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Caprine arthritis encephalitis virus (CAEV) replicates productively in cultured epididymal cells from goats

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ABSTRACT

The transmission of CAEV from male goats has not been well studied and the target cells that support viral replication are not well characterized. Epididymal epithelial cells (EECs) are important and play a key role in the fertility and motility of spermatozoa. During their transit, spermatozoa incorporate several EEC-produced proteins into their plasma membranes to stabilize them and prevent premature acrosomal reaction. This intimate interaction between spermatozoa and EECs may increase the likelihood of the infection of semen with CAEV if epididymal tissue is productively infected and sheds the virus into the duct. The aim of this study was to examine whether goat EECs are susceptible to CAEV infection in tissue culture. Cells were isolated from epididymides obtained from goats that were sampled from a certified-CAEV-free herd. Cultured cells were then inoculated with a molecularly-cloned isolate of CAEV (CAEV-pBSCA). Inoculated cells developed cytopathic effects (CPE), showing numerous multinucleated giant cells (MGC) in cell-culture monolayers. Expression of CAEV proteins was detected by immunofluorescence using an anti-p28, Gag-specific antibody. The culture medium of inoculated cells was shown to contain high titers (10^6 tissue culture infectious doses 50 per ml ($TCID_{50}$/ml)) of infectious, cytopathic virus when assayed using indicator goat synovial membrane (GSM) cells. Our findings clearly demonstrate that cells of the buck genital tract are targets of CAEV and are thus a potential reservoir that sheds infectious CAEV into the semen of infected animals. These data suggest the use of sperm from CAEV-free goat males for artificial insemination in genetic selection programs to minimize CAEV dissemination.

1. Introduction

CAEV is a member of the lentivirus genus of the Retroviridae family of single-stranded RNA viruses. CAEV causes progressive inflammatory lesions in several organs of infected goats. The high prevalence of CAEV is a major concern in industrialized countries. Transmission of the virus occurs mainly through a vertical route from infected

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dams to newborn or young kids following the ingestion of infected cells in the colostrum and milk [4,30]. Therefore, CAEV eradication programs are mainly based on the separation of dams from their offspring at birth. Kids are then reared on bovine colostrum and milk. Lateral transmission most likely occurs *via* droplets or infected cells discharged from the respiratory tract [42] and the practice of animals feeding from the pooled colostrums or unpasteurized milk [9].

Sexual transmission, which is considered less efficient, has become of interest because infected cells have been detected in the female and male reproductive tracts. Indeed, in nanny goats, the presence of CAEV proviral DNA has been clearly shown in the uterus, oviduct, ovary [10], and *cumulus oophorus* cells surrounding the oocytes [1]. Viral transcripts and proviral DNA have also recently been detected in the male genital tract tissues (*i.e.*, testis, epididymis, vas deferens, and vesicular gland) and semen from naturally infected male goats [2,8,31].

However, Ali Al Ahmad's study reported the resistance of spermatozoa to CAEV infection *in vivo*, suggesting that the gamete is not a source of infectious virus [16,22]. Possible sources are monocytes or macrophages, which are considered to be the principal target cells of virus *in vivo* or epithelial cells, which can commonly be found in the semen. The tropism of the goat lentivirus for cells of the epithelial lineage has been widely demonstrated in a large variety of animal tissues [3,19–21,23,45], and the epididymal epithelium has been shown to be a principal reservoir for small ruminant lentivirus expression [31]. The visna/maedi virus, an ovine lentivirus that is closely related to CAEV, was found in the epididymides of rams that had been experimentally infected with *Brucella ovis*, and its tropism for epididymal epithelial cells was responsible for the semen infection [33].

Because the goat lentivirus has been detected in the specialized epithelial cells that line the lumen of the epididymis of naturally infected bucks, the ability of epididymal epithelial cells (EECs) to harbor and replicate this pathogenic agent should be investigated.

The aim of this study was to determine whether EECs from virus-free bucks were susceptible to CAEV infection *in vitro* and able to productively replicate the virus.

2. Materials and methods

2.1. Animal and cell isolation

Epididymides were removed aseptically from anaesthetized, healthy, 3-year-old Alpine goats that were sampled from certified-CAEV-free French herds. Briefly, primary EECs were isolated from dissected epididymides as follows: each epididymis was excised and dissected to remove fat and large blood vessels. Epididymal tubule fragments (approximately 2–5 mm in length) were obtained from caput, corpus and cauda epididymis and transferred into 0.25% trypsin solution diluted in calcium- and magnesium-free PBS (Gibco-BRL, France) supplemented with 0.02% EDTA. After incubation at 37 °C for 30 min in a thermo-bath shaker (60 cycles/min), samples were centrifuged at 800 × *g* for 5 min, supernatant fluids were discarded and the cell pellets were resuspended in MEM supplemented with collagenase (1 mg/ml, type XI, Sigma, La Verpillère, France). After incubation at 37 °C for 40 min in a thermo-bath shaker as above, the samples were centrifuged at low speed (800 × *g*) for 5 min. The supernatant was discarded, and the cell pellet was resuspended in collagenase (0.25 mg/ml) solution diluted in 1× MEM. Single and aggregated epididymal epithelial cells were seeded in 25 cm^2 tissue culture flasks (Nunc, Polylabo, France) at a density of 1×10^6 cells and incubated at 37 °C in 5% CO_2 in a humidified atmosphere. To prevent the possible contamination of primary cultures with fibroblasts and smooth muscle cells [17], the supernatant containing aggregates of epididymal epithelial cells was transferred into a new culture flask after 8–12 h of culture. Under these conditions, non-epithelial cells adhere very rapidly to the surface of the initial flask, leaving EEC aggregates in suspension. The EEC aggregates then adhere to the new culture flasks to establish foci. The medium was replaced every 3 days until the cell monolayers reached subconfluence, and cells were then passaged following trypsin treatment.

Goat synovial membrane (GSM) cells, originally derived from an explanted, carpal synovial membrane from a colostrum-deprived newborn goat [25], were cultured in MEM supplemented with 10% FBS for the production of virus. GSM cells are highly susceptible to lytic, fusogenic infection by visna maedi virus and non-lytic, fusogenic infection by CAEV.

2.2. Virus

CAEV-pBSCA was produced by transfecting GSM cells with the pBSCA plasmid, harvesting the medium and titrating the released virus on fresh GSM cells, as described previously [24]. Virus stocks were titrated on GSM cells and macrophages. Titers were approximately 10^6 $TCID_{50}$/ml.

2.3. Cell infectivity assay and virus titration

EECs were seeded at 10^5 cells per well in 1 ml of media and cultured under standard conditions (37 °C and 5% CO_2 in a humidified atmosphere). When the monolayer became subconfluent, cells were inoculated with CAEV-pBSCA at a multiplicity of infection of 0.1 (MOI = 0.1) and incubated under standard conditions. Six days post-infection, infected cells were fixed in formalin (10%), stained with May-Grünwald-Giemsa, and examined under the microscope for the presence of cytopathic effects (CPE), such as giant multinucleated cells resulting from the fusion of several infected cells. To examine the kinetics of virus production, subconfluent monolayers of cells were inoculated with CAEV-CO at MOI of 1. At 24 h post inoculation cells were rinsed and replenished with fresh medium. Every 24 h during 6 days post infection, culture supernatants of infected cells were harvested, filtered through a 0.45 μm membrane and serially diluted in medium. Ten-fold dilutions were used to inoculate GSM cells in 24-well plates, and infected cells were maintained in culture for 6 days. CPE that developed in the monolayers were scored, and virus titers were calculated using the Reed–Muench method [34]

and expressed as tissue culture infectious doses ($TCID_{50}$) per ml of supernatant.

2.4. Immunofluorescence

EECs were grown to subconfluence in 6 well plates, infected with CAEV-CO at MOI = 0.1 and 24 h later transferred into 8-chamber slides (LabTek, Dutscher, France). After 24 h cells were fixed with cold acetone, rinsed in PBS for 5 min and then incubated in a buffered solution (PBS + 10% donkey serum) for 30 min at room temperature. Monoclonal antibodies directed against cytokeratin (Interchim-N75350) and CAEV Gag-p28 (CAEP5A1-VMRD), respectively, were diluted 1:100 in 1× PBS supplemented with 1% BSA and used to detect the specific marker of epithelial cells and the viral antigen following 1 h incubation at 37 °C. Cell monolayers were rinsed with 1× PBS and then incubated (1 h at 37 °C) with a secondary donkey-anti-mouse IgG antibody Alexa Fluor 488 (A21202-Invitrogen) or donkey-anti-mouse IgG antibody Alexa Fluor 555 for fluorescent staining of epithelial cells and CAEV, respectively. Cells were then rinsed with 1× PBS and counterstained with TO-PRO®-3 iodide (Life-Technologies, Saint Aubin, France) before mounting with Dako fluorescent medium (DAKO – S 302380). Unrelated murine antibodies of the same Ig class were used as negative controls, and appropriate epithelial and infected cells used as positive controls.

2.5. Nested PCR amplification of proviral DNA

Infected or control EECs and infected or freshly prepared GSM cells (10^5 cells) were lysed as previously described [7]. Total DNA was purified using a QIAamp DNA kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The presence of proviral CAEV DNA was examined using nested PCR [13]. CAEV gag sequences were amplified using primers GEX5 (5′ GAA GTG TTG CTG CGA GAG GTG TTG 3′) and GEX3 (5′ TGG CTG ATC CAT GTT AGC TTG TGC 3′) corresponding to bases 393–416 and 1268–1291 of CAEV-CO, respectively [36]. An initial denaturation at 94 °C for 3 min was followed by 35 rounds of amplification (denaturation: 94 °C for 1 min, annealing: 46 °C for 1.5 min and extension: 60 °C for 2.5 min). Five microliters of the resulting PCR product were used for a second round of amplification using the internal primers GIN5 (5′ GAT AGA GAC ATG GCG AGG CAA GT 3′) and GIN3 (5′ GAG GCC ATG CTG CTG CAT TGC TAC TGT 3′) located at positions 524–546 and 1013–1036 in CAEV-CO, respectively. Sample DNA integrity was confirmed by amplifying the β-actin gene using primers based on the human sequence [15]. Amplified bands were visualized by ethidium bromide staining after electrophoresis through a 1.5% agarose gel. This technique is able to detect less than 10 infected cells in total DNA isolated from 10^6 or 10^7 cells [7].

3. Results

3.1. Growth pattern of cultured EECs

Epithelial cells isolated from the caput, corpus and cauda of the epididymis were seeded at a density of 10^5 cells in 25-cm^2 culture flasks. They appeared as spherical-shaped aggregates and single cells at the time of plating. Within 48 h in culture, the majority of epididymal epithelial cells had firmly attached to the surface of the flask and formed colonies of fibro-epithelial or typical epithelial (polygonal) cells. Monolayers reached confluency after approximately 6 days. The initial monolayers were typically strewn with sparse, scattered macrophages, which were lost following the second or third passage, thus leaving homogenous epithelial cell culture monolayers. Cultures of EECs remained vigorous and healthy even after numerous passages (Fig. 1).

Cytokeratin-containing filaments of intermediate size are characteristic of epithelial cells and are restricted to cells of epithelial origin [12,27]. The great majority of cells used in this study expressed this cytokeratin marker in the early passages, and, during the passages, the cell populations became 100% positive for the cytokeratin marker regardless of the cell morphology (Fig. 3).

3.2. Detection of natural CAEV infection of EECs

To determine if these EECs were free from CAEV antigens and proviral genomes, we used an anti-p28 Gag antigen detection kit and nested PCR, respectively. Our results demonstrated that none of the monolayer cultures were positive for the Gag antigen, and, similarly, none of the DNA isolated from these culture cells contained the CAEV proviral genome. Taken together, these results indicate that epididymal epithelial cells harvested from bucks sampled from a certified-CAEV-free flock were free from natural infection with CAEV.

3.3. Susceptibility of epididymal epithelial cells to CAEV infection

To examine whether EECs are susceptible to CAEV infection, we derived primary cultures of EECs from the caput, corpus and cauda. The cell monolayers were then inoculated with CAEV-pBSCA at an MOI of 0.1. Inoculated cells were maintained in culture conditions and regularly observed for degeneration. Observation of the monolayers at 6 days post-inoculation revealed that all 3 types of cultures underwent multinucleated giant cell (MGC) formation (Fig. 1).

3.4. Amplification of viral-specific sequences from DNA of infected cells

To determine if the EEC monolayers that were inoculated with CAEV and underwent typical fusion have proviral genomes, we isolated total DNA that was then used as template for PCR amplification of CAEV *gag*-specific sequences. As shown in Fig. 2, a strong band corresponding to the expected 512-bp PCR product was detected in samples of CAEV-inoculated EECs and the positive control. In contrast, this band was absent in the DNA from uninfected cells used as a negative control. The 393-bp fragment corresponding to the product of PCR amplification of the endogenous β-actin gene was equally present in all

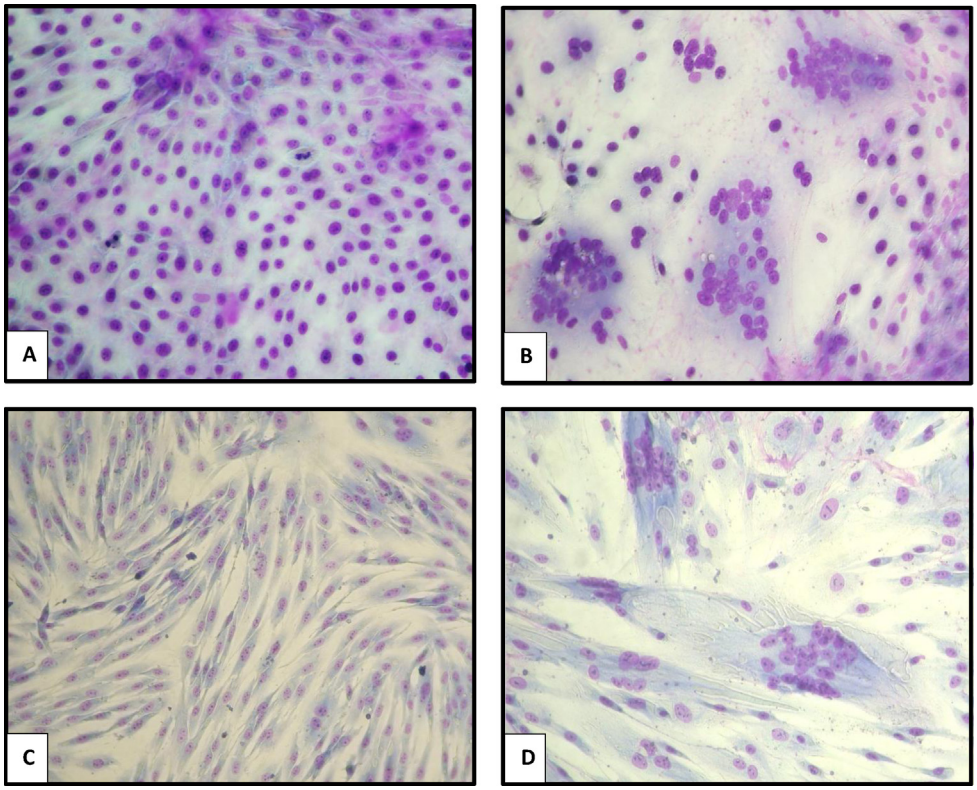

Fig. 1. Cytopathic effects of CAEV in goat EEC and GSM cells. Monolayers of cultured goat EEC and GSM cells were kept uninfected (a and c, respectively) or infected with CAEV-CO at MOI = 0.1 (b and d). At day 6 post-infection cells were stained with May Grunwald Giemsa and then observed under photonic microscope. Multinucleated giant cells are visible in both EEC and GSM infected monolayers. Magnification 200×.

samples. These data clearly show that the cytopathic effects observed in the monolayers following inoculation with CAEV are associated with the presence of CAEV provirus.

3.5. In situ detection of viral antigen expression in EECs by immunofluorescence

We used a specific anti-p28 mAb to examine if EECs inoculated with CAEV express viral antigens that can be detected by immunofluorescence *in situ*. As shown in Fig. 3, there were positive signals corresponding to the accumulation of viral antigens in the cytoplasm of all EECs cultures infected with CAEV, but not in the cytoplasm of uninfected control cells. This result indicates that inoculating EECs with CAEV induced the expression of the major capsid viral protein. Whether this protein was assembled into infectious viral particles remains to be determined.

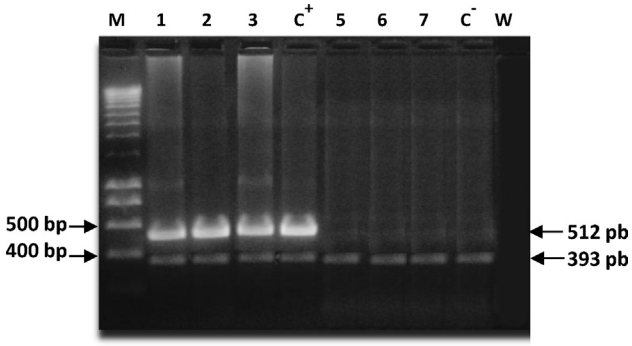

Fig. 2. CAEV proviral-DNA in infected EECs. DNA was isolated from cultured uninfected or CAEV-infected EECs and used to performed nested-PCR using specific sets oligonucleotide primers to amplify both the 512-bp CAEV gag and 393-bp-actin fragments. Nested-PCR products were separated in 1.5% agarose gel and bands visualized by staining with ethidium bromide. M: 100-bp DNA Ladder used as a molecular weight standard. Lanes 1–3: DNA isolated from CAEV-infected epididymal epithelial cells (Caput, corpus and cauda, respectively). C+: positive control (DNA from infected GSM cells). Lane 5–7: negative controls (DNA from non-infected caput, corpus and cauda EECs). C−: negative control (DNA from non-infected GSM cells). W: distilled water (Control lacking template DNA).

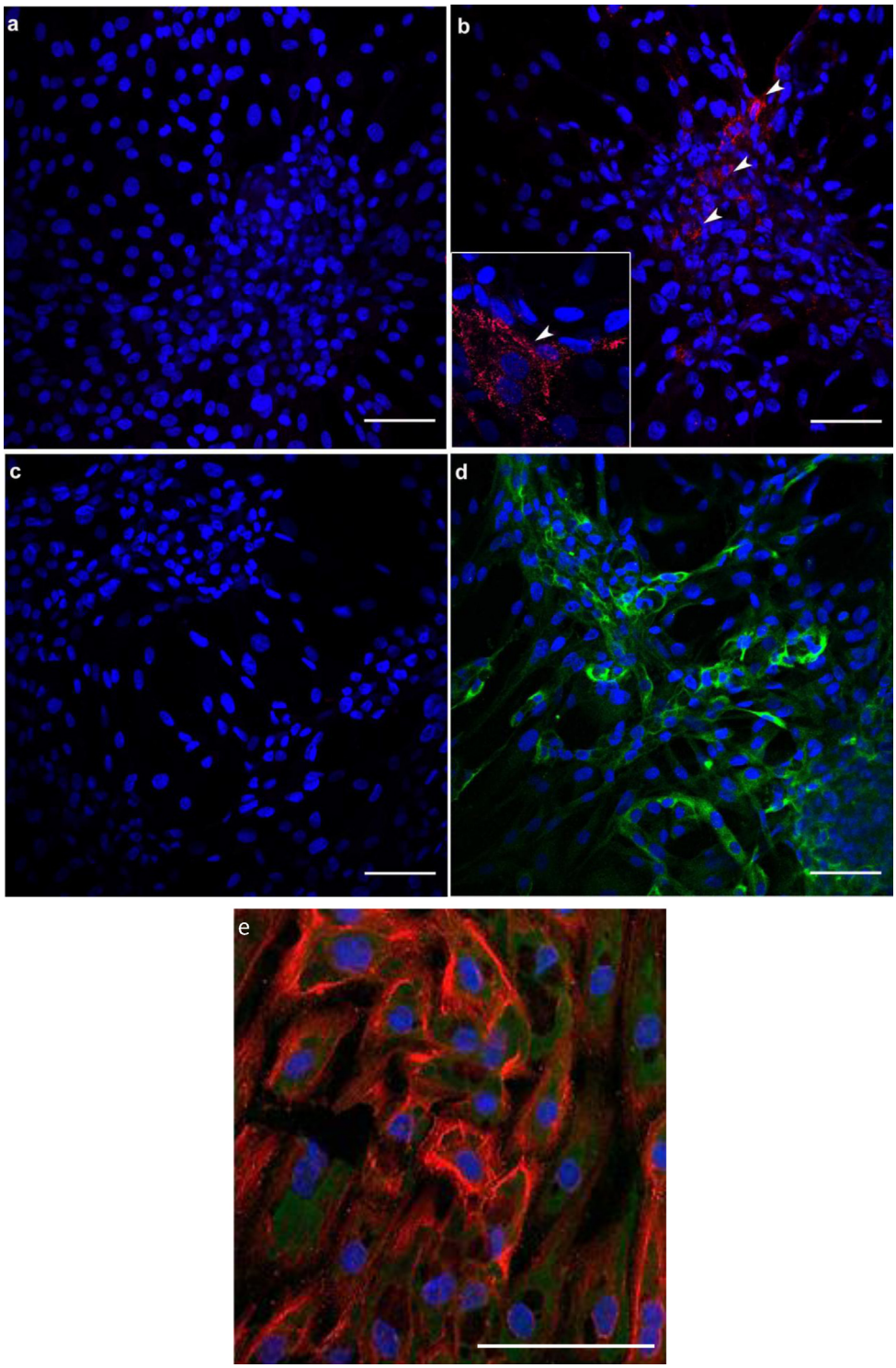

Fig. 3. Immunostaining of CAEV antigen and the endogenous cytokeratin epithelial marker in EECs. Monolayers of EEC cultures were infected with CAEV at MOI = 0.1, transferred into cell culture slide chambers and then fixed and stained as described in Section 2. (a and c) Cells stained with TO-PRO®-3 iodide only. (b) Cells were stained with anti-CAEV Gag p28 monoclonal antibody (mAb) and then counterstained with TO-PRO®-3 iodide. (d) Cells were stained with anti-cytokeratin mAb and then counterstained with TO-PRO®-3 iodide. (e) Cells were double labeled with anti-CAEV Gag-p28 and anti-cytokeratin mAbs and then counterstained with TO-PRO®-3 iodide.

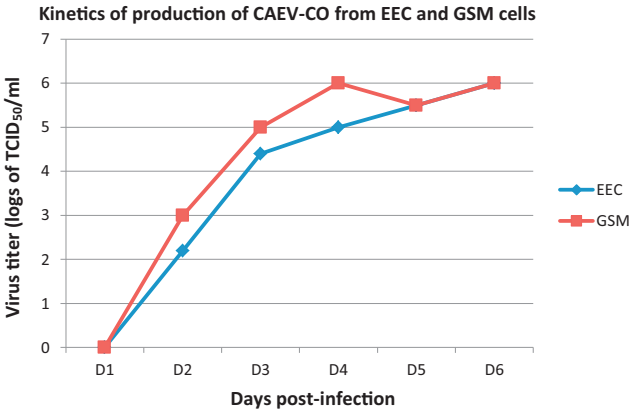

Fig. 4. Kinetics of CAEV production in EEC and GSM cells. Monolayers of EECs and GSM cells were inoculated with CAEV at MOI = 1 and progeny virus harvested every 24 h during 6 days. Titers of infectious cytopathic virus in the supernatants were determined following serial dilutions and inoculation of the indicator GSM cells as described in Section 2. Values of virus titers were used to plot the kinetic curves.

3.6. Productive replication and high titer production of CAEV in EECs

To examine if CAEV-infected EECs allow productive replication of the virus, we used serial dilutions of the culture medium that had been harvested at day 6 post-inoculation to inoculate fresh GSM cells. Inoculated cells were then observed and stained at day 6 post-inoculation. The results show typical cytopathic effects in inoculated monolayers of GSM cells, and the titration of this viral stock revealed the presence of 10^6 $TCID_{50}$/ml of CAEV regardless of the epididymal origin of epithelial cells. Moreover, to compare the kinetics of virus production in EECs and the highly permissive indicator GSM cells, supernatants of infected EECs and GSM cells were daily harvested (D1–D6) and used in serial dilutions to inoculate fresh GSM cells for virus titer determination. Results of these titrations are represented in Fig. 4, showing the ability of EECs to replicate the virus. These data clearly show that EECs are fully permissive to CAEV and replicate the virus with a similar efficacy to that observed in the highly permissive GSM cells and the natural target macrophages. These results suggest that these cells may play a role in virus amplification and/or serve as a reservoir *in vivo*.

Altogether, these data clearly demonstrate that cultured EECs are susceptible to CAEV infection and allow the virus to replicate productively and to high titers.

4. Discussion

Although there is no doubt that CAEV can infect the male genital tract tissues and the virus can be excreted into the semen from naturally infected bucks [2,8,31], there has been no characterization of target cells that could support viral replication. For this purpose, we performed an *in vitro* study to examine the susceptibility of EECs to CAEV infection. These cells line a portion of the male genital tract and are very important for the stability and motility of the spermatozoa.

The results of this study clearly show that, regardless of the morphology assumed in tissue culture, EECs from CAEV-free bucks expressed the epithelial cytokeratin marker and were susceptible to CAEV infection *in vitro*. In culture, cell monolayers were also found to harbor the proviral genome, which was detected by nested PCR. In addition, the spread of the virus to cells in the monolayer and the high titer of infectious virus released into the culture medium of infected EECs cultures clearly demonstrated a productive replication of the virus in these cells.

In ejaculates, we can distinguish different cells types other than sperm cells, such as macrophages, immature germ cells, and epithelial lining cells, among which are the epithelial cells of the epididymis [31]. These latter cells are important for the acquisition of fertilizing power and spermatozoa motility. Indeed, during their transit, spermatozoa receive several proteins from EECs that stabilize their plasma membrane and prevent a premature acrosomal reaction [40]. This intimate interaction between spermatozoa and EECs could increase the likelihood of semen infection if a productive CAEV infection of the epididymal tissue results in the shedding of the pathogen into the duct.

The results of our *in vitro* study are in favor of direct EEC infection. Indeed, following three serial passages, we obtained homogenous epithelial cell culture monolayers (high proportion of cytokeratin positive cells) that were free of macrophages. Despite the absence of macrophages, EECs were able to productively replicate the virus with high titers.

In vivo, the viral genome has been detected in tissue samples of epididymis [2,31], which could be implicated in the infection of semen. Indeed, the presence of CAEV proviral DNA in the epididymal semen of naturally [8,31] or experimentally infected male goats [43] has been reported. However, it was unclear whether the infection of epithelial cells in the epididymis tissue occurred directly by free CAEV particles or indirectly through cell-to-cell contact with infected macrophages, which are the main target of the goat lentivirus *in vivo* [16,22].

There are reports showing that macrophages and T cells found in rat epididymal epithelium are able to cross into the epididymal duct [11,35], and it is believed that HIV-1-infected $CD4^+$ T cells and macrophages from the epididymis are shed into the semen during the course of

HIV-1 infection [26,28]. Moreover, the detection of infected immune cells adhering to the epididymis of SIV-infected macaques suggests that virus particles and infected cells are susceptible to be released into the seminal fluid and contribute to its viral load [37]. Whether these infected permissive cells do transmit the virus to EECs and these latter participate in the viremia is still unclear.

All of these findings favor the infection of semen through infected immune cells, suggesting that the epididymis serves as a transit point for viruses harbored by these immune cells. It is well known that many lentiviruses replicate in epithelial cells. HIV-1 infection of mammary gland epithelium may contribute to milk-borne transmission [41], and initial infection might involve susceptible epithelial cells present in the reproductive tract [14]. Feline immunodeficiency virus [29], bovine immunodeficiency virus [44] and equine infectious anemia virus [6] have also been shown to infect epithelial cells, among others, and epididymis epithelium has been presented as a principal site of retrovirus expression in the mouse [18]. Transmission of these lentiviruses to epithelial cells from lymphocytes or monocytes could occur by a cell-to-cell contact mechanism [5,32], as reported for HIV-1 in the human reproductive tract [14,39]. While this mechanism was shown to occur for CAEV in cultured cells [38], it never has been demonstrated in goat reproductive tissues.

The present study reveals, for the first time, that cultured, goat epididymal epithelial cells are susceptible to CAEV infection and do replicate the virus. These results indicate that cells of the genital tract of bucks can be targets of CAEV and a likely source of CAEV proviral DNA in semen.

From an epidemiological point of view, this finding is interesting because it provides insight into the environment of the genital tract in which evolves the semen and supports the possibility that goat lentiviruses could be spread by ejaculation during mating or artificial insemination. Knowing that the classic management practices recommended for CAEV control are insufficient in CAEV eradication programs, the need to use tested, CAEV-free males as sperm donors for artificial insemination in genetic selection programs is justified.

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