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Original article

Mammary transmission of caprine arthritis encephalitis virus: a 3D model for in vitro study

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Abstract – Transmission of Caprine Arthritis Encephalitis virus (CAEV) from the mother to offspring is principally mediated by infected cells from colostrum and milk. The infection of the dam is often sub-clinical, and results in increased cellularity of milk, sometimes exacerbated by bacterial co-infections. Although monocytes are the major viral host cells, several other cell types, including epithelial mammary cells, fibroblasts and endothelial cells show low levels of in vivo infection. In vitro, however, all phenotypes of mammary gland cells are individually highly sensitive to CAEV infection. This suggests that local mechanisms act to control viral expression. Our goal is to analyse the mechanisms regulating local virus infection, including the physiological status of the mammary gland and bacterial co-infections. In this work, we present the development of a model for the in vitro reconstitution of mammary gland tissue using 3D cultures in Matrigel. Mononuclear cells from the blood are added to the 3D cultures in vitro. In these experimental conditions, the mammary cells spontaneously organize into mammospheres. Blood leucocytes migrate into the culture gel, and localize particularly at the periphery of the mammospheres. Mammospheres were susceptible to infection in vitro by CAEV, as shown by a cytopathic effect and expression of late CAEV antigen p30. This model will allow the in vitro study of virus expression, transfer of infection to mammary gland cells and interactions between the mammary gland cells, infected monocytes and immunocompetent cells. It will allow the study of mechanisms participating in the control of passage of pathogens into milk, according to the physiological and CAEV-infection status of the animal, microenvironment and the presence of bacterial co-infections.

mammary gland / goat / lentivirus expression / in vitro model

1. INTRODUCTION

Caprine Arthritis Encephalitis (CAEV) is a lentivirus that induces persistent infection in goats, and more than 50% of French herds are infected. Economic losses from CAEV infection are due to sub-clinical mastitis, the lowering of milk quality and limitations on international exchange of young breeding animals. Infection is mainly transmitted from the mother to young by infected cells in the mammary secretions. During the course of disease, periods where the virus is difficult to isolate from blood monocytes alternate with periods of reactivation of viral expression [1–3]. These periods of

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active viral expression occur at times when the mammary gland, the organ mainly responsible for contagion, is physiologically functional.

Like other lentiviruses, CAEV productively infects cells of the monocyte-macrophage lineage, which distribute the viral infection throughout the body. This lentivirus does not infect lymphocytes [4]. It is becoming clear that lentiviruses, including CAEV, can infect many non-leucocytic cells in vivo [5–7]. Examples include the following: microglial cells, astrocytes and oligodendrocytes; pneumocytes type I and II, and mammary fibroblasts as well as epithelial and endothelial cells, but the relevance of this infection to the expression of disease is still unclear. CAEV productively infects several types of endothelial and epithelial cells in vitro [8, 9]. We have established [10] that different cell types isolated from mammary tissue, including luminal epithelial cells, myoepithelial cells, endothelial cells and fibroblasts, are highly susceptible to in vitro infection. In vitro, the infected mature luminal epithelial cell acquires a capacity to attract adherent leucocytes similar to that of endothelial cells [10], which, if expressed in vivo, could modulate local cellular interactions. We have shown [10] that in vitro, the infection of myoepithelial cells is restricted to the integration of proviral DNA without viral expression. This restriction, if occurring in vivo, could maintain a reservoir of the viral genome in myoepithelial cells during mammary involution. We hypothesize that the viral genome could then be amplified during mammogenesis, and could be expressed according to the differentiation status and micro-environment.

Leucocyte transmigration can be amplified and modulated by CAEV infection of endothelial cells, as shown in vitro [11], and we have shown that the in vitro transmigration of leucocytes from CAEV-positive goats induces virus expression in monocytes carrying proviral DNA [12]. These various in vitro observations suggest that many different cells and mechanisms may be involved during the CAEV infection of the mammary gland in vivo. Our hypothesis is that the interactions between infected monocytes, mammary gland cells and immunocompetent cells influence the regulation of viral expression during natural infections, and thereby control the passage of infected cells into colostrum and milk. The study of the dynamics of cellular interactions during infection would strongly benefit from an in vitro model mimicking mammary tissue and allowing cell interactions in a controlled microenvironment. We therefore established a model using 3D cultures of caprine mammary gland cells that results in the in vitro organization of acinus-like structures.

2. MATERIALS AND METHODS

2.1. Animals

Tissue samples were taken from Saanen goats from breeding stocks with a sustained history of negativity for CAEV infection by both ELISA serology and PCR testing of blood leucocytes [13]. Housing and experimental procedures were approved by the ethics committee of the École nationale vétérinaire de Lyon. Samples of mammary tissue were obtained at autopsy from two animals, one at the end of gestation and the other at the end of lactation, under general anaesthesia with subsequent euthanasia (Dolethal, i.v. 200 $mg \cdot kg^{-1}$ body weight; Vetoquinol, Lure, France). Blood for the preparation of peripheral blood mononuclear cells (PBMC) was obtained from two other goats by jugular venipuncture onto heparin.

2.2. Preparation of mammary gland cells

For each specimen, four fragments of ca. 0.25 cm^3 were dilacerated with a scalpel blade in basal medium consisting of RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U per mL penicillin, 100 mg per mL streptomycin and 2.5 mg

per mL amphotericin B (Invitrogen). These were then incubated in 15 mL basal medium supplemented with collagenase type I (Gibco, Life Technologies, Cergy-Pontoise, France), 400 µg per mL, and trypsin (Gibco), 0.25g per 100 mL, in 25 mL conical tubes under intermittent agitation for 1 h 30 at 37 °C. The detached cells were then filtered through gauze and washed twice in basal medium, recovered by centrifugation (430 *g*, 10 min, 4 °C) and resuspended in basal medium supplemented with 10% fetal calf serum (FCS), 10% DMSO and epidermal growth factor (EGF; Sigma) 10 ng per mL, at a concentration of 2.10^6 cells per mL, and frozen in liquid nitrogen.

2.3. Supports for cell culture

Mammary gland cells were cultured in 2D on collagen-coated surfaces (Biocoat collagen I cellware, 25 cm^2 flasks or 96 well plates, Beckton-Dickinson). For 3D cultures, Matrigel (BD Matrigel matrix, phenol red free; Le Pont de Claix, France) diluted 1:2 in basal medium and maintained for 24 hours at 37 °C before use, was dispensed into cell culture inserts (0.4µm pore size PET track-etched membrane) in 24 well plates (Beckton-Dickinson) at 100 µL·well^{-1}, or directly into 96 well plates at 50 µL·well^{-1}.

2.4. Cultures of mammary gland epithelial cells

After thawing, mammary gland cells were washed twice and resuspended in basal medium supplemented with EGF (10 ng·mL^{-1}) and 10% FCS. The cells were then seeded onto 24 well inserts (3×10^5 cells·well^{-1}) or p96 plates (1×10^5 cells·well^{-1}). Every 3 days, 2/3 of the supernatant was gently removed and replaced with fresh medium.

2.5. In vitro hormonal stimulation

After 3 days of culture, the medium was replaced as above by basal medium supplemented with EGF 10 ng·mL^{-1}, insulin (Sigma) 5 µg·mL^{-1}, dexamethazone (Sigma) 0.1 µM, and prolactin (Sigma) 5 µg·mL^{-1}. The cultures were maintained for 7 further days, with one more medium change at day 3.

2.6. Preparation of peripheral blood leucocytes

Peripheral blood leucocytes (PBL) were prepared as previously described [11] by centrifugation on Histopaque density 1.073 (Sigma).

2.7. Labelling with CFSE

For some experiments, mammary cells or leucocytes were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Inc., Eugene, OR, USA) as described previously [11].

2.8. PBL migration in 3D cultures

PBL, labelled by CFSE or not, were suspended in medium and added to mammary gland cell 3D cultures during a standard medium change in a proportion of 50 000 PBL for p24 inserts and 30 000 PBL for p96 wells. The cultures were fixed after one further day of incubation at 37 °C, then examined by confocal microscopy or immunocytochemistry.

2.9. In vitro infection of 3D mammary cell cultures

CAEV-3112, a French field isolate from an arthritic goat, was used to infect 3D cultures in vitro at day three of hormonal stimulation. Viral suspension was added to culture supernatant of 3D cultures after a 3 day hormonal stimulation, at a dose of 0.5 tissue culture 50% infectious doses per cell in fresh conditioned medium. The evolution of cell cultures was followed during the 8 days of infection, then viral expression was investigated by immunochemistry using the CAEP5A1 monoclonal antibody to late

CAEV capsid antigen p30 monoclonal antibody (VMRD, Pullman, WA, USA).

2.10. Immunolabelling

Cell culture supernatants were cautiously discarded, then the cells were fixed at room temperature for 30 min in parafolmaldehyde (Sigma) 3.6% in PBS, followed by permeabilization for 30 min in 0.2% Triton ×100 (Sigma) in PBS. After washing, the cell cultures were incubated for 30 min at room temperature with primary antibodies as described previously (Milhau et al., 2003). Antibodies to cytokeratin (Sigma; monoclonal anti bovine cytokeratin K8.13), smooth muscle alpha-actin (Euromedex, Mundolsheim, France) and rabbit polyclonal serum anti human laminin 5 [14] were chosen for their cross-recognition of the corresponding caprine antigens. PBL were labelled by a mixture of antiCD4, anti-CD8 and anti-CD14 monoclonal antibodies, diluted 1/75 (VMRD; GC1A, CACT80C and MM61A). The secondary antibodies for confocal microscopy observations were (alexa fluor 488 goat anti mouse f(ab')2 and alexa fluor 633 goat anti rabbit f(ab')2 (Interchim, Montluçon, France). Immunocytochemistry was performed using a commercial kit (Envision, DakoCytomation; Trappes, France).

3. RESULTS

3.1. Evolution of mammary gland cell cultures on Matrigel

During the first 12 h following seeding on Matrigel, the cultures of primary mammary gland cells evolved from a single layer to the formation of islets (Figs. 1A and 1B). Already at day 1, the cells were organizing into mammosphere-like structures as illustrated in Figure 1C, where we observe cells directed axially towards the mammosphere. This presumably reflects cell migration during the formation of the organized structures. After 1–2 days of culture, the mammosphere-like structures became progressively more organized, as shown in Figures 1D and 1E.

From day 3 to day 7, the lumen of some mammospheres became lobulated, as shown in Figure 1F. Budding was also observed, with formation of secondary mammospheres (Fig. 1G). These acinus-like structures are limited by a basal membrane, as shown in Figure 1H. This membrane contains laminin 5, as established by immunolabelling with specific rabbit polyclonal antiserum.

As shown in Figures 2A and 2B, the junctions between some mammospheres could be observed from day 3–4. The initial phase consisted of the organization of isolated cells into a chain between the mammospheres, followed by the formation of what might represent the secretion of a connective membrane bridging the mammospheres.

3.2. Localization of epithelial and myoepithelial cells

All the cells forming mammosphere like structures were labelled by monoclonal anticytokeratin antibody (Fig. 3A). As shown in Figures 3B–3D, the cells in the outer mammosphere layer expressed smooth muscle alpha-actin, whereas those of the inner layer limiting the central lumen were cytokeratin+, but smooth muscle alpha-actin- (Fig. 3E). This indicates that the mammosphere-like structures were formed by a spherical structure of cytokeratin+, smooth muscle alpha-actin- epithelial cells, surrounded, totally or partially, by a layer of cytokeratin +; alpha-actin+ myoepithelial cells.

3.3. Response to hormonal treatment

By the seventh day after the addition of hormone enriched medium, 50 to 75% of mammosphere like structures expressed alpha lactalbumin, as established by a strongly-positive immunocytochemistry (Fig. 4A). In contrast, alpha lactalbumin was not expressed by most unstimulated mammospheres

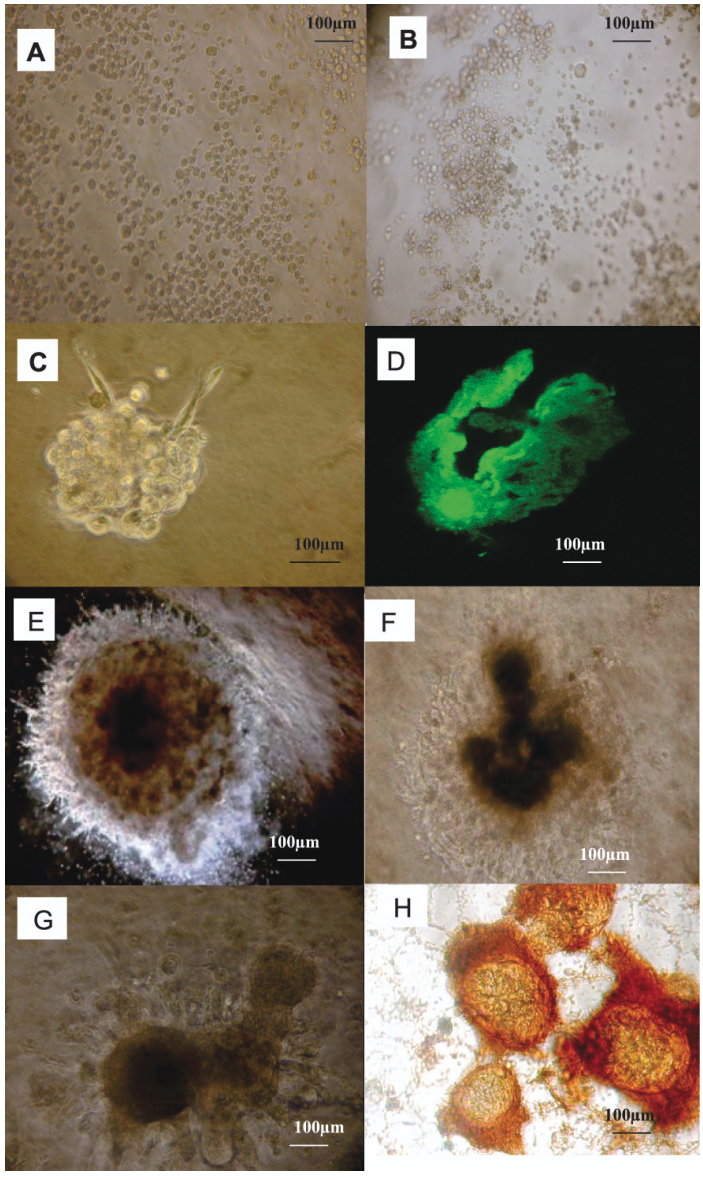

Figure 1. Organisation of mammosphere-like structures during culture in Matrigel. (**A)** Culture of mammary gland cells, 4 hours after seeding on Matrigel. (**B)** The same culture well, 10 hours after seeding. The cells are gathering in islets. (**C)** Organization of a mammosphere-like structure, one day after seeding.Two elongated cells are axially directed towards the centre of the mammosphere. (**D)** Mammosphere-like structure, at day 3 post seeding. CFSE labelled cells, confocal microscopy examination. (**E)** Mammosphere-like structure, at day 3 post seeding (PFA-Triton X100 fixation). (**F)** Mammosphere-like structure with lobulated lumen, at day 5 post-seeding. (**G)** Budding of a mammosphere-like structure into Matrigel, at day 7 post-seeding. (**H)** Mammosphere-like structure: labelling of basal membrane by anti-laminin 5 polyclonal antibody (immunocytochemistry, AEC). Size bars are ocular estimates. (For a color version of this figure, see www.edpsciences.org/rnd.)

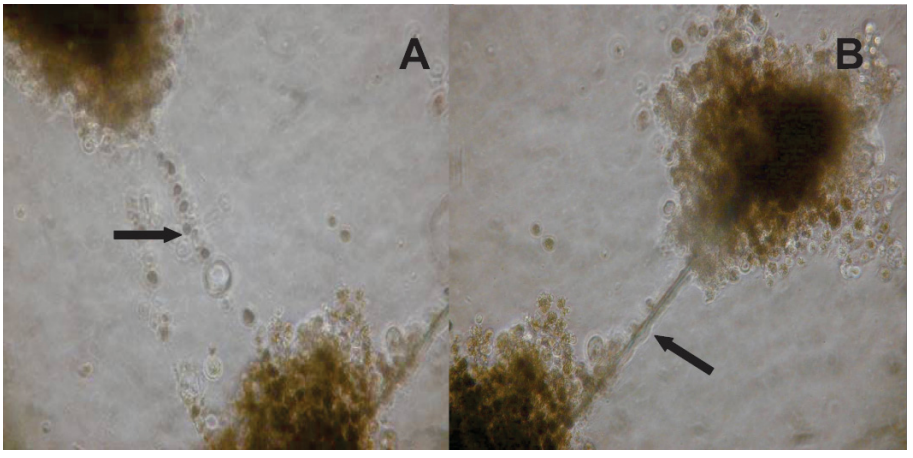

Figure 2. Formation of junctions between mammosphere-like structures. Culture of mammary gland cells on Matrigel, at day 5 post seeding. **(A)** Chain of isolated cells between two mammospheres (arrow). (**B)** Connective structure bridging two mammospheres (arrow). (For a color version of this figure, see www.edpsciences.org/rnd.)

(Fig. 4B), although a few structures at the periphery of the culture were weakly labelled.

3.4. Migration of peripheral blood leucocytes in 3D cultures

Leucocytes isolated from peripheral blood were added to 3D culture supernatants, and were examined after 24 h. Within 10 h after addition, the leucocytes were observed to be dispersed throughout the Matrigel. Immunocytochemistry and confocal microscopy were performed on the 3D cultures one day later. We observed mammospheres with leucocytes adhering in close contact to the epithelial cells (Figs. 5A–5C).

3.5. In vitro infection of 3D mammary cell cultures

The first observed effect of the in vitro infection of 3D mammary cell cultures was a modification of the 3D organization in a proportion of acinus-like structures. The p30 late CAEV antigen was detected in these structures at day 7–8 by immunolabelling, as shown in Figure 6A. About 15–25% of infected mammospheres presented, from day 5, alterations to their spherical morphology (Fig. 6B), ranging from minor deformation to almost complete disorganization.

4. DISCUSSION

The cells isolated from the goat mammary gland undergo three-dimensional organization in Matrigel, which begins in the first hours of culture by cell migration and formation of islets. Then, we observed formation of mammospheres, or acinus-like structures, morphologically similar to those described as deriving from mammary epithelial cell lines [15–17] or human breast tissue [18]. This was in accordance with the general property of the cells to develop architectural arrangements when cultured in three-dimensional matrices [19]. The acinus-like structures were limited by a basal membrane, as expected [15]. The synthesis of alpha-lactalbumin after in vitro stimulation with prolactin established that, as shown in other models [16], a basic physiological function is conserved in vitro. We

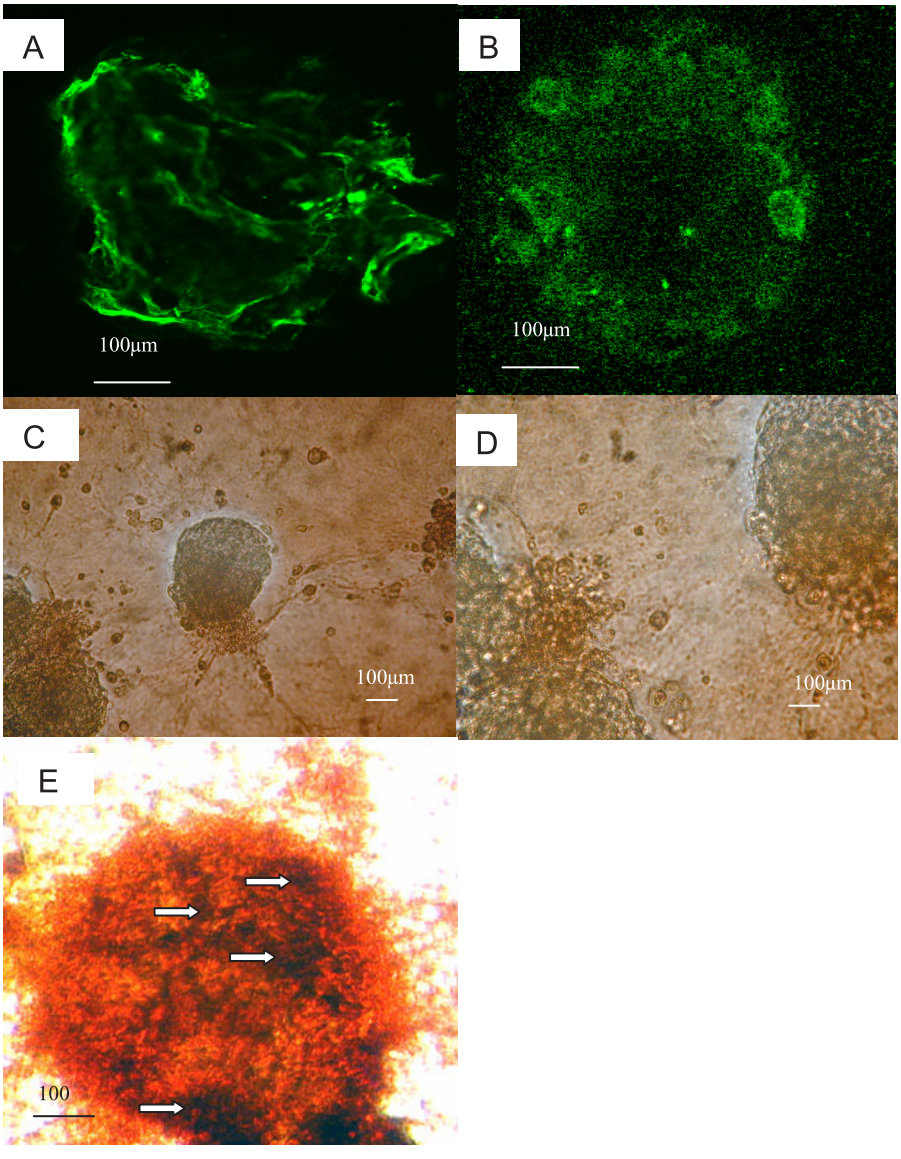

Figure 3. Characterization of mammosphere-like structure cells. (**A)** Mammosphere at day 4 of culture, labelled with anti cytokeratin mAb clone K 8.13; confocal microscopy examination (Alexa 488 nm). (**B)** Mammospheres at day 6 of culture, labelled with anti smooth muscle alpha-actin mAb; confocal microscopy examination (Alexa 488 nm). (**C)** and **(D)** Mammospheres at day 5 of culture, labelled with mAb anti smooth muscle alpha-actin, immunocytochemistry (DAB). See the layers of labelled cells. (**E)** Mammosphere at day 10 of culture. Double staining: anti cytokeratin clone K.8.13 (AEC-red) and anti smooth muscle alpha-actin mAb (DAB-brown) (arrows). (For a color version of this figure, see www.edpsciences.org/rnd.)

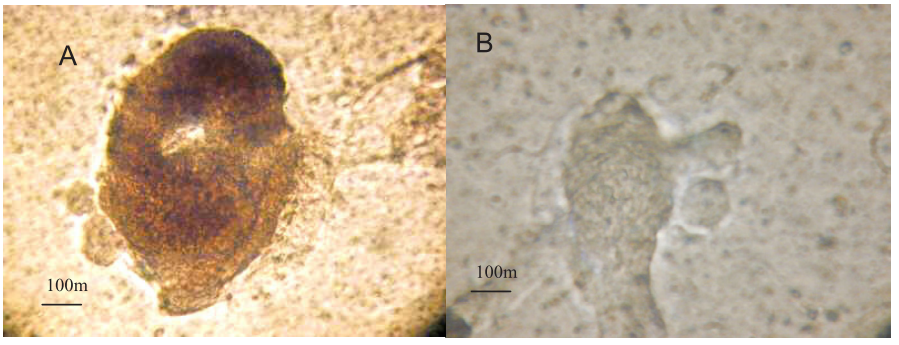

Figure 4. Alpha lactalbumin expression after in vitro stimulation with prolactin (Immuno cytochemistry, DAB). **(A)** Culture after 7 days hormonal stimulation. (**B)** Control, unstimulated culture. (For a color version of this figure, see www.edpsciences.org/rnd.)

characterized the cells forming the mammospheres, and established that the spherical organization of mammary epithelial cells was covered, in full or in part, by a layer of myoepithelial cells. This feature should be emphasized, as it shows the ability of different cell types to interact in vitro, reproducing the normal arrangement of cells in mammary tissue.

In our experimental conditions, Matrigel was diluted ½, and its initial structure could be modified by the organization and possible secretion of a cellular matrix between mammospheres (Fig. 1G). This culture model could differ from 3D cultures from cell lines, because the presence of different cell types leads to a more complex organization into tissue-like structures.

It is likely that a modification of culture conditions by the sequential addition of hormones and growth factors could further modify the development of the observed spatial organization, but our results already establish the formation, in vitro, of mammosphere-like structures, with myoepithelial cells in close contact with mammary epithelial cells. Mammospheres can be maintained as quiescent or, by the use of a prolactin supplemented medium, as lactating alveoli. Because three-dimensional organizations were obtained using cells which had been previously frozen, and with no additional culture steps before seeding into Matrigel, it will be possible to maintain mammary cell banks from the goats of different statuses for CAEV infection: uninfected, primary infection or reactivation.

Leucocytes isolated from peripheral blood migrate in Matrigel cultures, with a proportion coming into close contact with epithelial cells. This property will be used to infect mammary cell cultures by infected monocytes, after a first step of in vitro transendothelial migration in order to induce viral expression by the monocytes harboring viral DNA [12]. This model of culture also allows us to populate the mammary gland culture with immunocompetent cells after activating transmigration through the endothelium in vitro. In this work, we show that in vitro infection by free CAEV transmitted infection to acinus-like structures, as established by p30 late CAEV antigen expression. We used free, and not cell associated virus, so as to assess viral expression associated with 3D mammary gland cell cultures, as distinct from infected blood monocytes.

This model allowed us to mimic mammary tissue in vitro and to reproduce infection by cell-borne CAEV rather than by free

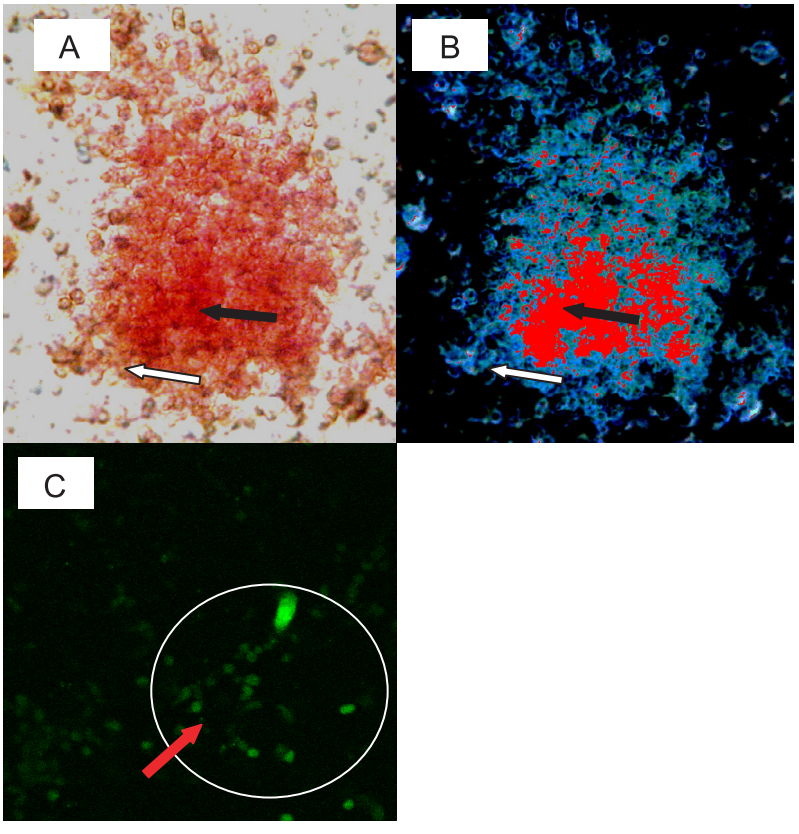

Figure 5. Localization of PBL in mammary gland cell Matrigel cultures. PBL were allowed to migrate for one day in Matrigel cultures of mammary gland cells. **(A)** Immunocytochemistry (Envision double stain), one day after migration of PBL in Matrigel cultures. Red (black arrow): epithelial cells (cytokeratin, K 8.13); brown (white arrow): leucocytes (CD4-CD8-CD14). (**B)** Same as 5.B, in false color. A typical labelled cell of each type was used to define the false-color range with tolerance set to 40 (PhotoFiltre Version 3.7, http://antonio.dacruz.free.fr). Red (black arrow): epithelial cells (cytokeratin, K 8.13); blue (white arrow): leucocytes (CD4-CD8-CD14). (**C)** Confocal microscopy examination. PBL were labelled with CFSE before seeding into the Matrigel cultures. PBL (green) are localized around an alveolar structure (circle). (For a color version of this figure, see www.edpsciences.org/rnd.)

virus. Furthermore, we were able to reconstitute the immunocompetent cell constituents of mammary tissue, offering a tool for the study of cellular interactions during CAEV infection. From a more general point of view, such 3D cultures of mammary gland cells could be employed for the study of the effect of mammary gland co-infections, and for in vitro screening of local immunostimulants.

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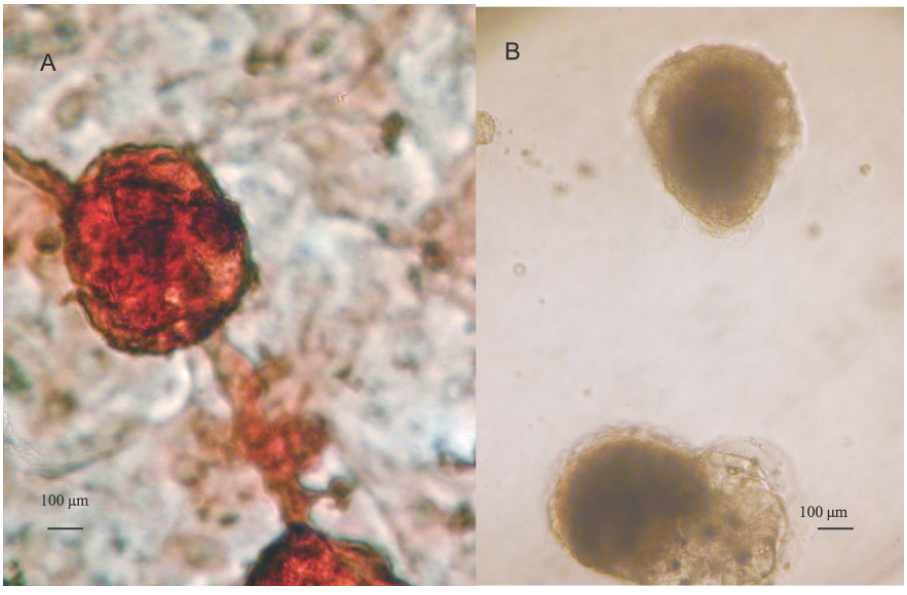

Figure 6. In vitro infection by CAEV. **(A)** 3D cultures at day 7 of infection by CAEV. Double staining: anti cytokeratine clone K.8.13 (AEC-red) and anti late CAEV antigen p30 (DAB-brown). **(B)** 3D cultures at day 7 of infection by CAEV. Modification of the morphology of acinus-like structures. (For a color version of this figure, see www.edpsciences.org/rnd.)

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