CSF virus-infected AOC cells.

classical swine fever / endothelial cells / cytokines / pro-coagulant activity

1. INTRODUCTION

Classical swine fever (CSF) is a major viral disease of domestic swine caused by a small enveloped RNA virus classified as a member of the *Pestivirus* genus within the Flaviviridae family [10]. The onset of widespread hemorrhages is one of the main characteristics of this disease, although the pathogenic mechanisms that are involved are not well understood. Porcine endothelial cells are susceptible to CSF virus infection, however the situation in vivo is not clear. The virus does not induce a cytopathic effect but during the infection course there is swelling of these cells due to their hydropic degeneration [22]. Therefore indirect immune and/or inflammatory mechanisms must be involved in the increased endothelium permeability and hemorrhages produced during the pathogenic process.

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The vascular endothelium plays a critical role in the regulation of coagulation through the constitutive expression and release of anticoagulants and pro-coagulant substances. Endothelial cells show important endocrine and exocrine functions, being able to respond to different stimuli by secreting substances with powerful effects [4, 26]. Cytokines released by immune and inflammatory cells and by the endothelial cells can also activate the endothelium and take part in different mechanisms of pathogenesis. In other hemorrhagic infections, such as the African swine fever or Dengue, involvement of endothelial cells in pathogenesis has been demonstrated [14, 23].

Most studies related with endothelial cells in CSF reported until now have shown a histopathological point of view [5, 18, 20]. A recent study performed in vitro with porcine primary endothelial cell cultures has shown that the replication of CSF activates cell inflammatory responses but at the same time suppresses IFN production and apoptotic pathways [3]. The availability of an immortalized porcine aortic endothelial cell line (AOC cells) [6] prompted us to investigate the effect of the CSF virus infection on the expression of swine relevant markers, cytokines and procoagulant activity. AOC cells maintain most phenotypical and functional characteristics of primary endothelial cell cultures and constitute a useful model for the study of CSF pathogenesis in vitro. In our experiments, infection was determined by flow cytometry, using monoclonal antibody (mAb) HC26 that recognizes the E2 protein of the virus [12].

2. MATERIALS AND METHODS

2.1. Virus

The CSFV strain Brescia, obtained from Dr A. Summerfield (Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland), was grown in the porcine kidney cell line PK-15, cultured in Eagle minimum essential medium (EMEM, Bio-Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS). The cultures were infected at a multiplicity of infection (moi) of 0.01. At 72 hpi, the culture flasks were submitted to three freeze-thaw cycles and the resulting lysates were centrifuged at 3 000 *g* for 10 min in order to eliminate cellular debris. As the control, mock infected cells were processed in the same way and UV-inactivated virus was prepared by exposing the lysates to a Sylvania G15T8 UV lamp at a distance of 10 cm for 20 min. Elimination of infectivity was tested by titration of the irradiated virus on PK-15 cells. Virus titers were calculated and expressed as TCID-50/mL [19]. The assessment of viral infection was performed by indirect immunofluorescence using mAb HC26 against the CSF virus glycoprotein E2 [12].

2.2. Cells

AOC cells, immortalized by transfection with a plasmid containing the complete genome of SV40 [6], were kindly provided by Dr Yelamos (Virgen de la Arrixaca Hospital, Murcia, Spain). These cells were plated onto flasks coated with 1% gelatin (Difco, USA) and cultured in RPMI 1640 (Bio-Whittaker, Verviers, Belgium), supplemented with 20% FCS and 2 mM L-glutamine.

2.3. Antibodies

Mabs to porcine leukocyte antigens used in this study are summarized in Table I. Mab 74-11-10 to porcine MHC class I antigens (SLA-I) were kindly provided by Dr J. Lunney (USDA, Beltsville, MD, USA), to CD29 (UCP1D2) by Dr F. Zuckermann (University of Illinois, Urbana, IL, USA), to CD61 (JM2E5) by Dr D. Llanes (University of Cordoba, Cordoba, Spain). Mab to CD80/86 (CTLA4-Ig) was purchased from Ancell (Bayport, MN, USA) and to porcine CD31 (LCI-4) from Serotec (Oxford, United Kingdom). Mab HC26 against the E2 protein of the CSF virus, a generous gift of Dr A. Summerfield (Institute of Virology and

Table I. Marker expression on AOC cells.

mAb	Antigen/cell characterizat.	24 h				48 h			
		Non-infected		Infected		Non-infected		Infected	
		%	MFI	%	MFI	%	MFI	%	MFI
74-11-10	SLA-I	90 ± 3	79 ± 14	91 ± 5	73 ± 18	88 ± 7	101 ± 18	91 ± 4	100 ± 19
BA1C11	SWC3	79 ± 7	23 ± 5	82 ± 5	20 ± 3	67 ± 9	26 ± 7	79 ± 8	33 ± 9
BA4B12	mono/MF	97 ± 2	96 ± 20	95 ± 2	95 ± 23	93 ± 4	117 ± 15	92 ± 6	106 ± 26
BA1H1	mono/MF	81 ± 1	49 ± 15	77 ± 4	48 ± 15	85 ± 4	37 ± 8	79 ± 15	23 ± 3
5A6/8	activation	91 ± 3	37 ± 7	87 ± 6	37 ± 7	86 ± 8	33 ± 6	86 ± 8	39 ± 10
2C12/10	myelomonoc.	75 ± 12	80 ± 18	68 ± 6	65 ± 16	48 ± 16	72 ± 9	41 ± 16	73 ± 3
UCP1D2	CD29	99 ± 1	95 ± 23	93 ± 4	80 ± 22	95 ± 2	88 ± 11	79 ± 12	81 ± 17
1C5	CD46	97 ± 1	138 ± 18	98 ± 1	155 ± 30	90 ± 5	154 ± 49	95 ± 4	175 ± 73
JM2E5	CD61	86 ± 9	80 ± 16	86 ± 9	80 ± 16	87 ± 8	79 ± 3	90 ± 6	71 ± 6
4C5/CR3	activation	85 ± 10	58 ± 20	87 ± 6	63 ± 20	80 ± 11	43 ± 17	77 ± 16	41 ± 21
CTLA4-Ig	CD80/86	94 ± 4	49 ± 16	86 ± 6	54 ± 21	94 ± 4	52 ± 13	84 ± 9	47 ± 12
LCI-4	CD31	94 ± 2	100 ± 30	95 ± 3	112 ± 36	89 ± 6	135 ± 41	80 ± 19	165 ± 64

Expression of different cell surface antigens on AOC cells infected or not with the CSF virus (moi 4) after 24 and 48 hpi in the conditions described in the "Materials and methods". The values are given as the percent of positive cells and mean fluorescence intensity (MFI) and represent the mean ± SD of 6 independent experiments.

Immunoprophylaxis, Mittelhäusern, Switzerland), was labeled with Alexa Fluor 488 dye, following the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). The rest of the mAbs used in this study were produced in our lab.

2.4. FACS analysis

AOC cells were detached from plastic with Trypsin-EDTA (Bio-Whittaker, Verviers, Belgium), washed with phosphate buffered saline (PBS), containing 0.1% bovine serum albumin and 0.01% sodium azide (fluorescence buffer, FB) and incubated in a V-shaped 96-well microplate (3×10^5 cells/well) with 50 µL of hybridoma supernatants for 30 min on ice. After two washes in FB, the cells were incubated for 30 min at 4 °C with 50 µL of FITC-conjugated rabbit $(Fab')_2$ anti-mouse Ig (Dako, Glostrup, Denmark) diluted 1/40 in FB. The cells were washed three times in FB and fixed in 0.3% paraformaldehyde prior to analysis in a Facscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). When the cells were permeabilized, they were treated with 200 µL of Permeafix (Ortho, Raritan, NJ, USA) diluted 1:2 in PBS, and after 50 min at room temperature, they were washed twice with FB before the addition of specific mAbs.

2.5. RT-PCR analysis for cytokine mRNA expression

Cytokine mRNA expression was evaluated as previously described [11, 15]. Total RNA was isolated using the Tripure Isolation Reagent (Roche, Basel, Switzerland), based on the method described by Chomczynski and Sacchi [9]. First strand cDNA was prepared from 10 µg of total RNA, previously denatured by heating 2 min

Table II. Primers used in PCR.

Primer		Sequence	Product size
IL-1α	5'	CTCTGAATCAGAAGTCCTTCT	566 bp
	3'	ATGGTTTTGGGTGTCTCAGGC	
IL-6	5'	CTGAACCTTCCAAAAAATGGCAG	318 bp
	3'	CAGGATGAGAATGATCTTTGTG	
IL-8	5'	AGCCCGTGTCAACATGACTTCC	357 bp
	3'	AGCCACGGAGAATGGGTTTTTG	
MIP-1β	5'	GCTCAGTTCAGTTCCAAGTC	283 bp
	3'	ACCATGAAGCTCTGCGTGAC	
G3PDH	5'	TGATGACATCAAGAAGGTGGTGAAGC	240 bp
	3'	TTACTCCTTGGAGGCCATGTGGGCCA	

All primers were designed from the corresponding porcine sequences, except for the G3PDH primers that were designed from the highest homology segments of humans, mice, rats and rabbits. Specificity was checked with the corresponding cloned cDNA when available.

at 65 °C and immediately placed on ice with 5 µL of RT reaction mix containing 10 × M-MLV RT buffer (Epicentre, Madison, WI, USA), 10 mM DTT, 0.5 mM oligodT, 50 µM each of dNTP, 12.5 U M-MLV RT, 20 U RNasin (Promega, Madison, WI, USA). The reaction mixtures were made up to 50 µL with RNAse free water and incubated for 1 h at 37 °C. For PCR, a variable amount of the cDNA (usually 2.5 µL) was used in a total volume of 25 µL of PCR mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.005% Tween 20, 0.005% (w/v) NP-40, 0.5 to 1 mM $MgCl_2$ (depending on the oligonucleotide pair), 50 µM each of dNTP, 10 pmol each of specific oligonucleotides (forward and reverse primers), and 1 U Dnazyme II DNA Polymerase (Finnzymes Oy, Espoo, Finland). PCR amplification was carried out for 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 90 s and extension at 72 °C for 2 min. PCR products were electrophoresed on 2% agarose gels containing ethidium bromide and analyzed by an image densitometer coupled to a computer program (TDI, Gelstation, Madrid, Spain). Under these conditions the intensity of the amplified bands was proportional to the amount of cDNA templates (data not shown). The primer sequences and the expected fragment sizes in base pairs are shown in Table II. The G3PDH expression was used as a reference and control for RNA content and integrity.

2.6. Pro-coagulant activity (PCA)

For this assay AOC cells were infected with the CSF virus at an moi of 4, incubated with an equivalent amount of UV-inactivated virus or with a medium, and then cultured in 12-well plates for 48 h. When the cells were stimulated, culture medium was withdrawn and 0.6 mL of PBS containing 1 µg of LPS was added to the cultures and maintained for 4 h at 37 °C. Then the supernatants were withdrawn and the cells were broken by three freeze-thaw cycles. The PCA was determined in the resulting lysate following a modification of the method described by Allavena et al. [1]. The lysate sample (0.2 mL) was placed in a pre-warmed plastic tube together with 0.2 mL of normal swine plasma. The reaction was triggered by the addition of 0.2 mL of 25 mM $CaCl_2$ and clot formation was monitored at different times in a Shimadzu 1240 spectrophotometer (Kyoto, Japan) at a wavelength of 600 nm.

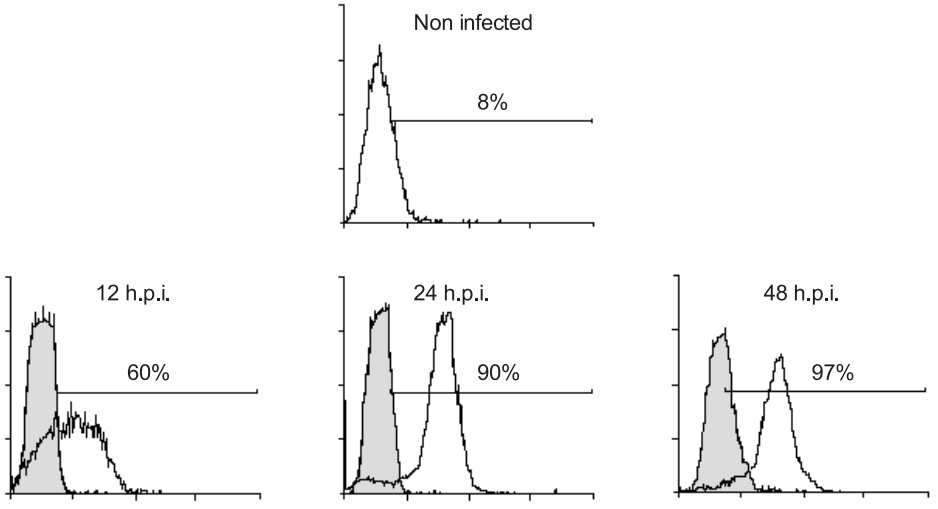

Figure 1. Flow cytometry of AOC cells infected by the CSF virus. AOC cells were infected with the CSF virus at a moi of 4 and at the times showed in the figure they were detached from plastic and permeabilized as described in the "Materials and methods". Then the cells were incubated with mAb HC26 against the E2 glycoprotein of CSF virus labeled with Alexa 488. The figure shows a representative of four independent experiments. The grey histograms represent the infected cells stained with an irrelevant mAb.

2.7. Statistical analysis

Comparisons of mean differences was done using the student t-test. Differences were considered significant if $p < 0.05$.

3. RESULTS

3.1. Infection of AOC cells with classical swine fever virus

We first investigated the susceptibility of AOC cells to infection by the CSF virus. Since this virus does not induce a cytopathic effect, infection was revealed by the use of a mAb directed against the E2 protein of the virus. This protein is not expressed on the cell membrane, so it was therefore necessary to permeabilize the cells. Several experiments were carried out in order to establish optimal infection conditions. When using a moi of 4, CSF-virus infection of the AOC cells progressed clearly. After 12 h, 60% of the cells were positive for the CSF-virus antigen. This percentage increased with time to 97% of the infected cells found at 48 hpi (Fig. 1); however, no morphological changes could be observed in infected cells by light microscopy.

3.2. Modulation of cell markers by CSF infection

The effect of virus infection on the expression of different surface antigens on AOC cells, including class I MHC antigens, molecules involved in adhesion of lymphoid cells to the endothelium (CD29, CD31, CD61), co-stimulatory molecules such as CD80/86 and other molecules recognized by mAbs generated in our laboratory (Tab. I). Some of these mAbs recognize activation antigens (5A6/8 and 4C5/CR3) or molecules also expressed by cells of the monocyte/macrophage or the myelomonocytic lineages (BA4B12, BA1H1, 2C12/10). The expression of these molecules was determined at 24 and 48 hpi and was compared

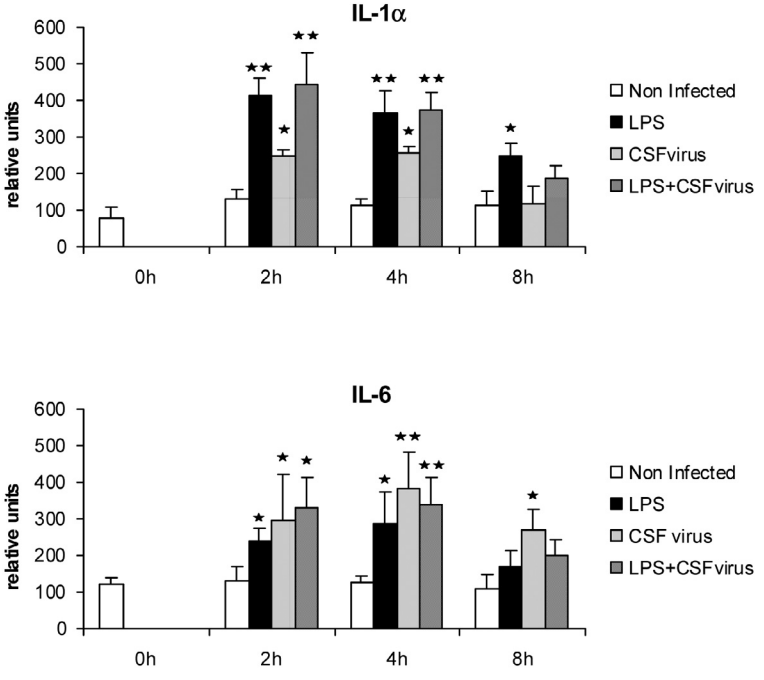

Figure 2. Effect of the CSF virus on the expression of cytokine mRNA. AOC cells were stimulated with 2 μg/mL of LPS or medium after being infected with CSF virus (moi of 4) or left uninfected. At the times indicated, RNA was purified, reverse transcribed, and amplified by PCR as described in the "Materials and methods". The data correspond to the ratio of the densitometry units of IL-1α and IL-6 to those of G3PDH bands × 100 and are expressed as relative units. The values represent the mean ± SD of 5 independent experiments. * $p < 0.05$; ** $p < 0.01$ compared with non-stimulated uninfected cells, as determined by the Student t-test.

with that of non-infected cells. Although more than 95% of the cells were infected, none of the markers studied underwent significant changes in their expression at any time, nor in their intensity of fluorescence (MFI) nor in the percentage of positive cells (Tab. I).

3.3. Effect of virus infection on cytokine mRNA expression

Comparative RT-PCR analyses were performed on RNA isolated from AOC cells that had been cultured in the presence of CSF virus (moi of 4) and/or stimulated with LPS (2 μg/mL). Total RNA was extracted at different times, reversed transcribed into cDNA and subsequently amplified with IL-1α, IL-6, IL-8 and MIP-1β specific primers (Tab. II). CSF virus induced the expression of IL-1α and IL-6 mRNA, with these transcripts clearly detectable at 2 hpi, reaching its maximum at 4 hpi and decreasing thereafter. IL-1α and IL-6 mRNA expression in mock-infected cells did not differ significantly from that of non infected cells (data not shown). LPS induced a clear increase in the expression of these mRNA, being more intense for IL-1α, but also significative for IL-6 (Fig. 2). However no synergistic effect was observed on these cytokines when the CSF virus infected cells and LPS stimulated cells were studied. With respect to IL-8 mRNA, its expression was constitutively

high in all cases and no apparent differences were noted among the different groups of the treated cells (data not shown). On the contrary, in the conditions tested for MIP-1β no transcripts were detected in any case.

3.4. Effect of virus infection on the PCA of AOC cells

The effect of CSF virus infection on the PCA or thromboplastin activity shown by AOC cells was investigated. The AOC cells were infected or not with CSF virus and 48 h later they were processed in order to determine their PCA. The PCA of endothelial cells can be assessed by measuring the time required by an endothelial lysate to promote clot formation. In order to evaluate clot formation we determined the optical density of a mixture of porcine plasma and AOC lysates at different times after the addition of $CaCl_2$ to trigger the reaction. In these conditions, CSF-virus infection of AOC induced a delay in clot formation with respect to uninfected cells, which reflects a decrease in the PCA. The difference between infected and non-infected cells was significant ($p < 0.05$) at 1 and 1.5 min after triggering the reaction. At longer times this difference decreased, tending to similar values (Fig. 3). No significant difference was found in PCA between non-infected cells and cells incubated with UV-inactivated virus.

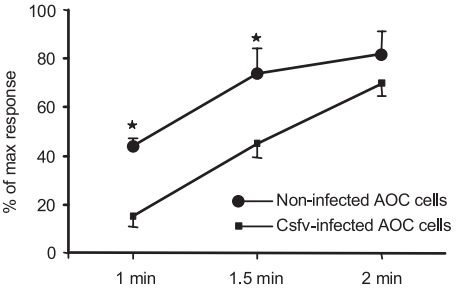

Figure 3. The effect of the CSF virus on the PCA or thromboplastin activity of AOC cells. AOC cells were infected for 48 h with CSF virus at a moi of 4. Then the cells were lysed and the PCA was determined in lysates by the evaluation of clot formation at the times shown using a spectrophotometer as described in the "Materials and methods". The figure shows the mean ± SD of 3 independent experiments. * $p <$ 0.05 as determined by the Student t-test.

4. DISCUSSION

One of the main characteristics of CSF pathology is the production of widespread hemorrhages. During the course of the disease, multiple defects of hemostasis develop with the infection of endothelial cells by CSF virus a relevant feature [22]. Endothelial cells which line blood vessels have a main role in hemostasis and in the immune response to infection contributing to numerous functions [4, 26]. Hemorrhages can be the result of degeneration and necrosis of endothelial cells by the direct action of the virus, as formerly described [8], however since the virus does not induce a cytopathic effect in vitro [13, 17], it appears more likely that necrosis does not only result from the direct effect of infection but also through different immune and inflammatory mechanisms produced during the disease. Other important factors affecting hemostasis are the consumption coagulopathy, the disseminated intravascular coagulation and the extreme thrombocytopenia produced in this disease [21, 25]. In this context, few reports can be found of the effect of the CSF virus on swine endothelial cells [3, 5, 18, 20, 22].

The aim of these experiments was to study the effect of CSF virus infection on the phenotype and function of endothelial cells using an established cell line of porcine aortic endothelial cells, immortalized by the transfection of the SV40 virus genome [6]. These cells named AOC, can be maintained in culture for at least 25 passages without signs of senescence and with preserved phenotypical and functional characteristics of primary endothelial cells [6]. These experiments confirmed that AOC cells constitutively express most classical markers of porcine endothelial cells, including Class I-MHC antigens, SWC3, the integrin CD29,

the adhesion molecule CD31 or PECAM-1 and the co-stimulatory molecules CD80/86. In our experiments these cells were clearly infected by the CSF virus, reaching higher percentages of infected cells than the cell lines used to grow the virus (data not shown). However, in spite of the high percentages of infected cells obtained, the expression of none of the molecules studied was affected by the virus.

The study of the effect of the CSF virus on the PCA or thromboplastin activity of endothelial cells constitutes an attempt to understand the mechanisms underlying the coagulopathy and the intravascular coagulation produced in CSF. Coagulation is a complex protease cascade involving about 30 interacting proteins and platelets. The cascade starts when thromboplastin is exposed to the bloodstream and forms a bimolecular complex with coagulation factor VII. The thromboplastin-factor VIIa complex can directly or indirectly activate factor X and hence generate thrombin [1, 26]. PCA can be measured by evaluating the time required by an endothelial cell lysate to promote clot formation [1]. We describe here a modification of the assay based on an easy and reliable way to monitor clot formation by optical density. In these conditions the CSF virus infection of AOC cells induced a significant delay in clot formation which reflects a decrease in PCA. This situation is similar to that described for the Dengue virus, another member of the Flaviviridae family. The Dengue virus infects endothelial cells and as a consequence the partial thrombosis time (PTT) is increased without perturbing pro-thrombin time (PT). The increase of PTT reflects the alteration of the coagulation intrinsic pathway [14]. Previous experiments performed in vivo have described that CSF infection induces the increase of PTT and thrombin clotting time (TCT), while PT remains unchanged [22]. Therefore, considering both the in vitro and in vivo results, it appears that the CSF virus exerts an anticoagulation effect on endothelial cells that affects either the extrinsic or the intrinsic coagulation pathways. On the contrary our results show that the CSF virus induces the expression of IL-1α and IL-6, two cytokines which characterize a proinflammatory state. These results agree with other studies performed with endothelial primary cultures, which describe that CSF infection induces a fast and maintained inflammatory response, although no type I IFN was found [3]. The failure to induce IFN has been related to the ability of noncytopathic viruses to persist in cultures. Although IL-1α and IL-6 promote procoagulant activity [16], a decrease in PCA caused by the CSF virus reflects the complex interactions that take place during a virus infection even in in vitro conditions. To this regard, endothelial cells posses several mechanisms to limit the response to injury, balancing the proinflammatory response and reducing the expression of the tissue factor (thromboplastin) [2]. On the contrary the situation during in vivo CSF virus infection may be more complex since the paracrine effect of proinflammatory cytokines on non-infected cells may result in the expression of pro-coagulant factors [7, 24], contributing to the disseminated intravascular coagulation characteristic of this infection [22]. It is noteworthy to note that in our experiments nearly all cells were infected.

Our experiments show how AOC cells constitute an interesting model for the study of CSF pathology in endothelial cells.

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