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Immortalized goat milk epithelial cell lines replicate CAEV at high level

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Abstract – Primary milk epithelial cells were isolated from CAEV-uninfected goats and three cell lines designated TIGMEC-1, TIGMEC-2 and TIGMEC-3 were established. The three cell lines retained their morphological characteristics of epithelial cells and expressed specific epithelial cytokeratin marker as well as the immortalizing SV40 large T antigen. The kinetics of growth of TIGMEC1, TIGMEC2 and TIGMEC3 cell lines showed a doubling time of 24-48 hours while the parental cell lines became senescent after the passage 6 in cell culture. Like the parental primary cells, the three cell lines were found to be highly sensitive to CAEV-pBSCA, an infectious molecular clone of CAEV-CO strain, and to a French isolate CAEV-3112. TIGMEC cell lines infected with CAEV-pBSCA became chronically infected producing high virus titers in absence of cytopathic effects. These cell lines may be useful for study of the possible physiological alterations in mammary epithelial cells infected with small ruminant lentiviruses and their consequences on milk quality. On an other hand, these cell lines can be used to study their role in virus transmission and pathogenesis.

CAEV / replication / epithelial cell line

Résumé – Des cellules épithéliales du lait immortalisées répliquent le CAEV avec une grande efficacité. Des cellules épithéliales primaires isolées à partir de chèvres non infectées par le CAEV ont été immortalisées pour dériver 3 lignées appelées TIGMEC-1, TIGMEC-2 et TIGMEC-3. Les cellules des 3 lignées ont conservé leurs caractéristiques morphologiques de cellules épithéliales et expriment la cytokératine, un marqueur spécifique des cellules épithéliales et l'antigène immortalisant T du virus SV40. La courbe de croissance des lignées TIGMEC-1, TIGMEC-2 et TIGMEC-3 montre une multiplication des cellules toutes les 24 à 48 heures alors que les cellules parentales ne

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se divisent plus après 6 passages en culture. Comme les cellules primaires dont elles dérivent, ces lignées se sont avérées sensibles à l'infection par le CAEV-pBSCA, un clone moléculaire infectieux de la souche CAEV-CO, et un isolat français le CAEV-3112. Les lignées TIGMEC infectées avec le CAEV-pBSCA deviennent chroniquement infectées et produisent des titres élevés de virus en l'absence d'effet cytopathogène. Ces lignées pourront être très utiles pour étudier les altérations physiologiques éventuelles liées à l'infection de cellules épithéliales par les lentivirus des petits ruminants et pouvant avoir des conséquences sur la qualité du lait. Par ailleurs, ces cellules pourraient être utilisées pour étudier le rôle des cellules épithéliales du lait dans la transmission du virus et l'induction de pathologie.

CAEV / réplication / lignées cellulaires épithéliales

1. INTRODUCTION

Caprine Arthritis Encephalitis virus (CAEV) is the natural lentivirus of goats, and induces mainly arthritis and mastitis in infected animals [9, 20]. CAEV mastitis presents a hardening of the udder during lactation period with diminution of milk production, and involves an important interstitial infiltration and accumulation of lymphocytes. Lymphocytes are not susceptible to infection by CAEV, which has been shown to target cells of the monocyte-macrophage lineage, but we have recently shown that primary mammary epithelial cells readily support CAEV infection and may be involved in the pathogenesis of mastitis [24].

Viral infection of epithelial cells in target organs often results in release of biologically active factors, which act as crucial components in the resulting pathology. Infection of bronchial epithelial cells by influenza A virus induces expression of interleukins IL-6, IL-8 and RANTES [1, 10, 22, 23] which attract monocytes, T lymphocytes, eosinophils and basophils, contributing to the development of airway inflammatory disease. Alveolar epithelial cells infected by respiratory syncitial virus contribute to acute episodes of respiratory obstruction [29, 33] and infected cell lines show increased expression of both intracellular – and vascular cell – adhesion molecules (ICAM-1 and VCAM-1, respectively) which contribute to inflammatory cell adherence [4, 33]. Such cultures also release IL-6 and IL-8 which may contribute to recruitment and activation of inflammatory and immune effector cells [2, 16].

Human immunodeficiency virus (HIV-1) has been shown to be responsible for induction of enteropathy by infecting intestinal epithelial cells [12, 13] and disregulating their function (for review, see [18]). Intestinal epithelial cells infected by HIV-1 show distorted differentiation [13] and altered glucose metabolism [23], ion exchange [12] and absorptive and secretory functions [3, 14].

In order to investigate the complex interactions between CAEV and mammary epithelial cells and their role in the development of mastitis, we needed permanent epithelial cell lines derived from the relevant tissues. We have therefore immortalized cells from three different preparations of milk epithelial cells from 2 goats using the SV40 large T antigen, and established permanent lines: TIGMEC-1, TIGMEC-2 and TIGMEC-3. All three lines conserved epithelial characteristics and constitutively expressed the large T antigen. Moreover they were found to be highly sensitive to CAEV infection and to replicate the CAEV-pBSCA strain in absence of cytopathic effect.

2. MATERIALS AND METHODS

2.1. Animals

Saanen goats were sampled from flocks in the area of Rhône-Alpes (France). These flocks were periodically tested for small ruminant lentivirus infection by Agarose gel immunodiffusion method . Sampled animals were first tested serologically using the ELISA test (P00301/04, Institut Pourquier, Montpellier, France). ELISA negative animals were tested for virus isolation from their monocyte-derived macrophage cultures [7] and by PCR [8] on DNA isolated from macrophage cultures. Only animals that were found negative by all the methods used were declared CAEV free.

2.2. Viruses

CAEV-pBSCA virus was produced from pBSCA, a plasmid carrying the complete CAEV genome of the CO strain [25]. pBSCA plasmid DNA was introduced into goat synovial membrane cells (GSM) cells by transfection, then culture medium containing released virus particles was harvested and titrated. CAEV-3112 French field isolate was obtained from explants of the synovial membrane of a naturally-infected arthritic goat [5]. Titers of virus stocks tested on GSM cells and macrophages were found to be around 10^6 Tissue Culture Infectious Dose (TCID50) /mL.

Inoculation of cells was performed at an M.O.I. of 0.1 on monolayer cultures seeded 24 hours before in 24 well plates and were maintained in culture for 6 days. The cells were then fixed, stained with May-Grünwald-Giemsa and examined for the presence of giant multinucleated cells. Titers were calculated using the Reed and Muench method [27] and expressed as TCID50 per mL of culture medium.

2.3. Indicator cell lines

GSM cells were originally obtained from an explant of carpal synovial membrane from a colostrum-deprived newborn goat [26]. The cells were expanded by cultivation in Minimum Essential Medium with 10% fetal bovine serum (FBS), then stored in liquid nitrogen. Typical monolayer cultures were passaged at 1:3 split ratios and used for 7-10 passages. GSM cells are highly susceptible to lytic and fusiogenic infection by Maedi-Visna virus, and non lytic fusogenic infection by CAEV.

2.4. Goat milk cell isolation and culture

Goat milk secretions were obtained from 2 healthy nannies 4 weeks post-parturition. Cells (2 to 3×10^6) were isolated from 100 milliliters of milk from each goat by centrifugation at $500 \times g$ for 10 min at 4 °C, then cell pellets were rinsed twice with Hank's balanced salt solution (HBSS) to remove the residual milk and fat globules. Cells were resuspended in 10 mL of R10 (RPMI medium supplemented with 10% FBS), seeded into two 25 cm^2 tissue culture flasks at a density of $2\text{-}5 \times 10^5$ cells/mL and incubated at 37 °C overnight. Non-adherent cells were washed off by two or three rinses with HBSS, then fresh R10 medium was added and cells were incubated at 37 °C, 5% CO_2. R10 medium was replenished every 3-4 days until sub-confluent cell monolayers developed. The initial culture monolayers contained mainly monocyte/macrophage cell types that differentiated into mature macrophages, as identified by morphological changes (enlargement of cells) and very strong adhesion. After 2 to 3 weeks in culture, foci of epithelial cells, morphologically clearly distinguishable from macrophages, emerged and, after two serial passages, homogenous epithelial cell culture monolayers were obtained [24].

2.5. Plasmid and DNA transfection

Goat milk epithelial cells (GMEC) were immortalized using pMK16-SV40-(ori) [17] as previously described [11] to generate large T antigen expressing GMEC (TIGMEC) cell lines. DNA was introduced into cells using LipofectAmine (Gibco BRL, Cergy-Pontoise, France). Briefly, goat milk epithelial cell cultures at passage 3 were dissociated by trypsin treatment, resuspended in R10 medium and seeded at 10^5cells/well into 6 well plates. After 24 hours, cells were rinsed 3 times with serum free medium and inoculated with LipofectAmine/DNA complex. For each well, 2 µg of DNA were mixed with 5 µg of LipofectAmine in 100 µL final volume and incubated 30 min at room temperature, then loaded into 1 mL of RPMI 2% FBS medium on the cell monolayer. Cells were incubated (37 °C, 5% CO_2) for 18 hours, washed 3 times with serum-free medium, dissociated with trypsin and transferred into 25 cm^2 flasks in R10. Medium was replaced every 3-4 days and subconfluent cell monolayers were passaged following trypsin treatment.

2.6. Immunocytochemistry

Cell monolayer cultures were developed on treated chamber slides (Merk Eurolab, Strasbourg, France). Cytokeratin, a specific marker of epithelial cell, was detected on acetone-fixed cells by immunocytochemistry using the specific monoclonal antibody clone K 813 (Sigma, L'Isle d'Abeau, France) diluted 1/50 in 1X PBS. TIGMEC cell lines were characterized by immunocytochemical detection of SV40 large T antigen using a specific monoclonal antibody (Ab-2, Oncogene Science, Paris, France) diluted 1/50. Typically cell monolayers were incubated with monoclonal antibody for 10 min at room temperature, then rinsed with 1X PBS and incubated 30 min at room temperature in a solution containing 1X PBS, 1% BSA and 0.5% of biotinylated goat anti-mouse Ig purified antibody (Dako, Trappes, France, Kit ref: K0377). Cells were again rinsed with 1X PBS, then incubated 30 min at room temperature in a 1X PBS solution containing streptavidin-biotin-peroxydase complex as recommended by the supplier. Following a 1X PBS wash, the cells were incubated 8 min with a solution of 1mg/mL of diaminobenzidine in 1X PBS. Appropriate positive and negative controls were set up for each antibody.

2.7. Kinetics of cell proliferation and dependence of TIGMEC lines on fetal bovine serum

TIGMEC lines and their parental primary GMEC were seeded at a density of 2×10^4 cells/well in 24 well plates and grown in R10 medium. Every 24 hours, quadruplicate wells were dissociated with trypsin and aliquots were counted to determine the total cell count for each well. The average number of cells, calculated every 24 hours, was used to plot kinetic proliferation curves for TIGMEC and MEC.

To study whether TIGMEC lines are dependent on high serum concentration, cells were seeded in 24 well plates at a density of 2×10^4 cells/well in RPMI medium containing 0, 1, 2, 5 and 10% FBS. Quadruplicate wells from each culture were dissociated with trypsin every 24 hours and counted to determine the total number of cells per well. The average daily values were used to plot proliferation kinetics for the different culture conditions.

2.8. Immunoprecipitation of virus specific proteins

Radio-immunoprecipitation was performed as previously described [7]. Briefly, cells were seeded into 6 well plates and, 24 hours later, monolayers were infected with CAEV-pBSCA or CAEV-3112 at an MOI of 0.1, then incubated at 37 °C, 5% CO_2 for 4-5 days. These monolayer cultures were then preincubated for two hours in MEM lacking methionine and cysteine,

and the proteins radio-labeled for 16 to 18 hours with 100 µCi of ^{35}S-methionine/cysteine (Amersham, France) in 1 mL of the same medium. Virus-specific proteins released into the supernatant or localized in the cell lysate fractions were immunoprecipitated using a hyper-immune serum (G9615) from a goat which had received several inoculations of a mixture of 3 different CAEV isolates and MVV-K1514 (Maedi Visna virus K1514). Clarified cell culture medium and cell lysates were incubated overnight at 4 °C in the presence of 10 µL of G9615 serum and protein A Sepharose. Immunoprecipitated proteins were then separated by SDS-PAGE and specific virus proteins were visualized by standard autoradiography.

3. RESULTS

3.1. Goat milk epithelial cell cultures and transfection

GMEC were isolated from 2 healthy nannies negative for CAEV infection (by serology, virus isolation and PCR) and transfected with Large T carrying pMK16 plasmid DNA. Transfected cells were cultured in R10 medium that was renewed every 3-4 days. Following serial passages, three epithelial cell lines were obtained and called TIGMEC-1, TIGMEC-2 and TIGMEC-3 .

3.2. Proliferation of immortalized epithelial cells

The immortalized cells retained the morphological and phenotypic characteristics of their parental GMEC. The ability of epithelial cells to form islets of regularly shaped cells was conserved in the TIGMEC lines.

We compared the growth kinetics of the TIGMEC lines to that of their parental primary GMEC (Fig. 1). The GMEC grew very slowly after 2 passages in culture and became completely senescent after passage 6-7. In contrast to this result, the TIGMEC cell lines showed persistent and active division every 24 to 48 hours and have been

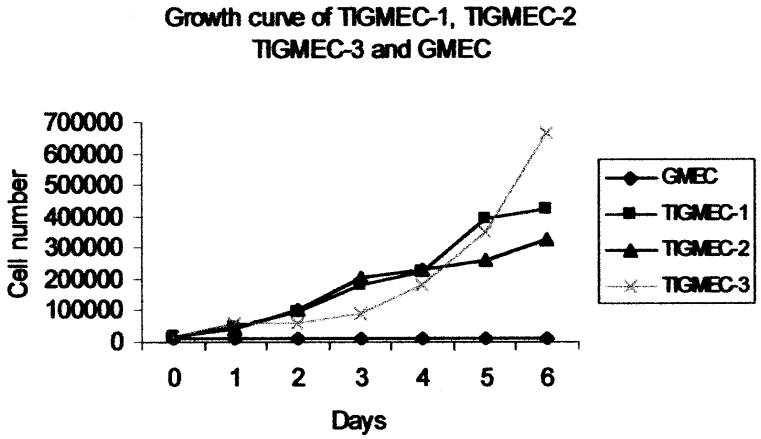


Figure 1. Growth curves of T-immortalized milk epithelial cell lines 1, 2 and 3 (TIGMEC-1, TIGMEC-2 and TIGMEC-3) and parental milk epithelial cells (GMEC). TIGMEC lines and their primary GMEC were seeded at a density of 2×10^4 cells/well in 24 well plates and grown in R10 medium. Every 24 hours 4 wells were dissociated by trypsin treatment and aliquots counted to determine the total cell count for each well. The average number of cells, calculated for each 4 wells every 24 hours was used to draw the proliferation kinetic curves for TIGMEC lines and GMEC.

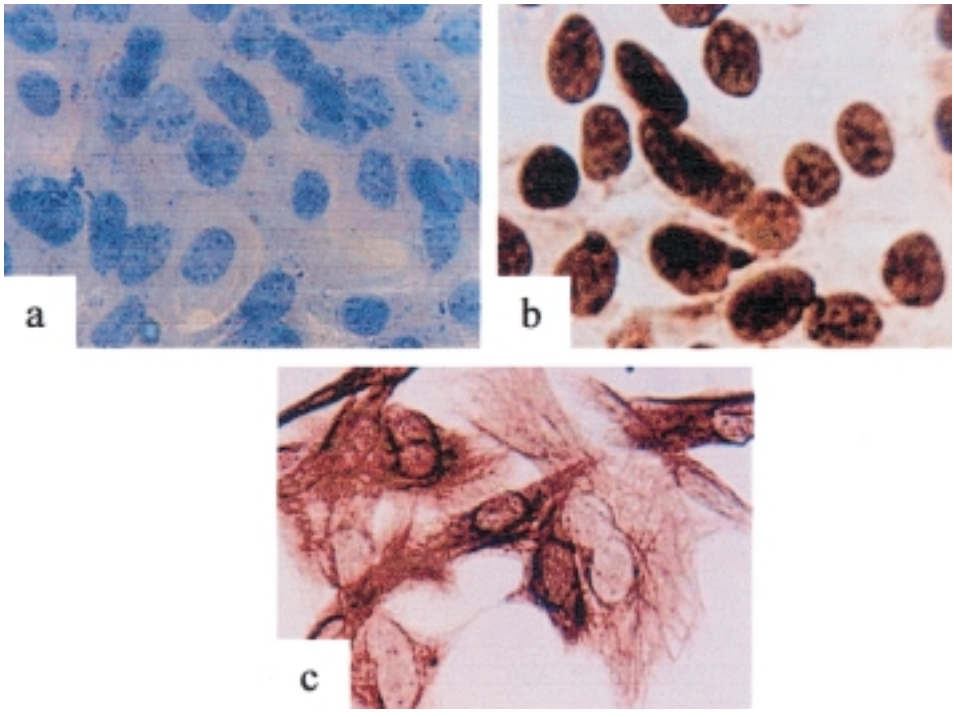


Figure 2. Detection of large T antigen and cytokeratin protein expression in TIGMEC-3. Immunocytochemistry was performed on TIGMEC-3 using the anti-large T monoclonal antibody (b) and the anti-cytokeratin specific antibody (c). Control (a) corresponds to TIGMEC-3 cells treated with PBS and showed no positive cells. TIGMEC-3 line stained with the K813 showed 100% staining both with the large T monoclonal antibody and with anti-cytokeratin antibody.
Original magnification 400 X.

propagated for more than one year without any sign of senescence. Dependence on growth factors that are present in the fetal calf serum was tested by culture of the TIGMEC lines in medium containing 0, 1, 5 and 10% FBS. Our results showed that minimum requirement was 5% FBS for all TIGMEC lines. In absence and in 1 % of serum the cells became non-adherent and died within 3-4 days.

3.3. Immunocytochemical characterization of TIGMEC cell lines (Fig. 2)

Specific monoclonal antibodies were used to detect the expression of the immortalizing T-antigen of SV40 and the cytokeratin specific marker. In the early passages (5-7) T antigen expression that was specifically localized in the nucleus, was observed only in individual foci, but by passage

9-12, 100% of cells in these cultures became positive for T-antigen (Fig. 2 b). In the early passages, the great majority of the cells expressed cytokeratin markers that were detected on the whole cell surface. In the later passage cultures 100% of the cells expressed cytokeratin (Fig. 2 c). These cells were found to be negative for vimentin and actin markers (data not shown).

3.4. In vitro infection of TIGMEC lines with CAEV

To determine whether the TIGMEC lines are permissive to CAEV, cells were inoculated with CAEV-pBSCA or CAEV-3112 virus strains, then the classical properties of CAEV infection were studied. Classical and typical giant multinucleated cells (MGC) and cell lysis were observed in monolayers of TIGMEC infected with CAEV-3112, 4 to 5 days post-infection. In contrast to these results, monolayers of TIGMEC infected with CAEV-pBSCA did not produce any MGC formation or cell lysis, even after serial passages of these infected cells. However, spontaneous cell fusion was observed within 4 to 6 hours following co-culture of CAEV-pBSCA-infected TIGMEC lines with the indicator GSM cells. Similar results were obtained with the three cell lines, those obtained with TIGMEC-3 cell line are presented in figure 3.

To verify that viral proteins were correctly expressed, processed and released from CAEV-infected TIGMEC, radioimmunoprecipitation was performed using our G9615 hyperimmune serum from an experimentally infected goat. The protein profiles obtained were similar to those observed after infection of GSM cells with CAEV-pBSCA or CAEV-3112 (Fig. 4 A and B). The major gag p25 protein was detected both in the cell lysates and in the supernatants of CAEV-pBSCA and CAEV-3112-infected TIGMEC, suggesting that virus was released into the supernatant of the infected cells.

We compared the kinetics of CAEV-pBSCA and CAEV-3112 production by a TIGMEC-3 line and by the commonly used GSM cells. Supernatants of TIGMEC-3 and GSM cells infected with CAEV-pBSCA or CAEV-3112 were harvested on days 1, 3, 5, 7 post-infection, and titers of infectious cytopathic virus were determined. A delay in virus production of CAEV-3112 was found in TIGMEC-3 compared to the GSM cells used as permissive cell controls (Fig. 5). At day 7 post infection virus production increased and was found to be similar for the two cell types. This delay was also observed with CAEV-pBSCA infected TIGMEC lines, with a dramatic reduction of virus production compared to GSM cells (Fig. 5). Later, the virus production rose to reach comparable levels (Tab. I).

Table I. CAEV-pBSCA and CAEV-3112 viral production on TIGMEC lines.

Days post-infection	*GSM*	*TIGMEC-1*	*TIGMEC-2*	*TIGMEC-3*
CAEV-pBSCA				
D5	$10^{5.25}$	10^{3}	10^{3}	10^{4}
D32	ND	ND	$10^{4.5}$	10^{4}
D98	ND	ND	ND	$10^{5.25}$
CAEV-3112	10^{6}	10^{4}	$10^{4.5}$	10^{5}

TIGMEC-1, TIGMEC-2, TIGMEC-3 and GSM cells were infected with CAEV-pBSCA molecular clone and CAEV-3112 field isolate viruses at MOI = 0.1. Virus titers were determined according to the Reed and Muench technique at different days post-infection.

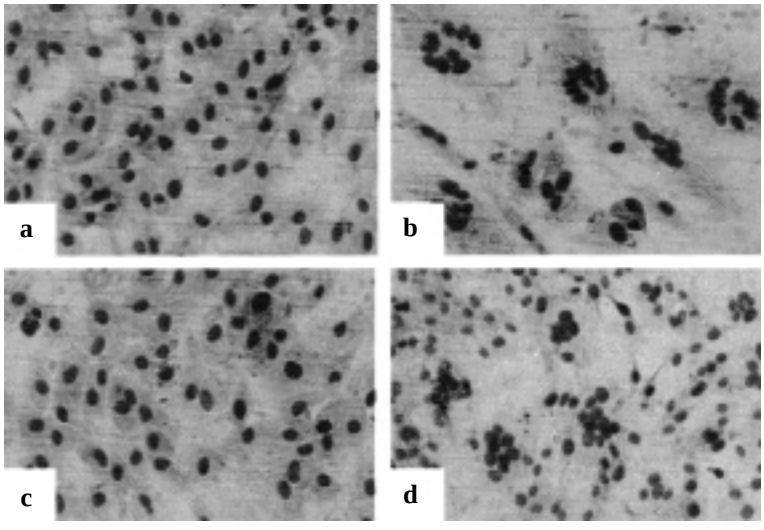


Figure 3. Cytopathic effect developed in TIGMEC-3 cell culture infected with two CAEV strains. Cells were infected with the molecularly cloned CAEV-pBSCA and with the CAEV-3112 field isolate at MOI = 0.1. Uninfected cell monolayer of TIGMEC-3 is shown in (a). Typical giant multinucleated cell formation observed in TIGMEC-3 inoculated with CAEV-3112 field isolate (b). No cytopathic effect was observed after inoculation of the TGMEC-3 with CAEV-pBSCA (c). Coculture of CAEV-pBSCA infected TIGMEC-3 cells with goat membrane synovial cells (GSM) developed giant multinucleated cell formation (d).
Original magnification 100 X.

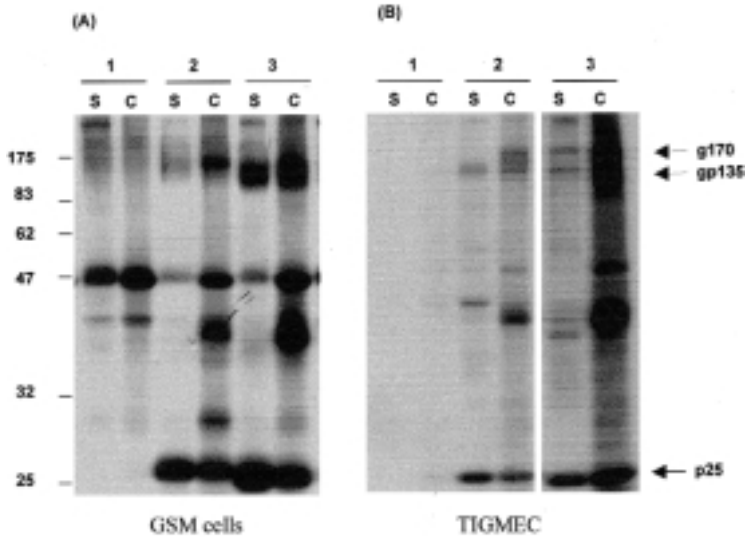
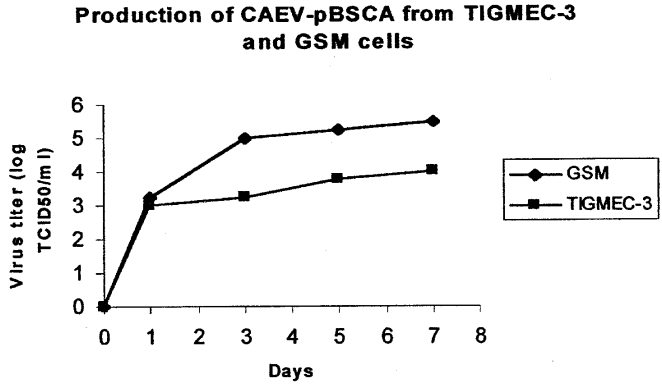


Figure 4. Radio-immunoprecipitation of viral proteins from cultures of TIGMEC lines infected with CAEV-pBSCA molecular clone and CAEV-3112 field isolate. A: Immunoprecipitation of viral proteins from goat synovial membrane cells used as positive control: uninfected (1), infected with CAEV-pBSCA (2) or CAEV-3112 field isolate (3). (S): supernatant, (C): cell lysate. B: detection of viral proteins on TIGMEC-3 line uninfected (1), infected with CAEV-pBSCA (2), with CAEV-3112 field isolate (3).

A



B

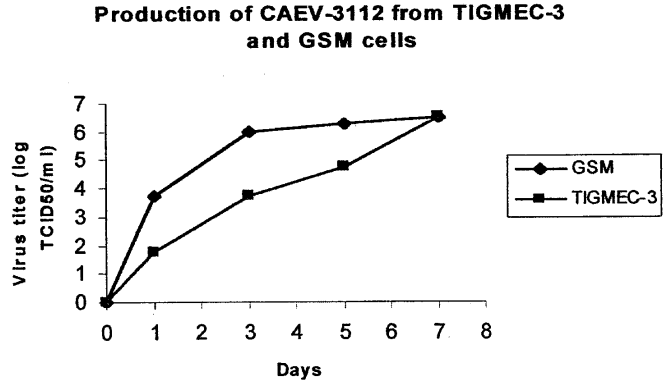


Figure 5. Viral growth curves comparing the production of CAEV-pBSCA and CAEV-3112 on TIGMEC and goat GSM cells. TIGMEC-3 line and the GSM cells were infected at a MOI of 0.1 with CAEV-pBSCA molecular clone and CAEV-3112 field isolate. Culture medium was harvested from infected cells at day 1, 3, 5, and 7. Viral titers were determined on GSM cells and calculated according to the Reed and Muench technique. A: kinetic curves of production of CAEV-3112 field isolate on TIGMEC- 3 and GSM cells. B: kinetic curves of production of CAEV-pBSCA field isolate on TIGMEC- 3 and GSM cells.

4. DISCUSSION

The present work describes new cell lines immortalized from primary goat milk epithelial cells. Primary goat milk epithelial cells were transfected with a plasmid expressing the SV-40 large T antigen, resulting in cell lines that were apparently endowed with unlimited proliferation capacity. All three TIGMEC lines conserved microscopic epithelial morphology and cytokeratin expression, a typical phenotypic characteristic. Parental GMEC rapidly lost any capacity of proliferation after only 5 or 6 passages in vitro, in contrast, serially maintained TIGMEC cells showed no sign of

senescence after more than 1 year, corresponding to more than 50 passages at their usual 24-48 hour doubling time.

All three TIGMEC lines are highly susceptible to infection by the molecularly cloned caprine lentivirus CAEV-pBSCA, and to the French field isolate CAEV-3112. The major Gag protein and the Env glycoprotein were easily immunoprecipitated both from lysates and from the supernatant medium of infected cells, suggesting that viral proteins were correctly processed and assembled into efficiently-released viral particles. Indeed, high titers of infectious and cytopathic viruses were harvested from the culture medium of infected cells.

Interestingly, CAEV-pBSCA replicated in these cell lines in absence of any cytopathic effect and led to a chronic infection with constitutive production of a high titer of virus. Lentivirus replication in epithelial cells in absence of cytopathic effects was also observed with certain human epithelial cells infected with HIV-1 [31, 32]. In contrast however, our results differ from those reported for HIV-1 replication in epithelial cells in at least two major points. First, all our TIGMEC lines and all primary cultures of GMEC we tested [24] are easily infected by CAEV, whereas four of eleven human primary epithelial cells and one of three cell lines were found resistant to HIV-1 infection [31]. These results may be explained by an inefficient usage of galactosyl-ceramide rather than the CD4 receptor by HIV-1 to enter these cells [15]. Secondly, the molecular clone and the field isolate of CAEV were found to replicate to high titer in TIGMEC cells, whereas, HIV-1 replicates only very poorly in human mammary epithelial cells [30, 31], as in other epithelial cells from different organs. Cytopathic effects were observed when the CAEV-CO infected TIGMEC cell lines were put in co-culture with goat synovial membrane cells (GSM), which are well known to be highly permissive to CAEV infection. A cell line derived by immortalization of mammary epithelial cells from a naturally-infected goat showed persistent infection by CAEV (data not shown). Similarly to TIGMEC infected in vitro with CAEV-pBSCA, this cell line also replicated the virus in absence of cytopathic effect. These results suggest that goat milk epithelial cells are resistant to the fusiogenic activity of CAEV envelope, or the fusion domain of the CAEV envelope transmembrane is not well exposed at the surface of these epithelial cells. This could result from usage of an unusual receptor(s) by CAEV to enter these epithelial cells. The receptor(s) used by CAEV to enter the cells is still unknown. In our recent studies of CAEV species tropism, we found that this virus is capable of infecting cells from a very large variety of mammalian species including moufflon, cattle and pig ([19], unpublished results). This suggests either that the receptor is widely expressed, or that CAEV can use various receptors to infect target cells. However, our recent data demonstrated that the lack of functional receptors for CAEV on human cells is the only barrier protecting these cells from infection [25].

This high permissivity of milk epithelial cells to CAEV infection suggests the possible implication of these cells in the persistence of viral infection in adult animals and in the efficient transmission of virus to new-born kids. Therefore, mammary and milk epithelial cells could serve as a reservoir of CAEV for persistent infection and directly contribute to virus transmission or by infecting macrophages in milk as has been suggested for other viruses like HIV-1, human T- cell leukemia virus type I (HTLV-I) and Bovine Leukemia virus (BLV) [6, 28]. CAEV-infected mammary epithelial cells *in vivo* may also be involved in the development of mastitis in infected goats. We demonstrated that CAEV-infected milk epithelial cells produce high virus titers in vitro [24]. Such high production in vivo may result in induction of an inflammatory process by recruitment and activation of inflammatory and immune effector cells. This type of mechanism has already been observed

with other viruses like influenza H3N2 and RSV in bronchial epithelial cells [29, 33].

CAEV infection of mammary epithelial cells may also interfere with their ability to produce milk. Indeed, in CAEV infected goats a decrease in milk production is commonly observed [20]. Such deregulation of epithelial cell functions has been also described for HIV-1 infection of the intestinal epithelial cells [12, 13, 14, 21].

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