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Infection of primary cultures of mammary epithelial cells by small ruminant lentiviruses

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Abstract – The caprine arthritis-encephalitis virus (CAEV) and Visna Maedi virus cause persistent infections with long latent periods and induce degenerative and chronic inflammatory lesions of the central nervous system, joints, lungs and udder. Monocyte/macrophage lineage is the main target cell for CAEV and Visna Maedi virus but we speculate that mammary epithelial cells may also be infected. Primary cultures of milk cells, mammary tissues of experimentally and naturally infected goats and ewes were used. Primary cultures of mammary tissue from ewes and goats were infected with the CAEV Cork strain. The lentiviral infection of the primary culture was demonstrated by a typical cytopathic effect in mammary epithelial cells and the presence of an infectious virus in coculture with permissive fibroblasts. To identify the epithelial cells in explants and demonstrate the antigenic expression of CAEV, primary cultures were immunostained with polyclonal anti-keratin and monoclonal anti-CAEV p30. Colocalisation studies under a UV fluorescence microscope and by epifluorescence microscopy showed the expression of specific viral antigens in mammary epithelial cells from the eight animals used. Infected mammary epithelial cells may act as a reservoir for the virus which may play an important role in the virus dissemination and in the pathogenesis of the mammary lentiviral disease. © Inra/Elsevier, Paris.

CAEV / mammary gland / epithelial cell / lentivirus / small ruminant

Résumé – **Infection de cultures primaires de cellules épithéliales mammaires par les lentivirus des petits ruminants.** Le virus de l'arthrite et de l'encéphalite caprine (CAEV) et le Visna Maedi provoquent des infections persistantes avec de longues périodes de latence et induisent des lésions inflammatoires et/ou dégénératives chroniques du système nerveux central, des articulations,

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du poumon et de la mamelle. Les cellules de la lignée monocyte-macrophage sont la principale cible des virus CAEV et Visna Maedi ; cependant nous faisons l'hypothèse que les cellules épithéliales mammaires sont également infectables. Des cultures primaires de cellules du lait et de tissu mammaire de chèvres et de brebis expérimentalement et naturellement infectées ont été utilisées. Des cultures primaires de tissu mammaire d'une chèvre et d'une brebis ont été infectées avec la souche de CAEV Cork. L'infection lentivirale des cultures primaires a été montrée par l'observation d'un effet cytopathique caractéristique dans les cellules épithéliales mammaires et la présence de virus infectieux dans les cellules en coculture avec des fibroblastes permissifs. Pour identifier les cellules épithéliales dans les explants et pour démontrer l'expression antigénique du CAEV, des immunomarquages ont été réalisés sur les cultures primaires avec un anticorps anti-CAEV p30. Des études de colocalisation sous microscope à fluorescence et par microscopie à épifluorescence ont montré l'expression d'antigènes viraux dans les cellules épithéliales mammaires des huit animaux étudiés. Les cellules épithéliales mammaires pourraient agir comme un réservoir de virus qui pourrait jouer un rôle important dans la dissémination du virus et dans la régulation de l'infection lentivirale dans la mamelle.

CAEV / cellule épithéliale / mamelle / lentivirus / petit ruminant

1. INTRODUCTION

The caprine arthritis-encephalitis virus (CAEV) and the Visna Maedi virus are lentiviruses, non-oncogenic retroviruses which cause persistent infections with long latent periods. They induce degenerative and chronic inflammatory lesions of the central nervous system, lungs, joints and udder [2]. Viral infection of the mammary gland is followed by an induration of the udders without individualised nodules but with a hypertrophy of the retromammary lymph nodes. The mammary tissue shows a diffuse infiltration with lymphoplasmocytes, lymph follicles around the lactiferous ducts and fibrosis [5, 6, 18]. The economic costs of CAEV infection arise from early culling, loss of milk production, poor growth of kids and increased replacement costs [13]. The disease also increasingly threatens the export of live animals.

Cells of the monocyte/macrophage lineage are the major host cells of the CAEV and Visna Maedi virus. Viral replication in these cells is dependent on cellular differenciation so that the replication in monocytes is restricted [2]. A broader range of cells may, however, permit the replication of CAEV and the Visna Maedi virus. In vivo, epithelial cells of the thyroid, kidney and small intestine of infected goats were shown to harbour CAEV RNA. The viral antigen is detected in cells of the central nervous system of sheep (including epithelial cells, fibroblasts and endothelial cells) [17]. Ovine aortic smooth muscle cells allow the replication of Visna Maedi in vitro [8]. Another member of the lentivirus family, HIV-1, can infect epithelial cells. The presence of HIV-1 in thymic epithelial cells has been demonstrated in vivo and certain HIV-1 strains induce a productive and persistent infection in these cells in vitro [1]. Epithelial cells derived from the human cervix incorporate HIV proviral DNA and produce new virus in vitro after infection with HIV-infected T-cells or monocytes [15].

Infected cells of colostrum and milk are responsible for the lactogenic route of CAEV transmission between dams and kids. With HIV and HTLV-1, transmission from the mother to child also occurs through breastfeeding. These viruses can be derived from blood by infected lymphocytes and monocytes. It is therefore possible that non-lymphoid cells of the breast gland are productively infected. Indeed, HIV-1 replication has been shown to be present in primary culture of ductal and alveolar mammary epithelial cells [16]. Furthermore, basal mammary epithelial cells are susceptible to

HTLV infection and transfer infection to normal peripheral blood lymphocytes [9]. Polymorphonuclear neutrophils, lymphocytes, macrophages and epithelial cells are present in goat mammary gland secretions. In this study, in order to investigate whether epithelial cells can be infected with CAEV, primary cultures of mammary gland tissue were obtained and infected cells were identified by immunostaining and colocalisation studies.

2. MATERIALS AND METHODS

2.1. Animals

Milk cells (*table* I) were obtained from three Saanen goats experimentally infected by intramammary inoculation of 10^6 cells infected with the Cork strain of CAEV [7]. After two lactations, the goats were killed by intravenous injection of sodium pentobartital (Dolethal, Vétoquinol, Lure, France) and the mammary glands were removed immediately after slaughter for mammary tissue culture. The goats were not lactating and not pregnant at the time of slaughter.

Mammary gland tissues were also obtained from naturally infected animals: two freshly slaughtered ewes from a local slaughterhouse and one goat (*table* I). They were in mixed physiological states at slaughter. After being excised, the mammary glands were kept at 20 °C and returned to the laboratory for dissection. All mammary glands were free from pathological changes when examined prior to tissue sampling.

2.2. Preparation of epithelial cells

2.2.1. Milk epithelial cells

Cells from defatted milk were cultured in a modified Eagle medium (MEM) (Seromed, Polylabo, France) supplemented with 10 % foetal calf serum (IBF, Villeneuve-la-Garenne, France) as previously described [12]. In order to eliminate all non-adherent cells, the medium was changed 24 h after inoculation. Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and the medium (MEM–10 % SVF) was changed weekly.

2.2.2. Primary mammary epithelial cells

A piece of mammary tissue (approximately 0.5 × 1 × 1 cm) was aseptically excised and placed in approximately 2.5 mL of modified Eagle medium, 10 % foetal calf serum supplemented with antibiotics: penicillin + strepto-

Table I. Presence of virus in milk cells and mammary tissue explants of goats and ewes.

	Milk epithelial cells			Mammary tissue explants		
	Cytopathic effect	Infectious virus	p30	Cytopathic effect	infectious virus	p30
Experimentally infected in vivo (three goats)	+	+	NT	+	+	+
Experimentally infected in vivo (one ewe, one goat)	NT	NT	NT	+	NT	+
Experimentally infected in vivo (two ewes, one goat)	NT	NT	NT	+	NT	+

NT: not tested.

mycin (100 UI/mL + 100 µg/mL; Boehringer Mannheim, France) and fungizone (1.25 µg/mL; GIBCO BRL, France). The sections were minced using scissors, transferred to four-well Lab-Tek slides (Nunc, Amilabo, France) and cultured with 0.2 mL of medium per well. The chambers were filled up with MEM after 24 h of incubation at 37 °C with 5 % CO_2. The medium was changed every 2 days during the first week of culture, then weekly.

2.3. In vitro infection of mammary cell culture

Cells growing from explants of mammary tissue of one ewe and one goat (*table* I) were infected with the Cork strain of CAEV. After 2 weeks of culture, when the cells covered about 50 % of the lab-Tek slide wells, the cells were infected at a multiplicity of infection (M.O.I.) of 3×10^3 syncytia forming units per well. The virus inoculum was allowed to adsorb for 8 h and then removed. The cells were washed with medium and incubated for 1 week in the presence of MEM supplemented with 5 % foetal calf serum. Uninfected cells were cultured in parallel in the same conditions.

2.4. Detection of viral infection in primary culture of mammary epithelial cells

After 3 weeks of culture, typical viral cytopathic effects were searched for in milk epithelial cells and cells growing from explants of mammary tissue after a May-Grünwald-Giemsa staining.

Milk epithelial cells and cells growing from explants were examined for the presence of infectious virus by coculture as previously described [7]. The cells were cocultured with permissive caprine fibroblasts and observed daily for the appearance of syncytia over a period of 10 days.

To confirm the epithelial nature of the cells and demonstrate the antigenic expression of CAEV, cells growing from explants of mammary tissue were analysed by indirect immunofluorescence after 3 weeks of culture. The cells were washed with PBS, fixed in cold (–20 °C) acetone and ethanol (1:1, vol/vol), then rinsed three times with PBS and incubated with primary antibodies: polyclonal rabbit anti-keratin (Monosan, Genzyme, France) and/or monoclonal mouse anti-CAEV p30 (V.M.R.D., Pullman, USA). All antibodies were used according to the manufacturers' recommendations and diluted in PBS (1:200 for anti-cytokeratin and 1:500 for anti-CAEV p30). Lab-Tek slides were incubated for 1 h at 37 °C in a humidified chamber. The cells were washed three times in PBS and incubated with secondary antibodies: anti-rabbit fluorescein isothiocyanate conjugate (Sigma, France) and/or anti-mouse rhodamine conjugate (Jackson, Interchim, France). Secondary antibodies (1:200) were used according to the manufacturers' recommendations. Lab-Tek slides were incubated at 37 °C in a humidified chamber for 1 h. Following extensive washings in PBS, the slides were mounted with a fluorescent mounting medium (Dako, France), examined under a UV fluorescence microscope and then with an epifluorescence microscope. Positive and negative controls included the CAEV Cork-infected or -uninfected caprine fibroblasts derived from synovial membranes.

3. RESULTS

The culture of milk cells included single and small islands of epithelioid cells, and a few macrophage-like cells. In explant cultures, after 1 week, several types of attached cells were present: fibroblasts and islands of small epithelial cells with a small amount of cytoplasm. Fibroblasts formed net-like bundles encircling the epithelial islands and grew faster, covering more culture surface than did the epithelial cells. Small epithelial cells had the greatest growth potential and gave rise, after 2 or 3 weeks in culture, to large squamous-like cells with a very large amount of cytoplasm and frequent vacuoles. These large cells appeared in or around the islands of the small epithelial cells. The immunocytochemistry with anti-rabbit cytokeratin antibodies confirmed that both small and large epithelioid cells from mammary tissue explants were positive for cytokeratin. In contrast, fibroblasts from mammary explants or derived from foetal goat synovial membranes were negative for cytokeratin.

A spontaneous cytopathic effect was observed after 3 weeks in milk epithelial

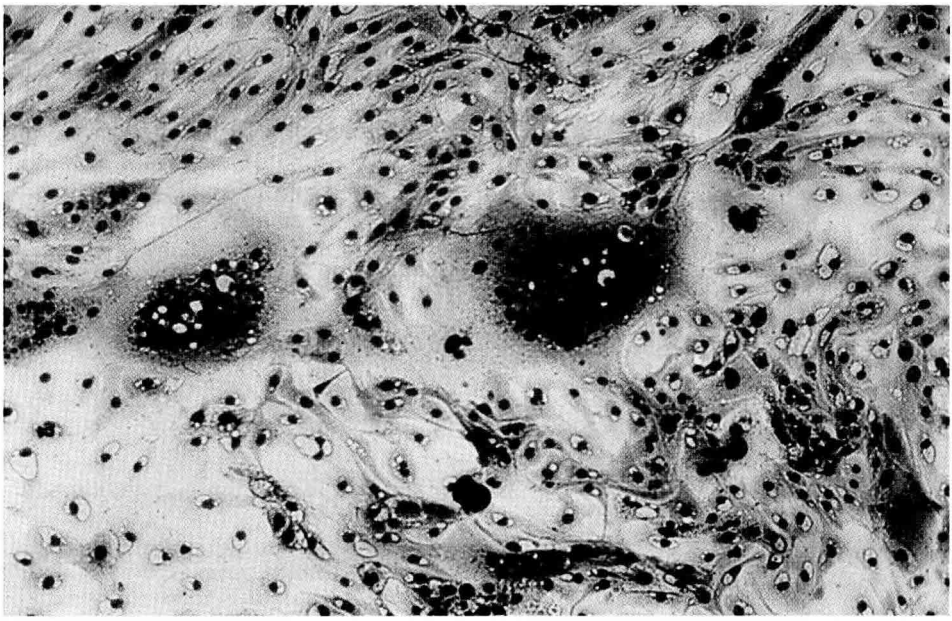

Figure 1. Cytopathic effect in mammary tissue explants from a goat experimentally infected with CAEV Cork. May Grünwald Giemsa stain. Original magnification × 370.

cells and in primary culture of mammary tissue (*table* I). In all mammary tissue explants, syncytia were mainly located in islands of epithelial cells and were smaller in mammary tissue culture of naturally infected ewes (three to five nuclei) than in experimentally infected goats (>10 nuclei) (*figure 1*).

The presence of an infectious virus induced a characteristic cytopathic effect in epithelial cells from milk and mammary tissue of experimentally infected goats after 8–10 days of coculture with permissive fibroblasts (*table* I).

Immunostaining and colocalisation studies were used to identify infected cells in primary mammary tissue cultures. The M.O.I. of 3×10^3 syncytia forming units per well induced the lysis of more than 80 % of the cells in primary cultures of the mammary tissue from the ewes. Many of the remaining cells showed an intense intracytoplasmic staining for cytokeratin and viral p30 antigens (*figure 2a*). Likewise, a positive intracytoplasmic viral p30 antigen staining is observed in epithelial cells of experimentally infected goat explants (*figure 2b*). In naturally infected ewes and the goat, a few cells, identified as epithelial cells stained for the viral p30 antigen, could also be observed with a slighter cytoplasmic staining (*table* I). In all mammary tissue cultures, mammary fibroblasts never showed CAEV p30 antigen staining. Caprine fibroblasts from synovial membranes infected in vitro with CAEV Cork as positive controls showed red staining for the viral p30 antigen and no green staining for cytokeratin. The specificity of the stainings was assessed by negative controls where staining was not observed.

4. DISCUSSION

Our findings demonstrate for the first time that mammary epithelial cells of ewes and goats can be infected in vitro with

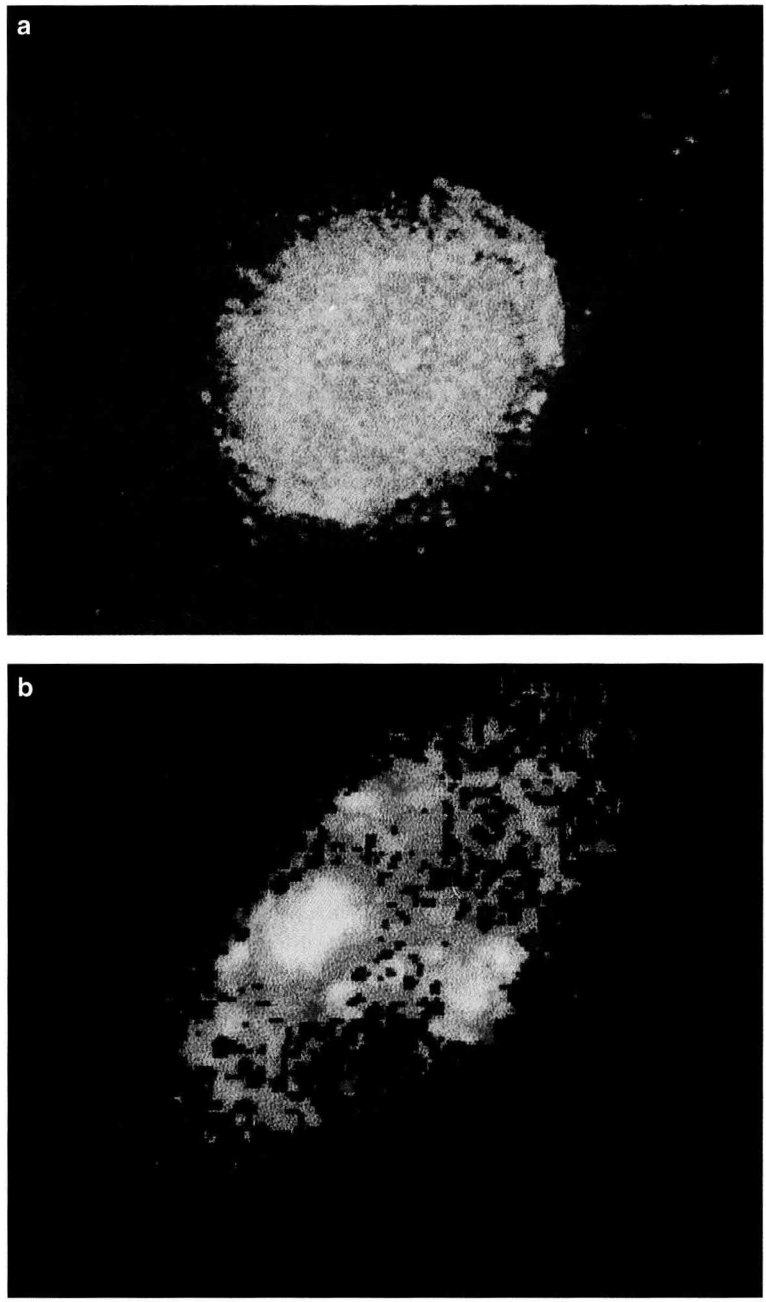

Figure 2. Immunofluorescence labelling of a mammary epithelial cell from a ewe infected in vitro with CAEV Cork (a) and mammary epithelial cell from a goat experimentally infected with CAEV (b). Cytokeratin is shown in green, colocalisation of cytokeratin and CAEV p30 in yellow. The micrograph was taken under epifluorescence illumination. Original magnification $\times 3\,000$.

CAEV Cork. Moreover, they express the CAEV p30 antigen in vitro after experimental or spontaneous infection of goats and ewes. We have, however, not been able to directly associate permissiveness of mammary epithelial cells with the release of the infectious virus. Indeed, although the explant cultures contain glandular epithelium, fibroblasts, endothelium and blood cells, milk is devoid of fibroblasts and endothelial cells but not of macrophages. Furthermore, the macrophages are the most permissive cell type for supporting the replication of small ruminant lentiviruses [3]. Therefore, one could argue that cultured mammary epithelial cells were CAEV positive only because they became infected by CAEV producing macrophages in milk or explant cultures. There are several aspects of our data, however, that suggest this was not the case and that the mammary epithelial cells were CAEV infected in vivo before being cultivated. First, CAEV replication in fibroblasts in vitro is rapid and lytic. However, we have not observed any cytopathic effect in fibroblasts of explant cultures. Second, during the first week of culture, most macrophages were eliminated from milk cells and explant cultures by selective trypsinisation. Third, and most importantly, data obtained by immunostaining indicated that the expression of the capsid CAEV p30 antigen, a late stage antigen, occurred only within mammary epithelial cells.

Mammary epithelial cells may act as a reservoir for CAEV in the mammary gland. It has been established that lungs are a reservoir for the Visna Maedi virus. In situ PCR and hybridisation have previously demonstrated the presence of Visna Maedi virus in bronchiolar epithelial cells in the lungs of infected sheep. The majority of these cells harbour a single copy of the viral genome in a transcriptionally silent state [14]. Likewise, lentiviruses could enter mammary epithelial cells but infectious virus could be produced only after cell activation. Such growth-arrested cells may provide an in vivo reservoir for the persistence of proviral CAEV and later reactivation of the cells by host factors, for example hormones, may result in the release of infectious viruses.

CAEV and Visna Maedi virus replicate efficiently in non-dividing cells. Rather than depending on cell division, they require activation and/or differenciation of the host cell for productive replication. CAEV infects cells of monocyte/macrophage lineage and viral replication in these cells is dependent on cellular differenciation so that replication in monocytes is restricted. Monocytes contain only proviral DNA. Transcription of viral RNA and production of viral proteins and mature virions does not occur until the monocytes localise in tissue and mature to macrophages [11]. Normal mammary tissue is hormone-dependent and undergoes structural and functional changes as a consequence of variations in the levels of secreted hormones [10]. Profound ultrastructural differenciation of the epithelium has been described immediately before and after parturition [4]. Virus recovery in milk and blood monocytes is increased at parturition and after an induction of lactation suggesting that hormone levels may regulate virus production and/or infectivity [12]. Hormones that stimulate DNA synthesis and consequently milk secretion, also enhance HIV replication in mammary epithelial cells [16]. Smaller epithelial cells observed in our study have the greatest growth potential and give rise to larger cells which represent the terminally differenciated phase of the cell maturation sequence. Differenciation–activation of infected mammary epithelial cells may activate viral gene expression at the end of pregnancy and the infectious virus is then produced in the mammary tissue and colostrum during lactogenesis. Therefore, to study viral production in the mammary gland, it would be better to use small, undifferenciated cells from mammary tissue rather than large, differenciated cells from milk. Moreover, the explants allowed the analysis of CAEV infectivity in situ, where tissue architecture

is conserved and the physical relationship between different cell types is preserved.

CAEV has traditionally been thought to be present only in the promonocytes and monocytes in the bone marrow and blood of infected goats as a silent infection. Productive virus replication is associated with maturation and differenciation of macrophages in target tissue. Our results show that CAEV is also present in the mammary epithelial cells and that these cells express the antigen CAEV p30 in vivo. Thus, CAEV infection in the mammary gland could impact directly on the process of milk-borne transmission of CAEV infectivity from dams to kids and contribute to virus dissemination as it occurs with HIV-1 and HTLV. While much remains to be understood about the events surrounding the pathogenesis of CAEV in the mammary gland, our findings provide a foundation to more fully define the mechanisms involved in the migration of leucocytes to mammary tissue and the relationship between CAEV infection and the hormonal status of the goat.

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