

Maedi-visna virus infection of ovine mammary epithelial cells

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Abstract – The aim of this work was to perform a complete study of maedi-visna virus (MVV) infected mammary glands of naturally-infected sheep, and to determine if cells other than macrophages undergo a productive viral infection in this organ. Fifteen seropositive and two seronegative ewes were selected from MVV-infected flocks on the basis of clinical indurative mastitis and three sheep from an MVV-free flock. Within the mammary gland, MVV-positive cells were located by immunohistochemistry in the stroma and the epithelial alveolar barrier, most likely the ovine mammary epithelial cells (OMEC) of the acini. In situ hybridization confirmed these findings. Ultrastructural studies showed the presence of lentivirus-like particles budding off the cell surface in the alveolar barrier and also free in the acinar lumen. The presence of mammary histopathological lesions and MVV together with clear indications of productive infection (demonstration of a cytopathic effect in OMEC cultures and infection of co-cultures) were observed in the 15 seropositive and one of the seronegative sheep from the infected flock. These findings demonstrate that the OMEC were infected in vivo and probably underwent productive infection when studied ex-vivo. The OMEC of MVV-free sheep, which had subsequently been infected in vitro with MVV, also showed productive infection when challenged in vitro, confirming the replication of MVV in OMEC in vitro. The presence of MVV-infected OMEC in the mammary gland from infected animals, the productive infection in these OMEC and the release of lentiviral particles to the acinar lumen may have relevance in the pathogenesis and transmission of MVV infection.

maedi-visna / mammary gland / epithelial cells / indurative mastitis / ovine

1. INTRODUCTION

Maedi-visna virus (MVV), a lentivirus closely related to the human immunodeficiency virus (HIV), causes a chronic wasting disease in sheep, characterized by pneu-

monia, mastitis, encephalitis and/or arthritis [7, 32]. Classically, the most important syndrome of the infection is interstitial pneumonia [7, 41] which is only seen in adult animals and shares common features with the respiratory affection observed in children

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infected by HIV [39]. The mammary affection was described in the early nineteen-eighties and seems to be as important as the pneumonic syndrome, at least in some geographical areas [22]. This type of mastitis is clinically difficult to detect and mainly consists in a diffuse hardening of the mammary gland often observed after parturition [12, 25, 26]. The main clinical sign is the lack of milk production, which is normally detected by a deficient growth of the lamb [34]. In all the target organs, lesions appear to be the consequence of viral replication in tissue macrophages [35]. Contrarily to HIV, MVV does not seem to replicate in lymphocytes *in vivo* and is unable to produce a patent HIV-type immunosuppression *in vivo* [11]. It has been assumed that tissue macrophages are the only target cells for MVV, but several studies have demonstrated that MVV can infect a broader cell range such as oligodendrocytes, astrocytes, fibroblasts [10], endothelial cells [6] and smooth muscle cells [21], among others. It is known that the epithelial cells from granulosa, goat oviduct and those present in goat milk are highly permissive *in vitro* to the caprine arthritis and encephalitis virus (CAEV) [14, 15, 27, 28]. Even early embryonic cells from *in vivo*-produced goat embryos transmit this virus [16].

In the sheep mammary gland, little is known on the location and replication of MVV although tissue macrophages have been proposed to be the cells harbouring the virus and responsible for the presence of MVV in milk [8, 19, 36]. However, ovine mammary epithelial cells (OMEC) can be CAEV targets, at least *in vitro* [20]. In a recent work using *in situ* PCR in association with immunohistochemistry on tissue sections of the mammary gland from naturally infected sheep as confirmed by their ELISA-positive status, Carrozza et al. [3] demonstrated the presence of the MVV genome in interstitial fibroblasts, acinar epithelial cells, macrophages, endothelial cells, adipocytes and desquamating epithelium or macrophages in the lumen of the acini.

The aim of this work was to carry out histopathological, ultrastructural and infectivity studies in order to investigate the location of MVV in the mammary gland of naturally-infected sheep, specifically in OMEC, and to determine if a productive viral infection occurs in these cells. The results obtained could have important epidemiological consequences in MVV transmission.

2. MATERIALS AND METHODS

2.1. Animals, infection and mammary gland status

The animals used in this study were of the Rasa Aragonesa breed and belonged to 6 seropositive flocks. A seronegative accredited Latxa breed flock was used for supply of control animals. The serological test for classification of flocks and animals was based on a recombinant ELISA test (Elitest[®], Hyphen BioMed, Neuville/Oise, France, formally known as Innotest[®], Innogenetics, Ghent, Belgium) [38, 44]. A standard agar gel immunodiffusion test (AGID) for MVV (CVL, Weybridge, United Kingdom) was also applied [46]. Seroprevalence to MVV among the seropositive flocks used ranged from 5 to 50% and all these seropositive flocks had sheep with MVV-induced indurative mastitis after parturition and sheep with interstitial pneumonia caused by MVV. Seventeen adult sheep were selected from the seropositive flocks on the basis of history or the presence of clinical indurative mastitis after parturition and three control animals were selected from the seronegative flock. Only animals free of bacterial mastitis were used, the absence of bacteria being verified by culturing samples of homogenized mammary parenchyma in tryptone-soy-broth and PPLO broth. The serological status of the seronegative flock was determined using the same tests and the results were negative for more than 15 years.

Table I. Study on mammary glands of sheep from maedi-visna virus (MVV) seropositive flocks (groups A, ELISA positive; and B, ELISA negative) and an MVV-free flock (group C). Serological status: determined by ELISA and AGID tests. Micro lesions: MVV-associated microscopical lesions in the mammary gland. CPE and MVV proteins: detection of cytopathic effect (CPE) and MVV proteins in cultured epithelial cells from explants and in co-cultured choroid plexus cells. MVV protein in tissue: immunocytochemistry for MVV proteins in tissue sections. ISH: presence of proviral DNA by in situ hybridization in tissue sections. TEM: presence of lentiviral-like particles by electronic transmission microscopy studies.

Group	Sheep no.	ELISA	AGID	Micro lesions	CPE/MVV proteins	MVV protein/tissue	ISH	TEM
A	1	+	+	++	+	+	NS#	NS
A	2, 3, 4	+	+	++	+	+	+	NS
A	5, 6, 10	+	+	++	+	+	+	-
A	7, 8, 9, 11, 12	+	+	++	+	+	+	+
A	13	+	-	+	+	+	NS	NS
A	14	+	-	+	+	+	+	NS
A	15	+	...*	++	+	+	+	NS
B	16	-	-	-	-	-	NS	NS
B	17	-	...	+	+	+	NS	NS
C	18, 19, 20	-	-	-	-	-	-	-

...*: Inconclusive serum by Agar Gel Immunodiffusion test (AGID).

NS#: specimen not studied.

The animals were distributed, on the basis of the ELISA results, into three groups (Tab. I): (A) seropositive, sheep Nos. 1–15; (B) seronegative, sheep Nos. 16–17; (C) control animals, sheep Nos. 18–20. The animals were killed by intravenous injection of barbiturate and exsanguinated. Post-mortem examination was carried out and samples were collected for further analysis.

2.2. Histopathology

A macroscopic study was performed in all animals, with samples from the mammary gland, lung and brain being processed for routine histopathology. Microscopically, lesions in the mammary gland were classified as negative (–) when having a normal pattern, moderate (+) when showing a remarkable number of lymphocytes within the parenchyma, and severe (++) when evidencing the characteristic MVV-induced pathology in the gland (diffuse accumulation of mononuclear cells spe-

cially lymphocytes in acini, as well as fibrosis and hyperplasia of lymphatic tissue, with follicle formation close to the lactiferous ducts and also within the alveolar parenchyma [45]).

2.3. Cultures of ovine mammary epithelial cells

The mammary gland was sectioned to expose the teat sinus and the glandular cistern. Cultures were carried out in the 20 animals under study as previously described [5, 13] and were examined daily for the development of a cytopathic effect (CPE) over a one month period. Detection of CPE was confirmed by Giemsa staining and viral proteins were detected by immunocytochemistry (ICC) using anti-p25 antibody (supplied by G.D. Harkiss, University of Edinburgh, United Kingdom).

Cultures of OMEC from sheep negative to MVV by AGID, ELISA, western blotting

(strips kindly supplied by Dr G. Harkiss) and histopathology/ ICC were also used to evidence the presence of MVV in vitro. Pure MVV-free OMEC were obtained from the three control animals as previously described [5, 13]. These cultures were 100% positive for cytokeratin by ICC, negative to CPE after at least 3 weeks and negative to RT-PCR for MVV, using primers for *pol* and *gag* regions of the EV1 strain of MVV and sheep Na, K-ATPase primers as internal control for RT-PCR amplification [47]. Confluent monolayers of these OMEC were infected with EV1 strain at 10^5 multiplicity of infection (MOI). Appearance of CPE was studied and RT-PCR was carried out in these infected cultures when CPE was detectable. Finally, the supernatant from these cultures was added to MVV-free ovine foetal choroid plexus cells (SCPC) in culture to allow viral replication in these permissive cells.

2.4. Productive MVV infection in epithelial cells from mammary gland explants

Virus specific proteins and induced CPE were examined in the OMEC cultures derived from the 20 sheep. In order to check productive MVV infection, SCPC from MVV-free foetuses were co-cultured with the cultures of OMEC from all sheep. The cultures were examined weekly for the appearance of syncytia and CPE. Cyto centrifuge smears were prepared to detect the presence of viral proteins by ICC (see Sect. 2.5).

2.5. Immunocytochemistry and immunohistochemistry (IHC)

Small pieces of mammary gland parenchyma were snap-frozen and stored at -70°C . In order to test different antibodies, 5 μm sequential sections were obtained with a cryostat. After being air-dried for at least 2 h at room temperature (RT), immunocyto/histochemical techniques [23] were used in two different kinds of samples: frozen tissue sections and cyto centrifuge preparations obtained from OMEC cultures

from all sheep. Also cyto centrifuge preparations obtained from SCPC cultures from the productive MVV infection studies were included for immunocytochemical detection. The monoclonal antibodies (mAb) used were against MVV proteins (MVVp15, mAb 415, supplied by Dr D. Houwers, University of Utrecht, The Netherlands; MVVp25 supplied by G.D. Harkiss, and MVVp27, supplied by Dr J. De Martini, Colorado State University, USA) and cytokeratin (mAb AE1-AE3, Boehringer Mannheim, Mannheim, Germany). The specificity of staining was demonstrated by including negative controls (bronchoalveolar lavage smears and tissue sections from MVV negative animals) and a known positive control (MVV-experimentally infected SCPC). Another control consisted on the omission or substitution of primary mAb.

2.6. In situ hybridization (ISH)

Digoxigenin-labeled probes were used for in situ hybridization, the fragment of DNA being a 512 nt product of the *gag* region [4] of the CAEV genome. This fragment had been shown to detect a number of strains of ovine lentiviruses and was produced by Nested-PCR. Labeling with digoxigenin was performed according to the manufacturer's instructions (DIG-High Prime; Boehringer Mannheim), the stain used being Pellet PaintTM Co-Precipitant (Novagen, Madison, WI, USA).

Tissue sections embedded in paraffin were used for ISH studies. Samples from the mammary gland parenchyma from sheep Nos. 2–12, 14, 15 (group A) and 18–20 (group C) were fixed in 10% buffered formalin for 24–72 h and embedded in paraffin. Cryostat sections were transferred onto coated charged glass slides (Hirschmann Laboratories, Eberstadt, Germany) and dried at 37°C . Paraffin was removed with xylene, the sections were cleaned with two 10 min washes of 100% alcohol and dried. The sections were permeabilized with pepsin for 15 min at 40°C in a humidified chamber. A wash in distilled water followed. The

sections were dehydrated through a graded alcohol series and air-dried. The probe was diluted into Probe Diluent (Biomed Corporation, Suite F Foster City, CA, USA), at concentrations ranging from 1 to 5%, using 100 μ L for sections. The probe-containing fluid was evenly spread over each sample by placing a large coverslip on top. The sections were heated in a thermal block at 90 °C for 3 min. Hybridization was performed overnight at 40 °C in a humidified chamber. Coverslips were removed and the slides were washed twice in 0.5 \times Probe Wash (Biomed Corporation) at 22–24 °C. An additional wash was performed with the same buffer at 65 °C for 2 h. The slides were rinsed in Tris-buffered saline (TBS) for 10 min at 22–24 °C and incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragment solution (1:200, Boehringer Mannheim), for 1 h in a humidified chamber at 22–24 °C, and finally washed in TBS three times for 10 min. The slides were incubated for 30–60 min in alkaline phosphatase substrate buffer (NBT/BCIP, Pierce Biotechnology, Rockford, IL, USA) in a humidified chamber at 22–24 °C. They were then rinsed twice with distilled water and counterstained with Nuclear Fast Red (Sigma Aldrich, St. Louis, MO, USA). The reaction was stopped with tap water and preparations were rinsed with distilled water, covered with Crystal Mount (Biomed Corporation) and dried at 60 °C. Once Crystal Mount was dry, permanent mounting was achieved by using DPX mounting medium (Sigma Aldrich). Cytospins of MVV EV1-infected choroid plexus cells and MVV positive tissues were used as positive controls. A non-related plasmid DNA was used as a negative control in each hybridization reaction.

2.7. Ultrastructural studies

Samples of mammary gland parenchyma and cistern for transmission electron microscopy (TEM) examination were obtained from sheep Nos. 5–12 and 18–20 and were fixed in Karnovsky fixative. The samples

were then sectioned in cubes of approximately 1 mm³, washed overnight in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide and dehydrated in an ascending grade of ethanol and embedded in epoxy resin (Epon 812, Fluka, Buchs SG, Switzerland). Ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and observed in a Philips CM-10 transmission electron microscope.

3. RESULTS

3.1. Serological observations

ELISA and AGID results are shown in Table I. In Group A, twelve sheep were positive in both tests, and three were positive by ELISA, but negative or inconclusive by AGID. In Group B, one animal was negative by both tests and the other was negative by ELISA and inconclusive by AGID. The animals of the control group were negative by both tests.

3.2. Clinical signs and pathological findings

All the animals from the infected flocks presented an indurative mastitis with a diffuse, bilateral hardening of the gland and with a firm and uniform section. Sheep were slaughtered within two months after parturition. All animals in group A showed microscopical lesions, although in two of the animals the lesions were only moderate. In Group B, sheep No. 17 also showed moderate lesions and sheep No. 16 was free of MVV-associated lesions but presented a marked and diffuse fibrosis of the gland. Remarkably, only one animal (No. 10) had MVV-related lesions in the lung. Control animals were free of lesions.

3.3. Productive MVV infection in epithelial cells from mammary tissue explants

Productive MVV infection in OMEC from mammary tissue explants (defined as

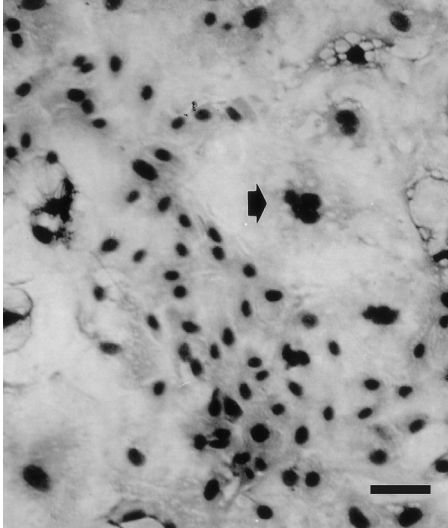


Figure 1. Ovine mammary gland epithelial cell (OMEC) culture from maedi-visna naturally infected sheep, showing the presence of cytopathic effect and syncytium formation (arrow). Giemsa staining. Bar: 32 μ m.

the presence of CPE, detection of MVV-associated proteins in cell cytoplasm, and infectivity by co-culture) was detected in the sheep of group A (with the exception of sheep No. 16) and in one sheep (No. 17) of group B but in none of the group C animals (Tab. I). These OMEC disclosed CPE after four weeks in culture (Fig. 1), with the MVV antigens being detected in these cells by ICC. Productive infection was finally confirmed by the appearance of CPE and the presence of MVV proteins in SCPC co-cultured with these OMEC. Cytokeratin-stained epithelial cells showed a diffuse, intense grey staining in the cytoplasm. The totality of the cells in OMEC preparations and none of the choroid plexus cells used as controls were stained by cytokeratin mAb. OMEC controls labeled with unrelated mAb were not stained. OMEC from the control group animals were free of MVV.

3.4. Experimental infection of MVV-free mammary epithelial cells cultures in vitro

OMEC cultures free of MVV (according to PCR and MVV protein staining) were experimentally infected in vitro with the strain EV1, developing CPE after five days. The RT-PCR amplicons obtained then with these cells had the expected size (142 nt for *gag* and 280 nt for *pol*) and were observed only in cultures showing CPE, confirming that OMEC had been successfully infected in vitro. ATPase amplicons demonstrated a correct RT-PCR amplification in all the samples. When supernatants of these infected OMEC cultures were added to cultured MVV-free fibroblasts, these cells developed CPE after four days, also being MVV positive by RT-PCR. These results further confirmed that productive infection of OMEC is achieved with experimentally infected OMEC.

3.5. Viral detection in tissue sections

The presence of the virus was observed by IHC and ISH within the mammary gland stroma mainly in cells morphologically compatible with macrophages that were located around the acini and lymphoid follicles, as well as in the interstitium. However, viral presence was also found in individual acinar cells morphologically compatible with epithelial cells by IHC and ISH, the strongest signal by IHC being located at the apical cell border (Figs. 2 and 3). Sections treated with unrelated mAb showed no staining by IHC and no reaction by ISH. Sheep Nos. 16 and 18–20 showed no positive reaction to viral antigens (Tab. I). Lentivirus-like budding particles were observed by TEM at the apical cell border of a few milk-secreting OMEC in sheep Nos. 7–9, 11 and 12, showing the characteristic thickening of the cellular membrane (Figs. 4 and 5). Particles were sometimes observed free in the acinar lumen. No particles were observed in the preparations from sheep Nos. 5–6, 10 and 18–20 (Tab. I).

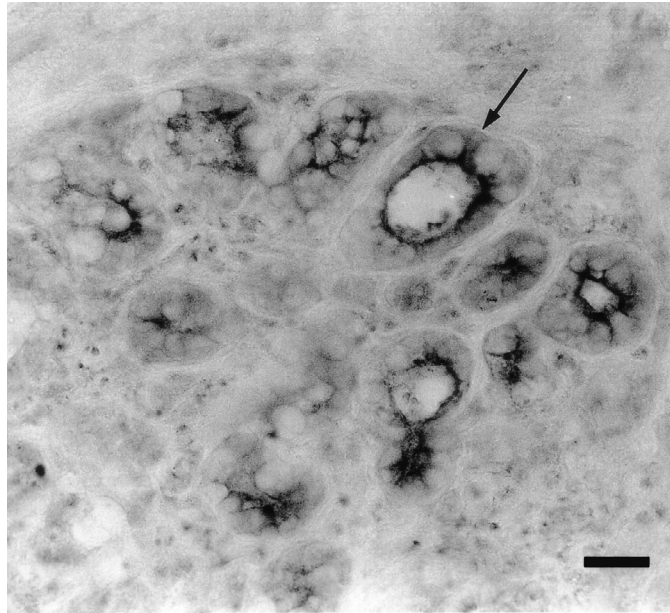


Figure 2. Tissue sections from maedi-visna naturally infected sheep showing positive signals to MVV in the alveolar lining cells by single immunocytochemistry (p25 antibody). Bar: 60 μ m.

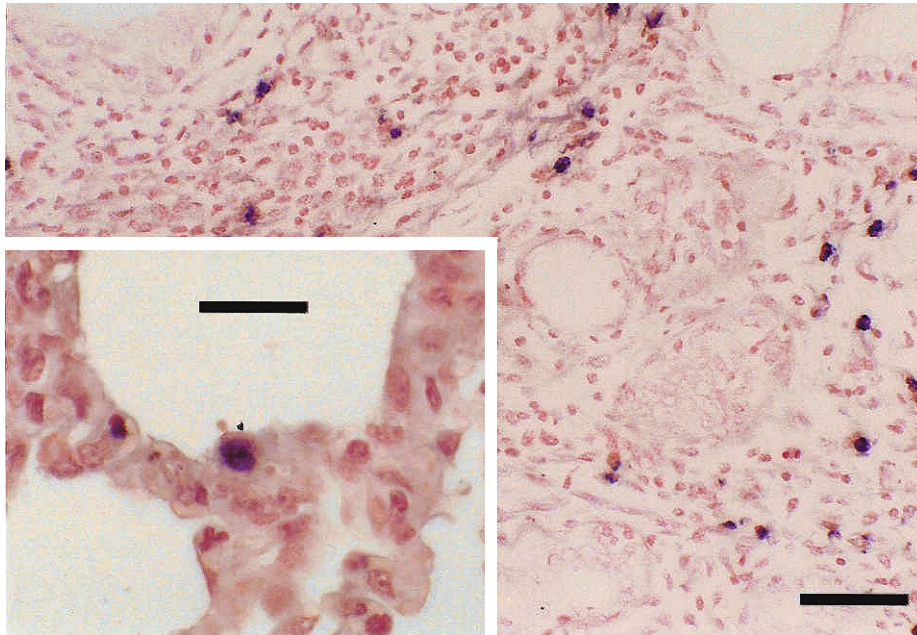


Figure 3. Tissue sections from maedi-visna naturally infected sheep studied by in situ hybridization. Positive cells are located within the inflammatory reaction. Bar: 40 μ m. Insert: A single positive cell morphologically compatible with an OMEC. Bar: 20 μ m.

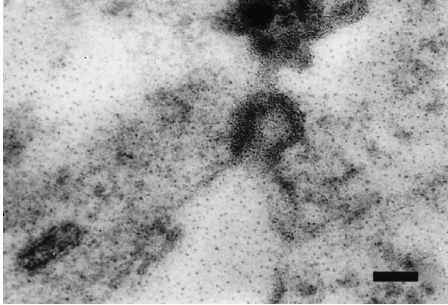


Figure 4. Mammary gland tissue section of maedi-visna naturally infected sheep. A lentivirus-like particle is observed at the apical cell border by transmission electron microscopy. Bar: 125 nm.

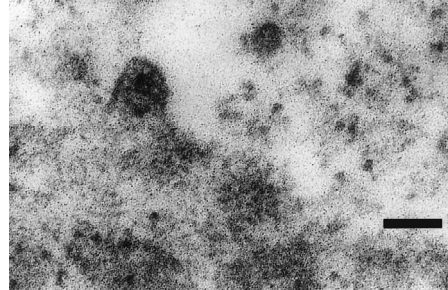


Figure 5. Characteristic budding particle with thickening of the cell membrane, shown by transmission electron microscopy. Bar: 100 nm.

4. DISCUSSION

Analysis of MVV-affected ovine mammary gland revealed in this study that OMEC undergo infection. Detection of viral proteins and viral nucleic acids in the OMEC indicates that cells undergoing MVV infection in the udder are not only macrophages but also OMEC. Additionally, the presence of budding particles at the OMEC cell membrane level strongly suggests that OMEC cells give rise to infective virus and, therefore, are productively infected; a fact that is further supported by the infection of susceptible cells in co-cultures with infected OMEC.

Previous *in vitro* studies demonstrated that lentivirus-free goat and sheep mammary epithelial cells can be experimentally infected with CAEV [20]. We further demonstrate that MVV can experimentally infect OMEC *in vitro*. These cells not only presented a CPE associated with the presence of MVV proteins, but also the corresponding culture supernatants were able to productively infect choroid plexus cells. A similar type of CPE have been reported to happen after 3 weeks of culture in goat mammary epithelial cells (GMEC) infected with lentivirus [20]. In addition, the co-culture of SCPC with the OMEC explants from

MVV naturally-infected sheep, show that these OMEC explants were also able to undergo productive infection during *in vitro* culture. The role of accessory cells as a source of MVV in cultures is unlikely to have happened because macrophages were removed by selective trypsinisation during the first week of culture and the presence of fibroblasts would have been detected by features such as earlier and more lytic CPE [20].

These observations underline that apparently no accessory cells are needed for MVV presentation and replication in OMEC. MVV can replicate *in vitro* in a wide variety of ovine cells [30], because the restriction that the virus presents under natural conditions is no longer maintained *in vitro* [29]. Similarly, CAEV, the caprine counterpart of MVV, can replicate in a variety of cells *in vitro* [14, 15, 20, 27, 28, 37] and, significantly, HIV-1 can also productively infect different cell lines *in vitro* including human OMEC [43].

IHC, ISH and TEM observations have demonstrated the *in vivo* presence of MVV in the acinar epithelia, indicating that OMEC are permissive for MVV infection *in vivo* and strongly suggesting that these cells may give rise to infective particles in natural conditions. *In vivo* MVV infection of the mammary gland is likely initiated by infected blood monocytes, which differentiate into macrophages in the mammary

tissue [8, 18]. Once differentiated, a direct macrophage-OMEC contact can be a mechanism of OMEC or macrophage infection within the mammary gland. It has been suggested from *in vitro* studies on CAEV infectivity that OMEC can be the targets for CAEV in the sheep mammary gland [20]. This is compatible with the proposal from *in vitro* studies that the myoepithelial cell may act as a reservoir cell and that, after infection, the mature luminal cell (OMEC) acquires the capacity to bind leukocytes and facilitates cellular interactions [24] and viral transmission. These observations are in agreement with our finding that not only macrophages but also OMEC may have a role in replication and as a reservoir of the virus in this organ and in MVV pathogenesis. A variety of epithelial cells are *in vivo* targets for lentiviruses and other retroviruses. MVV proviral DNA has been detected in MVV experimentally-infected bronchial epithelial cells [42], and CAEV proviral DNA has been observed in epithelial cells of the intestinal crypts [48]. Similarly, bovine leukaemia virus can replicate in OMEC in natural infection [2] and feline immunodeficiency virus (FIV) replicates in the salivary gland epithelium [33].

Virus-like particles reaching the acinar lumen, also observed by TEM in a previous work [17], are most likely present in milk, where MVV has been detected mainly in infected macrophages [31] that have traversed the epithelial barrier by a well-known mechanism [7, 8]. Furthermore, milk can also have OMEC [19], and some of these cells can be infected in MVV infected animals with clinical mastitis, confirming that milk may be a very important route of infection to the newborn lamb [8]. These findings are consistent with the widely accepted phenomenon of newborn lamb MVV infection through maternal milk at early stages of life [7, 9], and with previous observations that MVV can be isolated from mammary tissue [18] and milk [19]. Remarkably, CAEV appears to spread mainly through colostrum and milk [1, 9] and the feline immunodeficiency virus

(FIV) can also be transmitted via milk [40]. Moreover, MVV could enter the OMEC but infectious virus could be produced after OMEC activation. OMEC may provide an *in vivo* reservoir for the persistence of proviral MVV with the production and release of infectious viruses after cell reactivation by host factors, for example hormones [20]. It is important to underline that all positive animals studied in our work were in the post-partum period, and this could be linked to the detection and production of MVV in OMEC.

Milk can be an important fluid for MVV infection diagnosis. However, some of the MVV clinical mastitis animals do not produce milk, and serological methods may then be of higher diagnostic value. The association found in this study between clinical mastitis, seropositivity to MVV by ELISA and viral detection in the mammary gland was high, with one exception. Among the serologic tests used in this study to detect MVV antibodies, the increased sensitivity observed in the ELISA with respect to AGID was expected according to previous studies [38, 44] and confirmed by the presence of viral genome and proteins in histopathological observations.

In conclusion, the present study broadens the cellular spectrum of MVV distribution beyond the macrophage-monocyte lineage. The role of the OMEC as a reservoir of MVV *in vivo* provides new insight on MVV pathogenesis in the mammary gland and brings up new issues in the study of MVV replication and transmission to healthy cells, different tissues and uninfected animals. Additionally, our results support the role of milk as an important route of infection to the newborn lamb.

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