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Bovine herpesvirus 4 (BoHV-4) is tropic for bovine endometrial cells and modulates endocrine function

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Abstract

Bovine postpartum uterine disease, metritis, affects about 40% of animals and is widely considered to have a bacterial aetiology. Although the gamma herpesvirus BoHV-4 has been isolated from several outbreaks of metritis or abortion, the role of viruses in endometrial pathology and the mechanisms of viral infection of uterine cells are often ignored. The objectives of the present study were to explore the interaction, tropism and outcomes of BoHV-4 challenge of endometrial stromal and epithelial cells. Endometrial stromal and epithelial cells were purified and infected with a recombinant BoHV-4 carrying an EGFP expression cassette to monitor the establishment of infection. BoHV-4 efficiently infected both stromal and epithelial cells, causing a strong non apoptotic CPE, associated with robust viral replication. The crucial step for the BoHV-4 endometriotropism appeared to be after viral entry as there was enhanced transactivation of the BoHV-4 IE2 gene promoter following transiently transfection into the endometrial cells. Infection with BoHV-4 increased COX-2 protein expression and prostaglandin E_2 secretion in endometrial stromal cells, but not epithelial cells. Bovine macrophages are persistently infected with BoHV-4 and co-culture with endometrial stromal cells reactivated BoHV-4 replication in the persistently infected macrophages, suggesting a symbiotic relationship between the cells and virus. In conclusion, the present study provides evidence of cellular and molecular mechanisms supporting the concept that BoHV-4 is a pathogen associated with uterine disease.

Introduction

Bovine herpesvirus 4 (BoHV-4) was originally included in the *Betaherpesvirinae* subfamily and referred to as bovine cytomegalovirus, primarily because its biological properties in tissue culture most closely resembled those of human cytomegaloviruses (Storz *et al.*, 1984). However, molecular evidence has accumulated that indicate BoHV-4 is genetically more closely related to members of the *Gammaherpesvirinae* subfamily. This evidence includes large blocks of homologous genes arranged in the same order as two other gammaherpesviruses, Epstein-Barr virus and herpesvirus saimiri (Bublot *et al.* 1992). BoHV-4 replicates in a broad range of cell lines and primary cultures, causing a cytopathic effect (CPE) (Donofrio *et al.* 2002; Gillet *et al.* 2004; Peterson & Goyal 1988; Truman *et al.* 1986). However, like other herpesviruses, BoHV-4 can establish persistent infections in its natural host (Dewals *et al.* 2005; Dubuisson *et al.* 1989; Krogman & McAdaragh 1982; Osorio & Reed 1983) and experimental hosts such as the rabbit (Osorio *et al.* 1982).

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Although the presence of BoHV-4 has been demonstrated in many tissues, the cells of the monocyte/macrophage lineage are a common site of persistence in natural and experimental hosts (Dubuisson *et al.* 1988; Dubuisson *et al.* 1989; Naeem *et al.* 1993; Osorio & Reed 1983; Osorio *et al.* 1985). Cell lines persistently infected with gammaherpesviruses such as Epstein–Barr virus, herpesvirus saimiri, human herpesvirus 8, and murine gammaherpesvirus-68 have been established from cells isolated from infected hosts (Ceserman et al. 1995; Jung *et al.* 1999; Nilsson 1979; Usherwood *et al.* 1996). This process has likely been greatly facilitated by the growth-transforming ability of these gammaherpesviruses (Flore *et al.* 1998; Jung *et al.* 1999; Miller *et al.* 1997; Moses *et al.* 1999). In contrast, no evidence for growth-transformation has been obtained for BoHV-4. Each of the genes associated with transformation by other gammaherpesviruses is unique to each virus, and a homologous gene is not found in BoHV-4 (Lomonte *et al.* 1996).

BoHV-4 was first isolated in Europe from animals with respiratory and ocular diseases by Bartha and colleagues (Bartha *et al.* 1966) and later in the United States by Mohanty and colleagues (Mohanty *et al.* 1966). BoHV-4 has been isolated from a variety of samples and cells from healthy cattle and animals with metritis, abortion, pneumonia, diarrhoea, respiratory infection or mammary dermatitis (Bartha *et al.* 1966; Egyed 1988; Thiry *et al.* 1988). The pathogenic role of BoHV-4 remains unclear, and correlations between BoHV-4 and disease are unresolved even by experimental infection. Indeed, few investigators have successfully produced experimental disease (Thiry *et al.* 1988), and direct inoculation of the natural host only occasionally elicited respiratory and genital disease including abortion (Castrucci *et al.* 1987; Wellemans *et al.* 1986).

Abortion may follow infection with a variety of alpha-, beta- and gammaherpesvirus, but viral causes of uterine disease are seldom investigated in cattle. Although postpartum metritis affects up to 40% of cattle, causing considerable infertility and economic loss, it has been assumed that most disease is of bacterial origin and virus isolation or serology is rarely considered (Sheldon & Dobson 2004). The first reported isolation of BoHV-4 from a case of bovine metritis was in 1973 (Park & Kendrick 1973). Later several other isolates were obtained from cows with reproductive disorders from different countries, including Italy (Castrucci *et al.* 1986) and India (Mehrotra et al. 1986). In Belgium, BoHV-4 seroprevalence was associated with postpartum metritis, and chronic infertility of cattle (Czaplicki & Thiry 1998). Postpartum metritis has also been associated with BoHV-4 in the USA (Frazier *et al.* 2002; Frazier *et al.* 2001), Spain (Monge *et al.* 2006) and Serbia (Nikolin *et al.* 2007). There is a gap in knowledge about the direct correlation between viral infection and uterine pathology. Thus, the objectives of the present study were to determine the interaction, tropism and outcome of BoHV-4 challenge of endometrial epithelial and stromal cells.

Materials and Methods

Endometrial cell isolation and primary cultures

To better understand the interaction of BoHV-4 with the endometrium it was important to use purified populations of epithelial and stromal cells without contamination by leukocytes, because BoHV-4 function can be influenced by macrophages (Donofrio & van Santen 2001). Bovine uteri from post-pubertal non-pregnant BoHV-4 serum negative animals with no evidence of genital disease were collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology (Ireland *et al.* 1980). Genital tracts with an ovarian Stage I corpus luteum were selected for endometrial tissue and cell culture, and only the horn ipsilateral to the corpus luteum was used.

The endometrium was cut into strips and placed into serum-free RPMI-1640 (Sigma) supplemented with 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 2.5 μg/ml of Amphotericin B (Sigma), working under sterile conditions. The strips were then chopped into 1 mm^3 pieces and placed into HBSS (Sigma) and used as previously described (Asselin *et al.* 1996; Fortier *et al.* 1988), with the following modifications. Briefly, tissue was digested in 25 ml sterile filtered digestive solution, which was made by dissolving 50 mg trypsin III (Roche), 50 mg collagenase II (Sigma), 100 mg BSA (Sigma) and 10 mg DNase I (Sigma) in 100ml phenol-red free HBSS. Following a 1.5 h incubation in a shaking water bath at 37°C, the cell suspension was filtered through a 40 μm mesh (Fisher Scientific) to remove undigested material and the filtrate was resuspended in phenol-red free HBSS containing 10% FBS (Sigma) and 3 μg/ml trypsin inhibitor (Sigma)(Washing medium). The suspension was centrifuged at 100 × *g* for 10 min and following two further washes in washing medium the cells were resuspended in RPMI-1640 containing 10% FBS, 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 2.5 μg/ml of Amphotericin B. The cells were plated at a density of 1×10^5 cells in 2 ml per well using 24-well plates (Nunc). To obtain separate stromal and epithelial cell populations, the cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells (Fortier *et al.* 1988). The removed cell suspension was then replated and incubated allowing epithelial cells to adhere (Kim & Fortier 1995). Stromal and epithelial cell populations were distinguished by cell morphology as previously described (Fortier et al. 1996). The absence of immune cells in the uterine cell cultures was confirmed by RT-PCR for the CD45 pan-leukocyte marker as previously described (Herath et al. 2006). The culture media was changed every 48 h until the cells reached confluence. All cultures were maintained at 37°C, 5% CO_2 in air, in a humidified incubator.

Cell line cultures

To explore BoHV-4 tropism, the effect of viral challenge of the endometrial cells were compared with other cells, including: Madin–Darby bovine kidney (MDBK, ATCC, CCL-22), and from M. Ferrari (Istituto Zooprofilattico, Brescia, Italy) bovine embryo kidney (BEK), bovine embryo lung (BEL), and bovine turbinates (BT). The cells were maintained as monolayers in DMEM (Cambrex), containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin. The cells were incubated at 37°C with 5% CO_2 in air, in a humidified incubator and subcultured when growth reached 70–90% confluence every 3 to 5 days.

Virus

To test the susceptibility of cells to BoHV-4 infection, a recombinant BoHV-4 expressing enhanced green fluorescent protein was employed. Recombinant BoHV-4EGFPΔTK was obtained by insertion of the CMV/EGFP expression cassette from the pEGFP-C1 plasmid into the thymidine kynase (TK) locus of the DN 599 BoHV-4 strain (Donofrio *et al.* 2002). BoHV-4EGFPΔTK and the NADL strain of BVDV, were propagated by infecting confluent monolayers of MDBK at a multiplicity of infection (m.o.i.) of 0.5 tissue cell infectious doses/50 ($TCID_{50}$) per cell and maintained in MEM with 2% FBS for 2 h. The medium was then removed and replaced by fresh MEM containing 10% FBS. The virus was purified when approximately 90% of the cell monolayer exhibited a CPE, at approximately 72 h post-infection (P.I.). Cell-associated virions were freed by three cycles of freezing the flasks at −80°C and thawing. Cell debris was removed by low-speed centrifugation express as × g, and virions were pelleted through a 3 ml cushion of 30% sucrose in PBS, in a Beckman 70 Ti rotor at 35,000 rpm express as × g for 90 min at 4°C. Viral pellets were resuspended in cold MEM without FBS and $TCID_{50}$ were determined on MDBK cells by limiting dilution (Vanderplasschen *et al.* 1995).

Infection of primary cell cultures with BoHV-4

Stromal and epithelial cells were challenged once confluence had been reached with BoHV-4EGFPΔTK at the concentrations indicated in results, or 1 μg/ml O55:B5 lipopolysaccharide (LPS, Sigma) as a positive control. Viral infection was monitored every 12 h by observation of cell fluorescence using a fluorescence microscope (Axiovert S100, Zeiss). The supernatants were harvested and frozen at −20°C until used for prostaglandin radioimmunoassay, and the endometrial cells were collected immediately for RNA isolation and further analysis.

MTT cell survival assay

CPE is the morphological change associated with the detrimental effects of viral replication on host cell homeostasis that ends with cell death. For epithelial cells the CPE induced by BoHV-4 is characterized by swelling, whilst stromal cells shrink and detached from the surface of the culture flask. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell metabolic assay was used to measure the number of live cells. Briefly, 48 h after infection with BoHV-4EGFPΔTK, the cell cultures were incubated for 4 h with 100 μg/well MTT before addition of 100 μl of solubilisation solution (10% SDS in HCl 0.01 M), and further incubation for 16 h at 37 °C. The yellow tetrazolium MTT salt is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The optical density was measured at 540 nm, using 690 nm as the reference wavelength in an SLT-Lab microreader (Salzburg, Austria); for each cell type, a linear relationship between cell number and optical density had already been established. Each experiment was done three times and each treatment was performed with eight replicates. Statistical differences among treatments were tested by ANOVA.

Apoptosis assays and viral production

Apoptosis assays were performed because CPE by viruses such as BVDV is mediated by apoptosis. BVDV or BoHV-4EGFPΔTK infected confluent monolayers were gently scraped from 25-cm^2 flasks with a sterile scraper in the presence of culture medium and the cells pelleted by centrifugation at 12,000 × *g* for 1 h at 4 °C. The cell pellet was gently resuspended in 500 μl of extraction buffer (400 mM NaCl, 10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 0.1% NP 40), transferred to a polypropylene microcentrifuge tube and kept on ice for 30 min. The solution was centrifuged at 12,000 × g for 15 min, the supernatant recovered carefully in a fresh microfuge tube, and extracted with phenol. The aqueous phase was transferred to a microfuge tube and a 0.1 volume of 10 M ammonium acetate and 1 volume of isopropanol added, mixed and centrifuged at 12,000 × *g* for 5 min. The supernatant was removed and the white nucleic acid pellet washed with 500 μl of 70% ethanol and dissolved in 20 μl of TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.8) containing 20 μg/ml DNAase-free pancreatic RNAase (Sigma). Nuclear fragments were examined by electrophoresis in a 1.5% agarose gel and visualised by u.v. after staining with ethidium bromide. For propidium iodide staining, cells were washed with PBS, stained with 400 ng/ml propidium iodine for 30 s in the dark and fragmented nuclei visualized by fluorescence microscopy.

Viral production by BT, BEL, MDBK, BEK, endometrial epithelial and stromal cells was tested after infecting cells with 1 m.o.i. of BoHV-4EGFPΔTK. The viral inoculums' were removed after a 3 h absorption period and replaced with fresh media, and the viral titer quantified 48 h P.I.

Cell culture electroporation and viral reconstitution

To determine which step of the virus life-cycle is important for the expression of the tropic phenotype, a reconstitution viral assay was performed. MDBK, BT and BEK cells from a

sub-confluent 25 cm^2 flask were electroporated (Equibio apparatus, 270 V, 960 μF) with 2 μg of viral DNA purified as previously described (Gillet *et al.*, 1995) in DMEM without serum and antibiotics. Electroporated cells were returned to new 25 cm^2 flasks and fed with DMEM containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin. Endometrial stromal, endometrial epithelial and BEL cells from a sub-confluent 25 cm^2 flask were electroporated (Equibio apparatus, Opty-Puls, 300 V, 25 μF, 240 V, 1050 μF and 481 R) with 2 μg of viral DNA in DMEM with 10% FBS. Electroporated cells were returned to new 25 cm^2 flasks and stromal and epithelia cells were fed with RPMI-1640 containing 10% FBS, 50 IU/ml of penicillin, 50 g/ml of streptomycin and 2.5 g/ml of Amphotericin B and BEL cells with 90% DMEM containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin. The time necessary for the formation of viral plaques was monitored every 24 h by fluorescence microscopy

Recombinant IE2 plasmid construction and transfection

To further investigate the mechanisms associated with endometrial tropism, a molecular switch involving the viral immediate early genes (IE) was investigated. The IE genes are expressed immediately during cell infection, do not require prior viral protein synthesis for their expression, and their expression is mediated by the pool of transcription factors made by the cell, already present at the moment of infection and able to trans-activate at the transcriptional level the IE promoters. BoHV-4 immediate early 2 (IE2) protein (replication and transcription activator homologous, Rta) encoded by open reading frame 50 (ORF 50) is well conserved among gamma herpesviruses (Zimmermann *et al.* 2002). Rta expression plays a primary role in initiating viral lytic replication, not only during reactivation of latently infected non-permissive cells but also during de novo infection of permissive cells (Donofrio *et al.* 2004; Song *et al.* 2002; Sun *et al.* 1998; van Santen 1993). The capability of endometrial cells to transactivate the BoHV-4 IE2 promoter was investigated by transient transfection of a fluorescent labelled IE2 construct. A region of the BoHV-4 (DN599) genome corresponding to the (from nucleotide 61391 to nucleotide 62534, GeneBank accession number AF318571) (Zimmermann *et al.* 2002) was generated by PCR using total DNA isolated from BoHV-4 infected MDBK cells as template and a pair of IE2 promoter primers (sense: 5′-gggaattccatatggccagtgccaagcttttaag-3′; antisense: 5′-gggaactagctagcctgttgttctgctccctttta-3′) containing an artificial NdeI site on the 5′ end and a NheI site on the 3′ end, respectively. One microgram sample DNA was amplified over 35 cycles, each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and chain elongation with High Fidelity PCR Enzyme Mix (Fermentas) at 72°C for 2 min. PCR amplification was carried out in a final volume of 50 μl, containing 0.2 mM deoxinucleoside triphosphate and 0.25 μM of each primer. In the first cycle, the samples were denatured at 94°C for 5 min, and in the last cycle the extension step was increased to 7 min. The amplicon was column purified, NdeI, NheI digested and subcloned in pEGFP-C1 vector (Clontech), excised with NdeI and NheI to remove the CMV promoter and generate the pIE2prom.EGFP construct. Transient transfection using 25 μg of the plasmid DNA construct was performed by electroporation, as described above. Endometrial epithelial and stromal cells, BT, BEL, MDBK and BEK cells were electroporated with the reporter construct and monitored every 24 h by fluorescence microscopy for green cells.

Prostaglandin radioimmunoassay

Prostaglandins have a central role in many reproductive functions in mammals (Poyser 1995). Indeed, the duration of ovarian cycles and pregnancy depends on the presence of an active corpus luteum in the ovary, and the life span of the corpus luteum is regulated by the secretion of prostaglandin $F_{2\alpha}$ (PGF) and prostaglandin E_2 (PGE) from the uterine endometrium. Under physiological conditions, the uterine epithelial cells predominantly secrete PGF when stimulated with oxytocin, whereas the stromal cells produce PGE

(Asselin 1996). However, when epithelial or stromal cells are challenged with bacterial lipopolysaccharide (LPS), prostaglandin secretion is also stimulated (Herath *et al.* 2006). As BoHV-4 is isolated from animals with uterine disease, the effect on endometrial cell function of 24 h challenge with different amounts of BoHV-4 (10, 1 and 0.1 m.o.i. of virus) or a positive control of 1 ug/ml LPS, was determined by measuring prostaglandin E_2 (PGE) and prostaglandin $F_{2\alpha}$ (PGF) in culture supernatants by RIA, as previously described (Cheng *et al.* 2001; Leung *et al.* 2001). Samples were diluted in 0.05 M Tris buffer containing 0.1% gelatine and 0.01% sodium azide. Standards and tritiated tracers for the PGs were purchased from Sigma and Amersham International PLC (Amersham, UK), respectively. The antisera were a generous gift from Professor N.L. Poyser (University of Edinburgh, UK) and their cross-reactivity was: $PGF_{2\alpha}$ antiserum, 34% with $PGF_{1\alpha}$, 25% with $PGF_{3\alpha}$ and 0.54% with PGE_2; PGE_2 antiserum, 23% with PGE_1, 15% with PGE_3 and 0.47% with $PGF_{2\alpha}$ (Poyser 1987). The limit of detection for PGE_2 and $PGF_{2\alpha}$ was 2 pg/tube and 1 pg/tube, respectively. The intra-assay and inter-assay coefficients of variation were 4.4 and 7.8% for PGE_2, and 5.1 and 9.7% for 2 $PGF_{2\alpha}$ respectively.

Western blotting

PGF and PGE are synthesized from arachidonic acid (AA) under the regulation of cyclooxygenase 2 (COX2) isoenzymes in the endometrium (Arosh *et al.* 2002; Smith *et al.* 1996). Stromal cells in 6-well culture plates were serum starved overnight; infected with 5 m.o.i. of BoHV-4 in the absence of serum; and, the cells collected 2, 4, 6 and 6 h P.I. to measure COX2 protein expression by Western immunoblotting, with a positive control established by treating stromal cells with medium containing 20% FBS for 1 h. Cell extracts were prepared by adding 100 μl/well of cell extraction buffer (50 mM Tris-HCl, 150 mM NaCl and 1% NP-40; pH 8). Cell extracts containing 50 μg of total protein were electrophoresed through 12% SDS-polyacrylamide gels and transferred to nylon membranes by electro blotting. Membranes were incubated with rabbit anti-COX2 polyclonal antibody (AB5118; Chemicon International), probed with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G_1 (IgG_1) antibody (A0545, Sigma), and visualized by enhanced chemiluminescence (ECL Kit; Pierce, Rockford, IL).

The effect of stromal cells on a macrophage cell line persistently infected with BoHV-4

As BoHV-4 stimulated stromal cell PGE secretion, a scenario whereby PGE produced by stromal cells could activate BoHV-4 lytic replication in persistently infected macrophages was envisioned: (ì) Macrophages are the site of persistency of BoHV-4 (Donofrio & van Santen 2001; Lopez *et al.* 1996; Osorio *et al.* 1985) (ìì) persistently infected macrophages can easily reach the endometrium through the blood stream (ìíì) the small amount of BoHV-4 viral particles could infect stromal cells, which will release PGE and reactivate BoHV-4 in persistently infected macrophages. To test the above scenario, stromal cells were co-cultivated with a bovine macrophage cell line persistently infected with BoHV-4EGFPΔTK producing small amount of infectious viral particles (Donofrio *et al.* 2005). The persistently infected macrophage cell line (BOMAC/BoHV-4EGFPΔTK) was established as previously described (Donofrio & van Santen 2001) by infecting BOMAC cells, a cell line established from peritoneal macrophages by transformation with simian virus 40 DNA (Stabel & Stabel 1995). Confluent monolayers of BOMAC cells were inoculated (multiplicity of infection (m.o.i.) of 1 $TCID_{50}$/cell) with recombinant BoHV-4 (BoHV-4EGFPΔTK), by the third day after inoculation, more than 95% of cells had detached from the monolayer, leaving behind a small number of cells that did not exhibit cytopathic effect (CPE). Confluent monolayers established from surviving cells showed 100% infection, as indicated by the strong fluorescent signal, but without apparent signs of CPE. Also consistent with our previous observations (Donofrio & van Santen 2001), the persistently infected macrophages produced infectious BoHV-4; medium recovered from

BOMAC/BoHV-4EGFPΔTK cells inoculated onto BEK cells produced green plaques typical of BoHV-4EGFPΔTK. BOMAC/BoHV-4EGFPΔTK cells were sub cultured at a dilution of 1:2 every 3 days and their growth medium was stored at −80°C for viral titration. The yield of virus in the culture medium on the day the cells were sub cultured remained in the range of 4×10^2 $TCID_{50}$/ml throughout the first 20 passages. The macrophages were co-cultured with uninfected stromal cells or control BEK cells, using a transwell approach. After 48 h of co-culture, the stromal cells or BEK cells in the lower well were analyzed under fluorescence microscope for EGFP expression. In addition, the viral titre was measured in the medium from the upper well, where the persistently infected macrophages were located.

Results

Isolation of pure populations of bovine endometrial stromal and epithelial cells

The bovine endometrium principally comprises of epithelial cells and stromal cells but there are also sporadic leukocytes. To study the interaction of BoHV-4 with the endometrium it was important to use purified populations of epithelial and stromal cells without leukocytes, which can influence BoHV-4 function (Donofrio & van Santen 2001). Endometrial cells cultures were established by the fractional enzyme dissociation method described in Materials and Methods, and there was no mRNA expression of the CD45 pan-leukocyte marker in epithelial or stromal cells (data not shown) as previously described (Herath *et al.* 2006). The epithelial and stromal cell purity was greater than 95% as determined by microscopy (Fig. 1a and b) and the preferential secretion of prostaglandin $F_{2\alpha}$ and E_2, respectively, as described previously (Asselin *et al.* 1996; Herath *et al.* 2006).

Bovine herpesvirus 4 infects bovine endometrial cells and induces cell death

To test the susceptibility of bovine endometrial cells to BoHV-4 infection, epithelia and stromal cells were infected with 1 m.o.i. of a recombinant BoHV-4 (BoHV-4EGFPΔTK) expressing enhanced green fluorescent protein (Donofrio *et al.* 2002). A few epithelial cells showed fluorescence 12 h P.I. (Fig. 2a) and this became generalized by 24 h, with the CPE starting 48 h P.I. and complete by 72 h. In contrast, most stromal cells were infected by 12 h P.I. (Fig. 2b), and the CPE started at 24 h P.I. and was complete by 48 h. To quantify the cytopathic effect of BoHV-4 infection, cell survival after challenge with different viral doses was measured using a MTT assay. Epithelial and stromal cells were killed in a dose dependent manner 48 h after infection with BoHV-4 and the CPE was greater for stromal than epithelial cells (Fig. 2c). Thus, it appears that BoHV-4 infects both epithelial and stromal cells leading to a CPE, but the stromal cell infection seems to be more efficient.

CPE induced by BoHV-4 in bovine endometrial epithelial and stromal cells is not mediated by apoptosis but is associated with a productive infection

BoHV-4 replicates in cell lines or primary cell cultures from a broad spectrum of host species. The infection in some permissive cells leads to viral progeny and a CPE; in other cells, there is a CPE even though no viral progeny are produced; whereas in some non-permissive cells, there is persistent BoHV-4 infection with no effect on cell survival (Donofrio *et al.* 2000). As BoHV-4 infection induced a CPE in endometrial cells, the nature of the cell death and virus production was investigated.

Endometrial epithelial and stromal cells were infected with 5 m.o.i. of BoHV-4 and compared with 5 m.o.i. of BVDV, which is an established apoptotic virus (Schweizer & Peterhans 2001). The CPE induced by BoHV-4 was not mediated by cell apoptosis as determined by intranucleosomal DNA fragmentation (Fig. 3a) and propidium iodine staining

(data not shown), in contrast to the cells infected with BVDV where there was clear DNA fragmentation (Fig. 3a).

Endometrial epithelial and stromal cell viral production was compared with other fully permissive cell types (BT, BEL, MDBK and BEK cells) infected with 1 m.o.i. of BoHV-4EGFPΔTK. The viral titres 48 h P.I. were ~ one log higher for epithelial and two logs higher for stromal cells compared with the other cell types (Fig. 3b).

Post viral entry is a determinant step for BoHV-4 replication in endometrial cells

From the above data it appears that BoHV-4 has a striking tropism for endometrial cells or that endometrial cells are highly susceptible to BoHV-4 replication. However, viral attachment and penetration do not appear to be critical because many cell types were infected by BoHV-4. To determine which step of the virus life-cycle is important for the expression of the tropic phenotype, a reconstitution viral assay was performed. Endometrial epithelial and stromal cells, BT, BEL, MDBK and BEK cells were electroporated with purified BoHV-4EGFPΔTK genomic DNA and the time necessary for the formation of viral plaques was monitored by fluorescence microscopy. Green plaques started to appear by 24 h after electroporation in epithelial and stromal cells, in contrast to the other cell types where visible green plaques formed between 3 and 5 days after electroporation (Fig. 4a and b).

BoHV-4 Immediate Early 2 gene promoter is strongly trans-activated in endometrial cells

To further investigate the mechanisms associated with endometrial tropism, a molecular switch involving the viral immediate early genes (IE) was investigated using an EGFP labelled construct containing the IE2 gene (Fig. 5a) electroporated into endometrial epithelial and stromal cells, BT, BEL, MDBK and BEK cells. EGFP started to accumulate robustly as soon as 24 h after electroporation in the cytoplasm of stromal cells (Fig. 5b) and epithelial cells, in contrast to the other cell types where weak visible green cells appeared not before than 3 days post electroporation (Fig. 5c).

Low dose BoHV-4 infection stimulates PGE production in stromal cells

Under physiological conditions, the uterine epithelial cells predominantly secrete PGF when stimulated with oxytocin, whereas the stromal cells produce PGE (Asselin 1996). The capacity of the endometrial cells to produce PGs in response to BoHV-4 was investigated as BoHV-4 is frequently isolated from the uterus. The cells were capable of responding to pathophysiological challenge as LPS stimulated PGF (Fig. 6a) and PGE (Fig. 6b) secretion from the epithelial and stromal cells, respectively, as reported previously (Herath *et al.* 2006). Epithelial cells did not produce PGF in response to BoHV-4 (Fig. 6a). However, stromal cells challenged with 0.1 m.o.i. produced more PGE than untreated controls (Fig. 6b). In contrast higher viral doses suppressed PGE production, which is likely a consequence of the greater CPE induced by the virus replication. The rate limiting enzyme for PGE synthesis in the endometrium is COX-2 (Arosh *et al.* 2002; Smith *et al.* 1996). So, as BoHV-4 stimulated PGE secretion by endometrial stromal cells the induction of COX-2 by BoHV-4 was examined by Western blotting and COX-2 was up-regulated as early as 1 h P.I. (Fig. 6c).

Endometrial stromal cells enhance BoHV-4 replication in persistently infected macrophages

As BoHV-4 stimulated stromal cell PGE secretion, the effect of stromal cells on BoHV-4 replication in persistently infected macrophages was tested using a transwell approach (Fig. 7a). After 48 h of co-culture, the stromal cells had a higher level of infection and a higher viral titer in the upper well, than the BEK cells (Fig. 7a and b). These data support the

concept of a symbiotic relationship between the stromal cells and persistently infected macrophages.

Discussion

Bos taurus are particularly prone to uterine infection and metritis after parturition, which causes considerable infertility and economic loss (Sheldon & Dobson 2004). The most commonly recognized uterine pathogens are *Arcanobacterium pyogenes* and *Escherichia coli* (Bonnet *et al*. 1993; Griffin *et al*. 1974; Olson *et al*. 1984; Ruder *et al*. 1981; Sheldon & Dobson 2004). Although viral isolation or serology is not routinely performed in animals with uterine disease, BoHV-4 has been implicated in cases of bovine metritis (Czaplicki & Thiry 1998; Fortier *et al*. 1988; Frazier *et al*. 2002; Monge *et al*. 2006; Nilsson 1979; Park & Kendrick 1973). The present study demonstrated a viral tropism by BoHV-4 for the endometrium. The high efficiency of BoHV-4 replication in endometrial cells was associated with strong transactivation of viral IE genes. Furthermore, BoHV-4 increased stromal cell COX-2 protein and stimulated prostaglandin E_2 secretion. This viral induced stromal cell PGE secretion may be a mechanism by which viral replication is stimulated in macrophages, which are the usual repository for persistent bovine infections.

Viruses are restricted to using the metabolic and biosynthetic pathways of the cells that they infect. These pathways vary between cell types, lineage, stage of differentiation, and with the state of cell activation. There are many examples of viruses that replicate in specific cells and at particular stages of cell growth, differentiation, or activation. This includes the reactivation of cytomegalovirus when host cells differentiate into macrophages; initiation of papillomavirus replication by keratinocytes; and, replication of minute virus in testicular cells. The key mechanism mediating these effects is the regulation of viral gene expression at the transcriptional level by host cells factors. The present study identified a striking tropism of BoHV-4 for endometrial cells. BoHV-4 efficiently infected purified populations of bovine endometrial stromal and epithelial cells, leading to a non-apoptotic cell death and *de novo* viral production, which could be an important mechanism underlying the metritis associated with BoHV-4 infection in cattle. The lack of BoHV-4 apoptogenicity found in endometrial cells where BoHV-4 infection was highly productive, well fit with the BoHV-4 pro-apoptotic behaviors found in some cell types (Gillet *et al*. 2005) where, BoHV-4 infection is completely unproductive. In a complex multicellular organism, rapid apoptotic cell suicide on virus infection can be seen as an altruist response (Allops *et al*. 2000). If it occurs before complete virus replication, and assembly, it will be highly effective in limiting viral production. Many, and possibly most, large virus like herpesvirus that, due to their size and complexity, have relatively long replication time, encode antiapoptotic genes, as is the case for BoHV-4 (Gillet *et al*. 2004).

For many viruses, tropism and successful replication is determined by specific cellular receptors that must be engaged for virus binding and entry. However, BoHV-4 can enter many cell types from different species (Donofrio *et al*. 2002). Furthermore, successful replication of BoHV-4 seems to be governed primarily by post-entry events as shown by the fast viral reconstitution following the electroporation of nude viral DNA into the cells. The open reading frame (ORF) 50 gene product, also known as the replication and transcription activator (Rta), is an immediate-early gene which is well conserved among all gamma-2 herpesviruses. BoHV-4 IE2 RNA is the less abundant, spliced, 1.8 kb RNA, which is transcribed from the left to the right on the restriction map of the BoHV-4 genome and contained in the 8.3 kb Hind III fragment F (van Santen 1993). The predicted amino acid sequence, of the protein encoded by IE2 RNA, reveals that it could encode a 61 kDa protein with amino acid sequence homology to the Epstein-Barr virus transactivator R and its homolog including, herpesvirus saimiri, equine herpesvirus 2, murine herpesvirus 68 and

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Kaposi's sarcoma-associated herpesvirus. Transactivation studies have shown that BoHV-4 IE2 protein specifically transactivates expression of a reporter gene linked to the promoter regulatory region of the BoHV-4 early (E) gene encoding the BoHV-4 homologue of herpes simplex virus type 1 major DNA binding protein (van Santen 2003). The BoHV-4 ORF 50 homologues have been shown to play a pivotal role in regulating the lytic switch in a non-permissive cell line following his over expression (Donofrio *et al.* 2004). Indeed, in the present study IE2 promoter transactivation in endometrial cells triggered the BoHV-4 phenotype in those cells, as shown by the fast up-regulation of EGFP reporter gene expression driven by IE2 promoter. Interestingly, a preliminary analysis of the BoHV-4 IE2 promoter by Tfsitescan/dynamicPlus server, reveals 5 positive regulatory elements that are also found in the rabbit uteroglobin promoter and apparently specific for endometrial cells. The most surprising similarity was found for the responsive element close to the TATA box, which may indicate the existence of an endometrial-specific TATA box, (Misseyanni *et al.* 1991). A comparison with promoters of two other endometrium specific genes, the rat homologous to rabbit uteroglobin (Hagen 1990) and the pig uteroferrin gene (Simmen 1989) indicates that this TATA box element is well conserved. Further studies are needed to identify the nature of the factors in bovine endometrial cells and how they interact with BoHV-4 IE2 promoter.

Specific enhancers and transcriptional activators produced by host cells are important to initiate and maintain viral replication. In persistently infected macrophages, BoHV-4 replication is stimulated by the addition of exogenous PGE (Donofrio *et al.* 2005). Bovine endometrial cells induce COX2 and PGE production in response to a number of cell activators and inflammatory signals, such as lipopolysaccharide (Herath *et al.* 2006). Taken together these observations support a scenario where viral particles infecting the endometrium, could stimulate PGE production and establish a positive-feedback loop between PGE production and viral replication (Donofrio *et al.* 2005). That model of BoHV-4 induced metritis seems to be supported in the present *ex-vivo* study, by the observations that bovine endometrial stromal cells produced PGE following BoHV-4 infection and cells persistently infected with BoHV-4 were reactivated after co cultivation with endometrial stromal cells using a transwell approach. Infections by several viruses, including many herpesviruses, such as Hepes Simplex Virus, human cytomegalovirus, Epstein-Barr virus and murine gammaherpesvirus 68 have been reported to alter COX-2 expression (Reynolds & Enquist 2006). In fact, rhesus cytomegalovirus even encodes a COX-2 homolog in its genome, emphasizing the importance of this enzyme (Hansen *et al.* 2003). In addition, many studies have examined the regulation of COX-2 expression and PGE_2 production during viral infection as well as the effect of PGE_2 production on viral replication and virulence (Steer & Corbett 2003). Prostaglandins are potent mediators of many critical physiological and inflammatory responses, and they modulate the host defence against various pathogens. They suppress some innate immune factors, including nitric oxide (NO) production, and have effects on the acquired immune response, specifically by suppressing the Th1 response. For instance, PGE_2 can inhibit the production of gamma interferon by activated human T cells in vitro (Snijdewint *et al.* 1993) and that of Th1 cytokines such as interleukin-12 in vivo (Kuroda *et al.* 2000; Newberry *et al.* 1999). In addition to inhibiting the production of Th1 cytokines, PGE_2 switches the immune response toward a Th2 response, which is less effective in mounting an antiviral response (Betz & Fox 1991; Kuroda *et al.* 2000). PGE is one of the most potent and abundant PGs present during inflammatory reactions (Appleton *et al.* 1996). The very early host responses to viral infections are usually non specific and include the induction of cytokines such as interferons and tumour necrosis factor alpha. Nitric oxide synthase (NOS) is an interferon-inducible protein that is activated during innate immune responses (Reiss & Comatsu 1998). When present at high concentrations after expression of the inducible isoform of NOS (iNOS), NO functions as a cytotoxic molecule, reacting with proteins or H_2O_2 to form a highly toxic

compound called peroxynitrite ($ONOO^-$) (Reiss & Comatsu 1998). Nitric oxide is also thought to participate in the antiviral response to infection by attenuating the replication of both DNA and RNA viruses (Reiss & Comatsu 1998). The products of COX and NOS enzymes, PGs and NO, have been shown to share an antagonistic relationship with one another. The inhibition of COX activity in vesicular stomatitis virus (VSV) infected cells causes a reduction in viral propagation and a concordant increase in extracellular NO levels. Treatment with an iNOS inhibitor, L-NAME, or exogenous PGE_2 in the presence of COX inhibitors can restore VSV growth and decrease NO production, underscoring a role for PGs in counteracting the antiviral effects of NO (Chen *et al.* 2000). Besides their role in immunomodulation and counteraction of the antiviral effects of NO, PGs have been shown to be involved in modulating transcription from viral promoters. The human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) contains sequences that are important for DNA integration, as well as signals, such as an internal polymerase II promoter, that are necessary for transcription of the integrated retroviral DNA. PGE_2 can increase transcription driven by the HIV-1 LTR in T lymphocytes (Dumais *et al.* 1998). Transcription of one of the immediate-early genes (IE2) of HCMV was reduced in cells that were treated with COX2 inhibitors. Therefore, a potential role for COX induction in the context of a virus infection is the activation of transcription from viral promoters via PGs.

In the case of BoHV-4 persistently infected animals, stromal cell PGE secretion may reactivate viral replication, which in turn would lead to endometrial inflammation. Bacterially induced metritis in cattle persistently infected with BoHV-4 could possible be exacerbated or become chronic following the recruitment from the blood stream to the site of inflammation of macrophages persistently infected with BoHV-4. This theory could explain the fact that BoHV-4 can be isolated from healthy animal too, where in the absence of inflammation the pathogenic potential of BoHV-4 is ameliorated.

In conclusion, the present study indicates that BoHV-4 is a likely pathogen associated with uterine disease. The virus is trophic for endometrial epithelial and stromal cells, causing a rapid CPE. It appears that the endometrial cells rapidly induce IE gene expression and viral replication. Furthermore, there was a symbiotic relationship between stromal cell PGE secretion in response to BoHV-4 and reactivation of viral replication in macrophages. These data provide evidence of cellular and molecular mechanisms underlying the observation of uterine disease in animals with BoHV-4.

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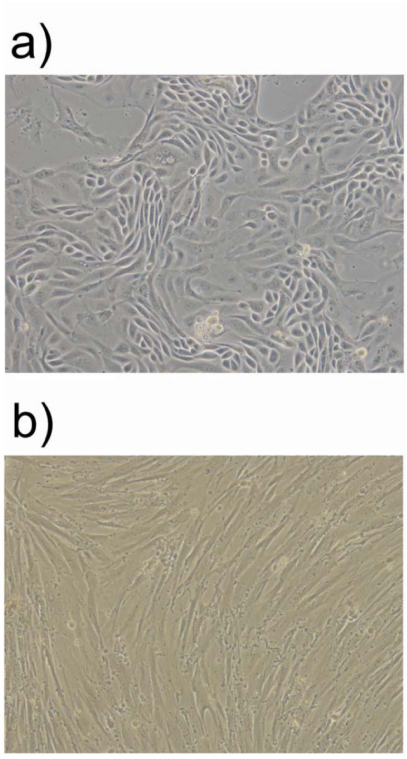

Fig. 1.
Representative phase contrast images (10X) of pure population of bovine endometrial epithelial (a) and stromal cells (b).

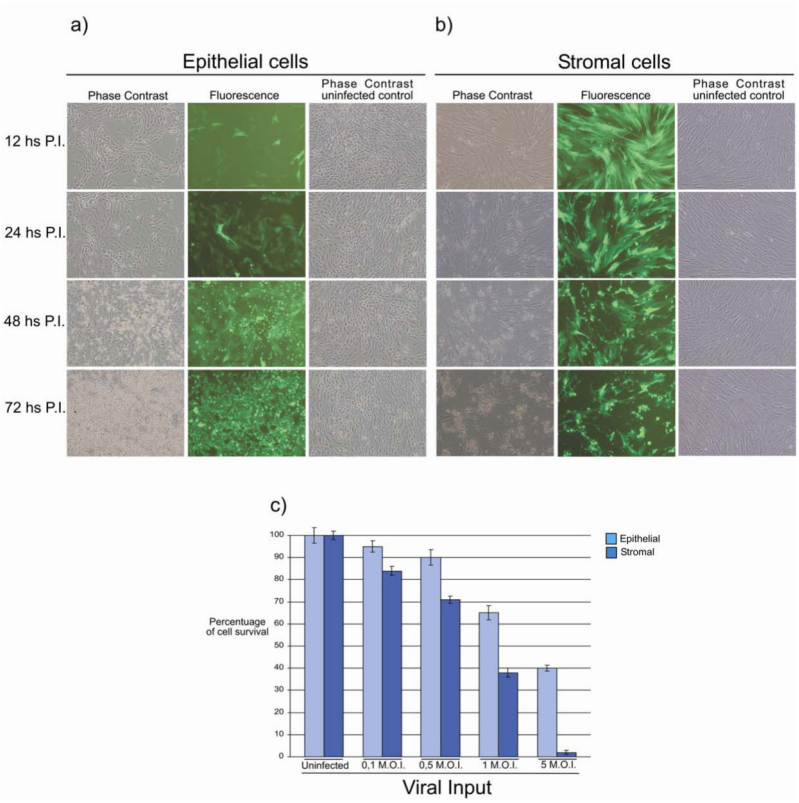

Fig. 2.

Representative fluorescence (green) and phase contrast images (10X) of bovine endometrial epithelial (a) and stromal (b) cells at different time (12, 24, 48, 72 h) post infection (P.I.) with 1 m.o.i. of BoHV-4 EGFPΔTK and the respective phase contrast images of uninfected control. Spreading of the infection can be observed by the green colour invading the field during the time and the CPE is morphologically appreciable by the change of the cell shape, where the cells tend to shrink, becoming roundest and detaching the flask surface. The experiment was repeated three times giving the same result. c) Assessment of cell survival measured by the reduction of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) at 48 h post infection. Hundred percent of survival was considered the uninfected control. Values are the mean ± S.D. of three independent experiments (n= 8 per treatment, P≤0,009).

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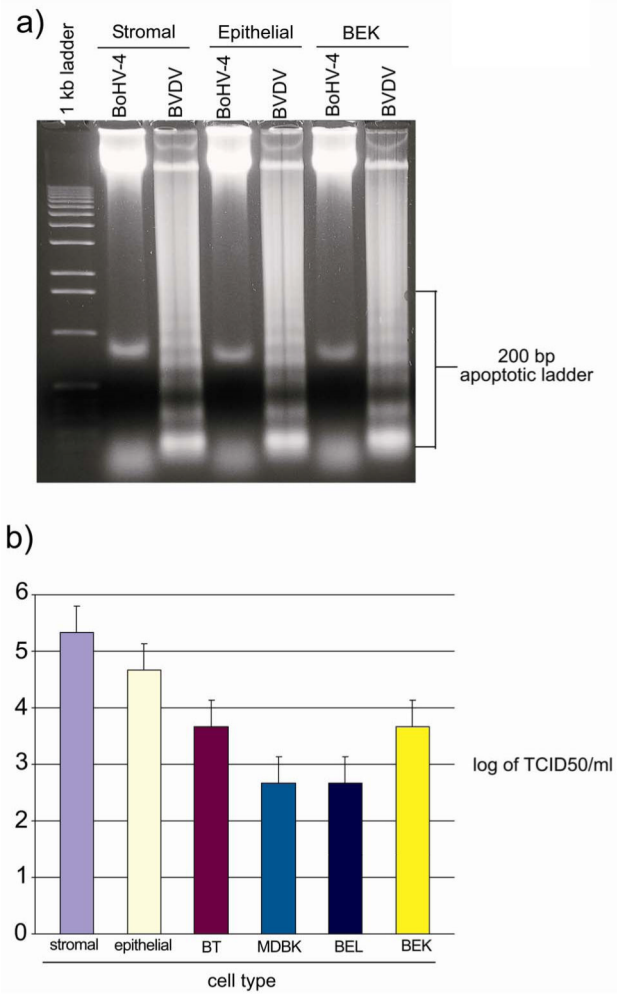

Fig. 3.

a) 1, 5 % agarose gel electrophoresis of DNA extracts from bovine endometrial epithelial, stromal and control BEK cells, infected with 5 m.o.i. of BoHV-4 and BVDV control virus, at 72 h post infection. b) Titers of BoHV-4 particles released by different cell types at 48 h post infection. Values are the mean ± S.D. of three independent experiments (n= 3 per treatment, P≤0, 05).

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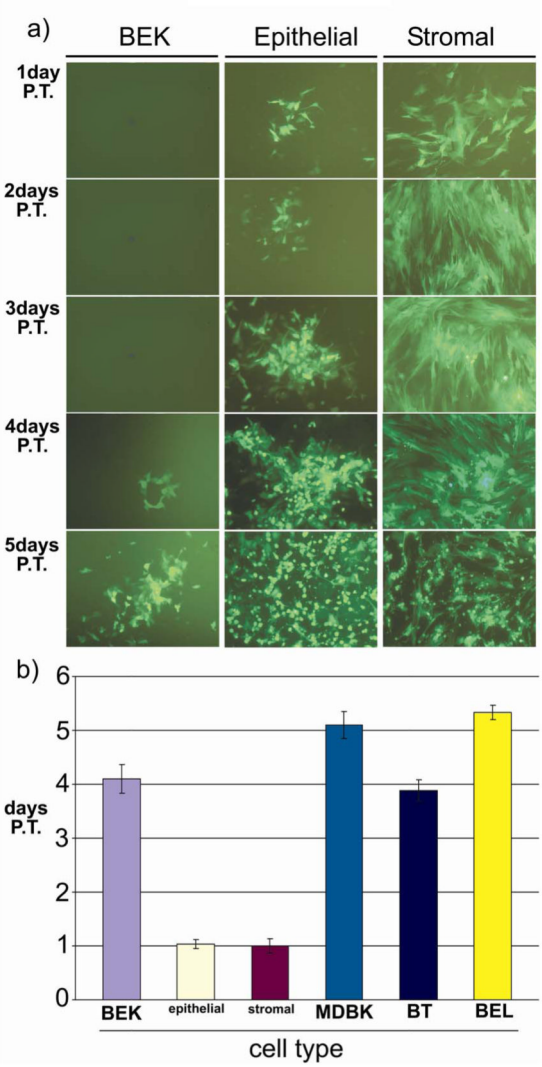

Fig. 4.

a) BoHV-4 plaques obtained at different time (1, 2, 3, 4, 5 days) post electroporation (P.E.) of nude BoHV-4EGFPΔTK DNA in different cell types. Images are representative for bovine epithelial, stromal and BEK cells. b) Graph bars, summarizing the time necessary for plaques formation in different cell types (endometrial epithelial cells, endometrial stromal cells, BT, BEL, MDBK and BEK cells). Values are the mean ± S.D. of three independent experiments (n= 3 per treatment, P≤0, 05).

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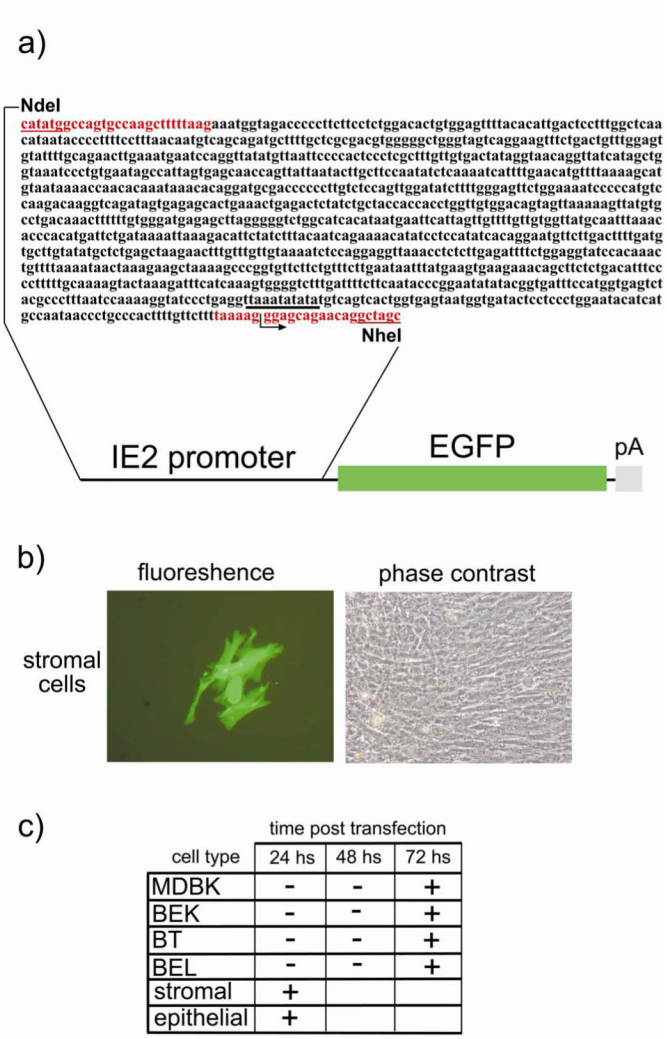

cell type	time post transfection 24 hs	48 hs	72 hs
MDBK	-	-	+
BEK	-	-	+
BT	-	-	+
BEL	-	-	+
stromal	+		
epithelial	+		

Fig. 5.

a) Diagram showing the 1,143 bp IE2 promoter sequence containing the putative TATA box (underlined in black) and the first 15 non coding nucleotides of the first exon. Sense and antisense primers used for the PCR amplification are in red and contain the NdeI and NheI restriction sites (underlined in red) respectively for sub-cloning of the amplicon in front of the EGFP ORF (green box) followed by the bovine growth hormone polyadenylation signal (pA (grey box)). b) Representative fluorescence and phase contrast images of transfected endometrial stromal cells with the above described construct and expressing EGFP 24 hours post transfection. c) Summarising schema of the time necessary for EGFP accumulation into the different cell types (endometrial epithelial cells, endometrial stromal cells, BT, BEL, MDBK and BEK cells), following transfection with the above described construct. Values are the mean of three independent experiments (n= 3 and P≤0, 05).

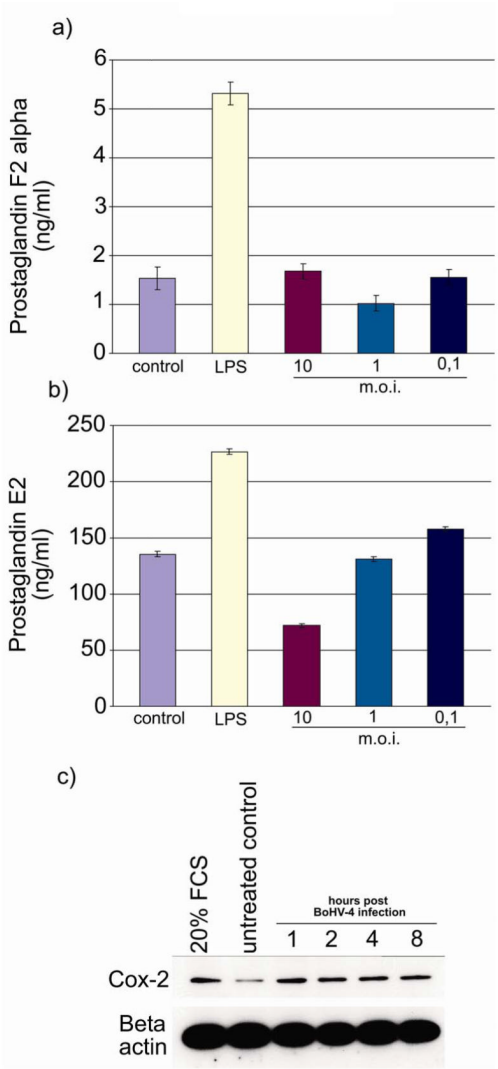

Fig.6.

Prostaglandin production by (a) epithelial and (b) stromal cells after challenge with LPS (1 μg/ml) or BoHV-4 at the different m.o.i. indicated. After 24 h in culture, supernatants were harvested and prostaglandin production was measured by RIA. Values are presented as the mean ± S.D. of three independent experiments. Differences were statistically different at $P < 0.05$ compared with a media control. c) Western immunoblotting of endometrial stromal cells extracts and infected with BoHV-4 EGFPΔTK. Positive control was established treating cells with medium containing 20% of FBS. Beta actin as the loading control. The same experiment was repeated three times, giving the same results.

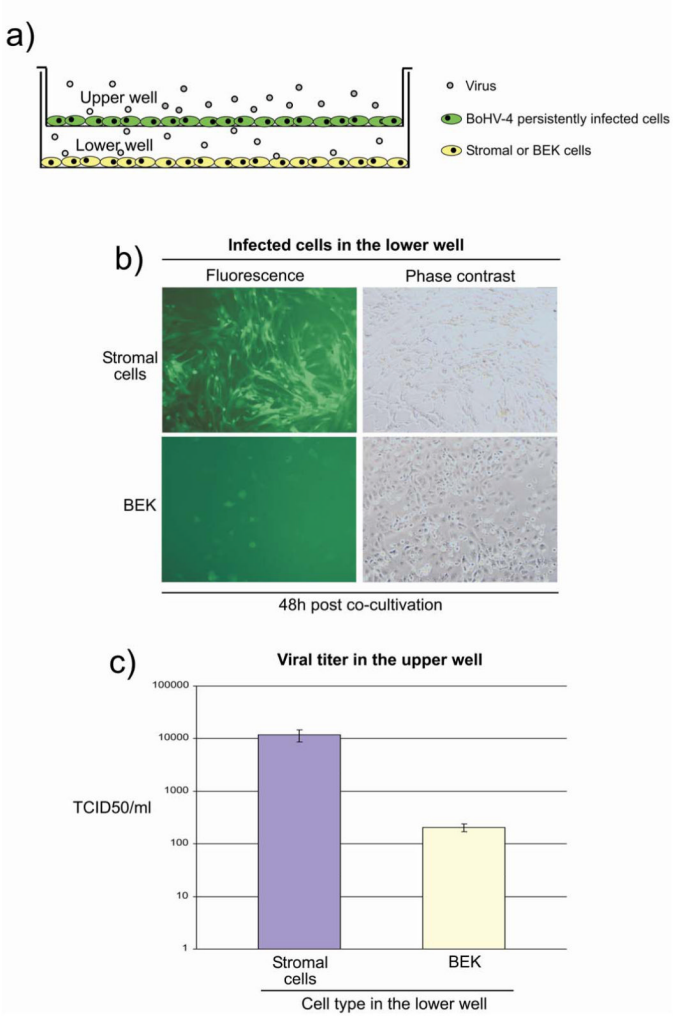

Fig. 7.

a) Schematic illustration of the transwell model used for co-culturing BoHV-4EGFPΔTK persistently infected bovine macrophage cell line (in green in the upper well) producing low amount of infectious virus (grey dots) with bovine endometrial stromal or control BEK cells (in yellow) seated in the lower well. The transwell consists of a permeable membrane and allows diffusion of the viral particles. b) Fluorescence and phase contrast images of bovine endometrial stromal and control BEK cells seated in the lower well at 48 hours (48h) post co-cultivation. c) Graph bar of the viral titer in the upper well. Values are the mean ± S.D. of three independent experiments (n= 3 and P≤0,05).