Detection of Visna Maedi virus in mesenteric lymph nodes and in other lymphoid tissues of sheep three years after respiratory infection

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ABSTRACT: Visna/Maedi virus (VMV), a small ruminant lentivirus responsible for lymphoproliferative pneumonia, encephalitis, arthritis and/or mastitis in sheep, has been detected in different non-lymphoid organs. However, only a few investigations have been carried out in lymphoid tissues. In this study, some lymphoid tissues and lymph node draining or non-draining VMV target organs from five sheep infected experimentally by the respiratory route three years previously were investigated. Archival samples of spleen, red bone marrow, caudal mediastinal lymph nodes, mammary lymph nodes, popliteal lymph nodes and mesenteric lymph nodes were tested by PCR for the presence of proviral DNA. Popliteal and mesenteric lymph node samples were tested also by immunohistochemical staining of the viral capsid antigen p28. The proviral DNA was detected by PCR in all the lymphoid tissue samples from the infected sheep. The viral antigen was stained in mononuclear cells in popliteal and mesenteric lymph nodes of the infected sheep. Although the lymph nodes draining the classical target organs seem to be more infected than the others, both the viral capsid antigen and the proviral DNA were present also in lymph nodes draining non-target organs, such as the mesenteric lymph nodes. These findings show the presence of VMV in different lymphoid tissues in the late stages of infection and suggest a potential role of these tissues as a site for viral reservoir and replication, even three years after infection.

Keywords: Visna/Maedi virus; sheep; lymphoid tissue; immunohistochemistry; PCR; experimental infection

Visna/Maedi virus (VMV) is a small ruminant lentivirus belonging to the Retroviridae family. It causes pneumonia (Maedi), encephalitis (Visna), arthritis and/or mastitis in sheep (Van derMolen and Houwers 1987; Houwers et al. 1988; Narayan and Clements 1989). The infection is mainly transmitted by the respiratory route and by colostrum. Unlike other lentiviruses, VMV is considered to have a tropism for monocyte/macrophages and not for lymphocytes and does not cause immunosuppression. Lymphoproliferative lesions have been reported mainly in lungs, the central nervous system, udder and joints (Pepin et al. 1998). However, the virus has been detected also in other organs, such as the kidney, the liver, the heart and the third eyelid (Capucchio et al. 2003; Angelopoulou et al. 2006; Brellou et al. 2007). There is a paucity of data regarding VMV infection in lymphoid organs. Research has been focused usually on lymph nodes draining target organs (Zink et al. 1987; Storset et al. 1997; Brodie et al. 1995; Niesalla et al. 2008). In our previous work, the VMV capsid antigen p28 has been detected in the mediastinal lymph nodes, in the spleen and in the bone marrow of eight sheep infected intratracheally three years earlier (Preziuso et al. 2003). These samples were not tested by bio-molecular methods.

The aim of this work was to investigate the localization of VMV in different lymphoid tissues of sheep three years after the respiratory infection, with particular focus on lymph nodes not draining VMV target organs.

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MATERIAL AND METHODS

Samples. Archival samples from seven out of ten ewes collected during a previous experiment were used in this work. Five ewes (numbers 1, 3, 4, 6 and 8) were inoculated intratracheally with the VMV strain 130 and two uninoculated sheep (numbers 9 and 10) were used as controls (Preziuso et al. 2003). The sheep were maintained under the same environmental conditions for three years. The archival samples were embedded in paraffin or stored at –20 °C and were collected from spleen, red bone marrow, caudal mediastinal lymph nodes, mammary lymph nodes, popliteal lymph nodes and mesenteric lymph nodes. Samples from three sheep (numbers 2, 5 and 7) inoculated with VMV were unavailable for this study. Preliminary results on the popliteal lymph node of sheep 1 were excluded from this experiment because they had been used in a previous preliminary study together with popliteal lymph node samples from sheep 2, 5 and 7 (Preziuso et al. 2009).

DNA extraction and PCR. DNA was extracted from 25 mg of frozen bone marrow and lymph node samples and from 10 mg of frozen spleen samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. The DNA was eluted in 100 µl AE buffer and immediately used for PCR. Tubes containing water were used as negative controls during DNA extraction.

A new nested PCR protocol was used to amplify a tract of the LTR gene. In the first reaction, the primers Fex- 5'-TGA CAC AGC AAA TGT AAC CGC AAG-3' and Rex 5'-CCA CGT TGG GCG CCA GCT GCG AGA-3' were used to amplify a highly conserved region of the LTR (Rosati et al. 1995). The first-round of PCR was performed in a reaction mix volume of 50 µl with deoxynucleoside triphosphates at a final concentration of 200µM each, $MgCl_2$ at 2.5mM, Taq DNA polymerase at 0.04 IU/µl (Taq PCR Core Kit, Qiagen, Hilden, Germany), each primer at a final concentration of 0.5µM and 2 µl of the template DNA. The reaction conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s, and a final extension of 72 °C for 7 min. In order to increase the PCR sensitivity, 1 µl of the product of the first reaction was used as a DNA template in the second round of PCR, which used the primers Fin: 5'-AAG TCA TGT A(G/T)C AGC TGA TGC TT-3' and Rin: 5'-TTG CAC GGA ATT AGT AAC G-3' (Ryan et al. 2000). The reaction composition and the amplification conditions were the same as in the first-round PCR but the annealing temperature was 50 °C and 1 µl of the product of the first PCR was used as a template.

Both first and second reaction products were analysed by electrophoresis of 10 µl of reaction products in 2.5% agarose gels containing 0.5 mg/ml of ethidium bromide with Tris-borate-EDTA buffer (89mM Tris, 89mM boric acid, 2mM EDTA pH: 8.3). Images were visualized on a UV transluminator and captured by the Kodak Gel Logic 100 system.

Histology and immunohistochemistry. In this study, mammary lymph nodes, popliteal lymph nodes and mesenteric lymph nodes were tested by immunohistochemistry (IHC), while the other lymphoid samples were already tested in other studies (Preziuso et al. 2003, 2009). The popliteal and mesenteric lymph nodes were also examined for histological lesions, while the histopathological findings in the mammary lymph node samples have been described previously (Preziuso et al. 2003).

Sequential slides (3 µm) cut from the paraffin embedded samples were used for haematoxylin-eosin staining and for IHC. The anti-p28 Capsid Protein MoAb CAEP5A1 (VMRD Inc., Pullman,WA, USA) was used to detect the viral capsid antigen by an avidin-biotin complex as described previously (Preziuso et al. 2003).

RESULTS

The proviral DNA was detected by nested PCR in all samples from the infected sheep but not from the uninfected sheep (Table 1). All mammary and mediastinal lymph node samples were positive by both first-round and second-round PCR, demonstrating a high amount of proviral DNA in the samples. All bone marrow samples were positive only by second-round PCR, suggesting a lower presence of the proviral DNA. Some samples of mesenteric lymph nodes, spleen and popliteal lymph nodes were positive by the first-round PCR, while the others were positive by the second-round PCR, indicating varying amounts of proviral DNA in the samples (Table 1).

Histopathological examination of the popliteal lymph nodes did not show any significant lesions. In the mesenteric lymph nodes of sheep 1, 4 and 6 moderate hyperplasia was observed. The cortex

Table 1. PCR results from spleen, red bone marrow (RBM), mediastinal lymph nodes (MedLN), mammary lymph nodes (MamLN), popliteal lymph nodes (PLN) and mesenteric lymph nodes (MesLN) collected from five infected sheep (No. 1, 3, 4, 6, 8) and from two uninoculated control sheep (No. 9 and 10)

Sheep No.	Spleen	RBM	MedLN	MamLN	PLN	MesLN
1	sPCR	nPCR	sPCR	sPCR	*	sPCR
3	sPCR	nPCR	sPCR	sPCR	sPCR	nPCR
4	nPCR	nPCR	sPCR	sPCR	sPCR	sPCR
6	nPCR	nPCR	sPCR	sPCR	nPCR	nPCR
8	sPCR	nPCR	sPCR	sPCR	sPCR	sPCR
9	neg.	neg.	neg.	neg.	neg.	neg.
10	neg.	neg.	neg.	neg.	neg.	neg.

neg. = negative; sPCR = positive in first-round (single PCR) and in second-round PCR (nested PCR); nPCR = positive only in second-round PCR (nested PCR)
*sample tested in a previous work (Preziuso et al. 2009) and positive by sPCR

was moderately expanded and some active secondary follicles with evident germinal centres were observed, especially in sheep number 4 and 6.

The p28 antigen was detected by IHC in the mammary lymph nodes, popliteal lymph nodes and mesenteric lymph nodes from the infected sheep but not in the samples from the two uninfected controls. The staining was mainly observed in the large cytoplasm of big round-to-oval or lightly elongated cells resembling macrophages. In the popliteal lymph nodes (Figure 1), the positive cells were mainly in the cortical area and were more numerous in sheep 4 and 6 than in sheep 3 and 8. A few stained cells were scattered in the cortex of the mammary and mesenteric lymph nodes.

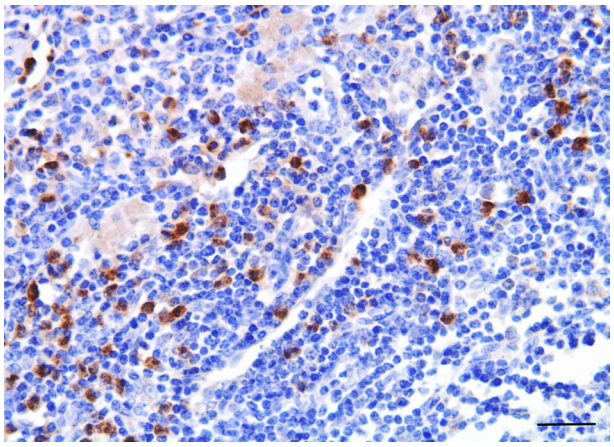

Figure 1. Popliteal lymph node, sheep No. 4. Numerous positive cells in the cortical area (anti-p28 IHC staining; bar, 70 μm)

DISCUSSION

Lymph nodes are well known target organs for some lentivirus infections, such as HIV and FIV. Little information is available on VMV infection in these sites. Popliteal efferent lymphatic cannulation experiments in sheep infected subcutaneously at several sites within the drainage area of the cannulated lymph node has demonstrated VMV replication and cell-associated dissemination some days post-infection (Bird et al. 1993). The lymph nodes proved to be an important CAEV reservoir, with moderate virus replication relative to what is reported for lentiviruses of primates (Ravazzolo et al. 2006). CAEV has been occasionally isolated from mammary lymph nodes, spleen and bone marrow (Surman et al. 1997). The Ovine Lentivirus has also been isolated from the bone marrow (Brodie et al. 1995), where viral replication has been demonstrated by combined immunocytochemistry and *in situ* hybridization in two lambs six or 16 weeks after virus inoculation (Gendelman et al. 1985). Small ruminant lentivirus RNA or capsid proteins have occasionally been detected in macrophages or macrophage-like cells in the spleen (Zink et al. 1990; Gendelman et al. 1985; Zink et al 1990; Ravazzolo et al. 2006).

In this work, we detected the DNA and capsid antigen of VMV in different lymphoid tissues three years after infection. These findings suggest that the lymphoid tissues could play a significant role in the pathogenesis of VMV infection and could be an important site of viral replication and reservoir, even some years after infection.

The finding of both the proviral DNA and the viral capsid antigen in the mesenteric lymph nodes of sheep three years after infection by the respiratory route was unexpected. In only one case was a weak positivity in these lymph nodes reported in two lambs a few weeks after pulmonary inoculation (Gendelman et al. 1985). Mesenteric lymph nodes have been suggested as a reservoir of HIV (Estaquier and Hurtrel 2008). In our previous work, the viral DNA and the capsid antigen have been found in the mesenteric lymph nodes of lambs fed with the colostrum of their mothers experimentally infected some years earlier (Preziuso et al. 2004). However, these findings were considered normal because the route of infection was the alimentary tract, the mesenteric lymph nodes were draining target organs and the tissues were examined a few hours after feeding infected colostrum.

Taken together, these results complete our histological and immunohistochemical preliminary results presented previously and suggest a diffuse infection of VMV in the lymphoid tissues at a late stage of infection. The lymph nodes which drain target organs seem to be more infected, although the virus is present also in other lymphoid tissues. These tissues harbour not only the viral antigen, but also the proviral DNA, suggesting a potential role as a long-term reservoir. The involvement of the lymphoid tissue in HIV and FIV infection is known, but it is not fully investigated in infections caused by lentiviruses with a different cell tropism, such as equine and small ruminant lentiviruses. Our work shows that the VMV capsid antigen and the proviral DNA are detectable in different sheep lymphoid tissues, including lymph nodes which do not drain target organs, three years after the respiratory infection.

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