Safety and immunogenicity of myxoma virus as a new viral vector for small ruminants

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Myxoma virus (MYXV), a leporide-specific poxvirus, represents an attractive candidate for the generation of safe and non-replicative vaccine vectors for other species. With the aim of developing new recombinant vaccines for ruminants, we evaluated the safety and the immunogenicity of recombinant MYXV in sheep. In vitro studies indicated that ovine primary fibroblasts were not permissive for MYXV and that infection of ovine peripheral blood mononuclear cells occurred at a low rate. Although non-specific activation significantly improved the susceptibility of lymphocytes, MYXV infection remained abortive. Histological and immunohistochemical examination at the inoculation sites revealed the development of an inflammatory process and allowed the detection of sparse infected cells in the dermis. In addition, inoculated sheep developed an antibody response directed against MYXV and the product of the transgene. Overall, these results provide the first line of evidence on the potential of MYXV as a viral vector for ruminants.

INTRODUCTION

As most ruminant vaccines use attenuated strains of pathogens, naturally infected and vaccinated animals cannot easily be differentiated. Development of recombinant vaccines for ruminant species would be helpful for the implementation of vaccine policies. The family Poxviridae, a family of large DNA viruses, represents attractive tools for vaccine vector development against a broad spectrum of diseases. This is particularly because of their ability to combine safety and immunogenicity. Their large genome size and their ability for homologous recombination allow as much as 25 kb of foreign DNA to be inserted. This enables the expression of genes and/or gene clusters, inducing a strong immunity against these target antigens (Moss et al., 1996). Moreover, they replicate exclusively in the cytoplasm of infected host cells (Moss, 2001).

The prototype poxvirus, vaccinia virus (VACV), has been used extensively because of its well-defined molecular characteristics (Moss, 2001) and its success in the vaccination programme to eradicate smallpox (WHO, 1980). Nevertheless, this vector is not considered to be safe enough for use, as virus replication is not host-restricted and allows dissemination (Redfield et al., 1987). Since host-restricted vaccine vectors are assumed to be safer, the VACV MVA strain or avipoxviruses (fowlpox and canarypox viruses) have been studied extensively, leading to the development of non-replicative vectors in different species (Boyle & Coupar, 1988; Taylor & Paolletti, 1988; Taylor et al., 1988; Sutter & Moss, 1992; Tartaglia et al., 1993; Carroll & Moss, 1997). New host-restricted poxviruses are also attractive. For instance, lumpy skin disease virus, a capripoxvirus with a host range limited to ruminants, was successfully evaluated as a non-replicative vaccine vector for non-ruminant species (Aspden et al., 2003).

Among the poxviruses, myxoma virus (MYXV) is another attractive candidate for vaccine development. This virus specifically infects Leporidae and causes the lethal disease myxomatosis in European rabbits (Oryctolagus cuniculus) (Fenner & Ross, 1994). Recombinant attenuated strains of MYXV have been shown to be efficient for rabbit vaccination against both myxomatosis and rabbit haemorrhagic disease (Bertagnoli et al., 1996; Barcena et al., 2000). Its potential for acting as a vaccine vector in non-leporide species has also been demonstrated by vaccination of cats against feline calicivirus (McCabe et al., 2002; McCabe & Spibey, 2005).

In view of the emergence of ovine infectious diseases in Europe, such as new outbreaks of bluetongue in 2006 and 2007, we examined the vaccine potential of recombinant MYXV for small ruminants. With this aim, we studied MYXV tropism in ovine cells, as very little information is
available for ruminant species (Pignolet et al., 2007), although different studies have indicated previously that MYXV tropism in vitro is not restricted to rabbit cells (Lalani et al., 1999; Sypula et al. 2004; Johnston et al., 2005; Wang et al., 2006). In this work, we investigated the multiplication of MYXV, both wild-type strain T1 and cell-cultured attenuated vaccine strain SG33 (Saurat et al., 1978; Bertagnoli et al., 1996), in ovine primary fibroblasts and peripheral blood mononuclear cells (PBMCs). The subsets of ovine PBMC preferentially infected by MYXV were also identified. Finally, in vivo analysis using recombinant virus was performed to evaluate its safety and immunogenicity in sheep.

Overall, our results provide the first indications of the safety and immunogenicity of recombinant MYXV for ovine species, suggesting that MYXV could be used as a non-replicative viral vector for small ruminants.

**METHODS**

**Cell culture and media.** Rabbit kidney cells (RK13; ATCC CCL-37) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL) supplemented with 10 % fetal calf serum (FCS). Culture medium was supplemented with 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹.

Primary ovine dermal fibroblast cells were isolated from the skin of a sheep. Briefly, skin tissue fragments were softly scraped and incubated for 15 min with trypsin (3 g l⁻¹) at 37 °C and then diluted in 20 % FCS and DMEM. The solution was filtered through a 100 µm cell filter (Falcon) and centrifuged at 800 g. Cells were harvested and cultured in DMEM supplemented with 10 % FCS, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹.

Ovine (Lacaune breed) and rabbit (New Zealand) blood was collected in EDTA tubes. For the isolation of PBMCs, PBS-diluted blood (1:2) was loaded on a density gradient (Ficoll-Paque Plus; Amersham) and centrifuged at 870 g for 10 min. Culture of PBMCs was performed at 37 °C in 5 % CO₂ in RPMI 1640 with GlutaMAX, 5 mM HEPES (Gibco-BRL) supplemented with 10 % FCS, 1 % sodium pyruvate (Gibco-BRL), 1 % non essential amino acids (Gibco-BRL), 1 % β-mercaptoethanol (Gibco-BRL), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Where indicated, cells were activated using 125 ng phorbol myristate acetate (PMA) ml⁻¹ and 50 ng ionomycin ml⁻¹.

**Viruses.** All recombinant viruses were derived from the wild-type Toulouse 1 (T1) and the vaccine attenuated SG33 (Saurat et al., 1978) strains and modified in the laboratory. The T1–Serp2–GFP recombinant virus was constructed by cloning the green fluorescent protein gene (gfp) fused to the 3' end of the serp2 gene. The T1-TK::lacZ recombinant virus contained the Escherichia coli lacZ gene driven by the vaccinia virus P11 late promoter inserted into MYXV thymidine kinase (TK) gene. The SG33–VP60 recombinant virus contained the rabbit haemorrhagic disease virus (RHDV) capsid (VP60)-encoding gene under the control of the strong early/late vaccinia virus P7.5 promoter inserted into the M11L–MGF locus (Bertagnoli et al., 1996). The SG33–GFP recombinant virus expressed enhanced GFP (eGFP) driven by the P7.5 promoter. The P7.5–eGFP cassette and the E. coli xanthine–guanine phosphoribosyl transferase (gpt) gene (Boyle & Coupar, 1988) under the control of P7.5 promoter were inserted instead of the M11L–MGF locus.

**Infection and virus growth curves.** Single-step analysis was performed on RK13 or ovine primary fibroblasts by infecting 10⁶ cells at an m.o.i. of 5. Adsorption was performed at 4 °C with inoculum containing 25 mM HEPES. After 90 min, the inoculum was removed and the cells were washed three times and incubated at 37 °C, 5 % CO₂.

For growth analysis in PBMCs, 2 x 10⁶ cells were infected at an m.o.i. of 1. The inoculum was allowed to adsorb for 90 min. Virus was then removed and each well was washed three times with culture medium and incubated with medium supplemented with 5 % FCS at 37 °C, 5 % CO₂.

At each time point of interest (as indicated in the figures), cells were frozen. To release virus from infected cells, each collected sample was subjected to three freeze–thaw cycles before titration.

For titration, dilutions of lystate were added to RK13 cells and adsorbed for 90 min. The inoculum was removed and fresh RK13 medium, supplemented with 5 % FCS, was added. After 48 h, the medium was removed and replaced by solid medium containing minimal essential Eagle’s medium supplemented with 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 2 % FCS, 25 mM HEPES, 0.2 % NaHCO₃ and 1 % low-melting-point agarose (Invitrogen). After 2 days, plaques were counted by macroscopic examination.

**X-Gal staining and analysis.** Rabbit and ovine cells (90–95 % confluency) were infected with T1-TK::LacZ virus (m.o.i. of 0.1 and 1). At 12 hours post-infection (p.i.), they were fixed with 2.5 % glutaraldehyde for 15 min at room temperature and stained with 2 mg X-Gal ml⁻¹ in 2 mM MgCl₂, 5 mM K₃Fe(CN)₆·3H₂O, 5 mM K₅Fe(CN)₆ in PBS for 4–10 h and observed by microscopy.

**Flow cytometry analysis.** Resting or activated PBMCs were infected with SG33–GFP (m.o.i. of 0.5–10) or mock-infected. Cells were harvested and washed in PBS with 0.5 % BSA, 2.5 mM EDTA, in 96-well plates. Cells were then incubated for 20 min at 4 °C with appropriate dilutions of Alexa Fluor 647-labelled anti-CD2, anti-CD5, phycoerythrin-labelled anti-CD4, -CD8 and -DR/DQ, biotin-conjugated anti-WC1, or isotype control (Seraclone). Phycoerythrin-conjugated streptavidin was added for anti-WC1 labelling. Cell viability was determined by the addition of propidium iodide (BD Biosciences Pharmingen) at 1 µg ml⁻¹ just before acquisition. Acquisition was performed using a FACS Calibur (Becton Dickinson). Dead cells and debris were excluded by appropriate gating and 30 000 events were collected. Analysis was performed using FlowJo or CellQuest Pro software.

**Intradermal inoculation of sheep and in situ examination.** Two adult sheep were inoculated in the flank with three doses (10⁵, 10⁶ and 10⁷ p.f.u.) of SG33–VP60 recombinant virus with six points of intradermal (i.d.) injection for each dose (around 7 cm between each injection point). Inoculation sites were sampled using biopsy punches at 6, 12, 18, 24, 48 and 72 h p.i. for each dose tested. As a control, cell culture medium was also injected and sampled at each time point. Animals were cared for following EU recommendations for animal welfare. After fixation in 10 % neutral formalin, tissues were routinely processed into paraffin blocks, sectioned at a thickness of 4 µm and stained with haematoxylin and eosin (H&E) for microscopic examination. Histological lesions were assessed and graded as follows: +, minimal; ++, light; ++++, moderate; ++++, marked; +++++, severe (according to Armed Forces Institute of Pathology recommendations). Sections were also treated with 0.1 % trypsin in PBS (pH 7.6) for 30 min at 37 °C and viral antigens were detected as described elsewhere (Guerin et al., 2001) using a rabbit polyclonal anti-MYXV serum as primary antibody and a secondary antibody (biotinylated goat anti-rabbit IgG; Dako).
Horseradish peroxidase–streptavidin complex was added and revealed using 3,3'-diaminobenzidine tetrahydrochloride. Nuclei were counterstained with Mayer’s haematoxylin. Negative controls comprised sections incubated either without specific primary antibody or with pre-immune serum in place of the specific primary antibody. A third negative control was samples injected with cell culture medium and processed using the same procedure as inoculated samples. No immunohistochemical staining was detected in the controls.

**Immunization of sheep with SG33–VP60 recombinant virus.**

Ten sheep were injected i.d. twice with 0.1 ml virus (2 × 10^6 p.f.u.) per sheep with a 21-day interval. Animals were monitored daily for temperature and clinical signs. Blood samples were taken on days 0, 21, 35 and 42 after the first immunization. The serological responses against MYXV and RHDV VP60 protein were evaluated by ELISA (using recombinant purified VP60 and semi-purified MYXV as antigens) (Bertagnoli et al., 1996). Briefly, ELISA plates (Falcon) were coated with 1 µg recombinant baculovirus-purified VP60 or 1 µg semi-purified MYXV overnight at 4 ºC. Binding of the RHDV or MYXV antibodies was visualized by incubation with donkey anti-sheep IgG serum conjugated to horseradish peroxidase (Serotec). Twenty minutes after the addition of substrate solution (tetramethylbenzidine at 2.4 mg ml^−1; Sigma), the reaction was stopped with 0.5 M sulphuric acid. The A_450 of each sample was then measured.

**Seroneutralization titration.** Sera were serially diluted in duplicate in Opti-MEM (Invitrogen) in 50 µl final volume per well. T1-TK::lacZ recombinant virus (50 µl) was added at a concentration of 50 p.f.u. per well. The plate was incubated for 1 h at 37 ºC and then overnight at 4 ºC. Freshly prepared RK13 cells (5 × 10^4) in Opti-MEM supplemented with 15 % FCS were added. The plates were incubated for 48 h at 37 ºC, 5 % CO_2, X-Gal (50 µl at 160 µg ml^−1) in Opti-MEM was then added. The results were read visually after about 12 h of incubation, with neutralization titres corresponding to the last dilution without a blue colour.

**RESULTS**

**Ovine primary fibroblasts are not permissive to MYXV infection**

MYXV is reported to be restricted to rabbits in vivo and to replicate in vitro in some non-leporide cells and some human cancer cells (Lalani et al., 1999; Sypula et al., 2004; Johnston et al., 2005; Wang et al., 2006), but no information concerning interactions between MYXV and ovine cells is currently available. Using an i.d. route of infection, fibroblasts might be among the main cell type initially infected by the virus at the primary site of inoculation before it possibly disseminates to other peripheral tissues. Thus, we investigated the infection of primary ovine dermal fibroblasts.

We infected both RK13 and ovine primary fibroblasts with recombinant T1-TK::lacZ virus expressing the lacZ gene under the control of the late P11 promoter. These cells promoted expression of the lacZ gene when infected at the two m.o.i. tested (0.1 and 1; Fig. 1a), suggesting that they supported virus entry and late gene expression. However, MYXV could not replicate and spread within the ovine fibroblast monolayer, as only sparse labelled cells were observed over time, whichever m.o.i. was used, and no diffusion to neighbouring cells was observed, even at 48 h p.i., in contrast to the results observed for RK13 infection (Fig. 1a).

We confirmed the non-replication of MYXV in ovine primary fibroblasts by a single-step growth analysis. Cells were infected with T1 or SG33 virus strain at an m.o.i. of 5 and virus production was determined by virus titration.
over a 72 h period (Fig. 1b). At all time points, samples were titrated in RK13 cells. The control infection of RK13 cells showed growth curves closely resembling a classical poxvirus replication curve, with a minimum reached at 4 h p.i., followed by a continuous increase until 48 h p.i. (Fig. 1b). No marked difference was observed between the two virus strains. By contrast, no viral production after T1 or SG33 infection of ovine fibroblasts was observed (Fig. 1b).

Taken together, these results indicated that ovine primary fibroblasts are not permissive for MYXV infection, whichever virus strain is used.

**Activated ovine leukocytes can be infected by MYXV**

PBMCs are one of the key cell types implicated in virus dissemination and the generation of an immune response. To evaluate MYXV infection of these cells, infected ovine and rabbit PBMCs were compared. Cells were infected with two recombinant MYXV, T1–Serp2–GFP expressing the Serp2 protein fused to eGFP and SG33–GFP virus expressing GFP under the control of the strong early/late vaccinia virus P7.5 promoter, and analysed by flow cytometry. The fusion between Serp2 and GFP did not alter the virulence of the T1 strain (data not shown).

Infections by T1–Serp2–GFP and SG33–GFP viruses were tested at an m.o.i. of 1 on non-activated PBMCs. Cells were collected at 16 h p.i. and infection levels were determined by gating live GFP-positive cells. We observed that 45.5% of rabbit cells expressed eGFP when infected with T1–Serp2–GFP and 16.1% when infected with SG33–GFP (Fig. 1b). By contrast, only a small fraction of ovine PBMCs was susceptible to MYXV infection. Approximately 0.4 and 2% of GFP-positive ovine cells were detected after infection with T1–Serp2–GFP or SG33–GFP, respectively (Fig. 2a).

Activation may be required to allow poxvirus infection (Chahroudi et al., 2005), PMA/ionomycin-activated rabbit and ovine PBMCs were then infected at the same m.o.i. (Fig. 2a). In activated rabbit PBMCs, infection levels rose to 54 and 37.6% for T1–Serp2–GFP and SG33–GFP, respectively. Only 5.1% and 20.3% of activated ovine PBMCs were GFP-positive when infected with T1–Serp2–GFP and SG33–GFP, respectively. We observed a mean of 8% GFP-positive cells after infection with recombinant T1–Serp2–GFP with a range of 2.6–14.2%, and a mean of 25% GFP-positive cells after infection with SG33–GFP, with a range of 9.1–39.3% (Fig. 2d). These results showed that activation of PBMCs significantly increased the rate of infection in ovine PBMCs.

As we planned to use MYXV as a vaccine vector, we next decided to focus on the attenuated SG33 strain.

To examine whether a higher m.o.i. increased the infection levels, various m.o.i. from 0.5 to 10 were tested with SG33–GFP virus (Fig. 2b). In activated ovine PBMCs, the increase in infection rate was initially proportional to the m.o.i. but quickly reached a plateau. Fig. 2(b) shows a representative experiment: 2.67% cells were GFP-positive at an m.o.i. of 0.1 compared with 18% at an m.o.i. of 10. This maximal infection level was reached at an m.o.i of between 1 and 2, depending on the experiment.

Finally, we analysed GFP expression relative to time of infection in resting and activated ovine PBMCs after infection with SG33–GFP at an m.o.i of 1. The highest percentage of GFP-positive cells was 16.4%, detected around 16 h after infection. This was followed by a slow
decrease in the number of GFP-positive cells down to 11.7 % at 32 h p.i (Fig. 2c). Resting cells contained a maximum of 3.5 % GFP-positive cells at 8 h p.i. (Fig. 2c).

Both B and T cells are the targets of MYXV in ovine PBMCs

To determine the cellular targets of MYXV among ovine PBMCs, peripheral leukocytes were infected with the SG33–GFP recombinant virus and stained with monoclonal antibodies (mAbs) specific to different leukocyte subsets. Thus, T lymphocytes were recognized with anti-ovine CD2, anti-ovine CD5 and anti-WC1 (γδ subset) mAbs. Subsets of CD2+ T cells were stained with anti-CD4 and anti-CD8 mAbs. Anti-CD5 mAbs stained both T and B cells and MHC II mAbs stained activated T cells and CD5− CD21+ B cells.

After 16 h of infection at an m.o.i. of 2, the infection rate was 3 % in resting cells (data not shown) and around 31 % in activated cells (mean of the percentage of GFP-positive cells in repeated experiments; Fig. 3a). PMA/ionomycin activation enhanced the infection rate in every target cell. In activated culture, more than one-third of GFP-positive cells was composed of T cells, as 31 % were CD2+ and 8 % were γδ T cells (Fig. 3a and b). Both CD4+ and CD8+ T cells were infected and their proportions were 24 and 3 % of the GFP-positive cells, respectively (Fig. 3a). The remaining GFP-positive cells were B cells, as we detected 45 % MHC II+ and 46 % CD5− among the GFP-positive cells (Fig. 3a).

We next investigated the susceptibility of each PBMC subset for MYXV infection. The frequency of GFP-positive cells differed widely among cell types. For example, 18 % of CD2+ cells, 15 % of CD4+ cells, 27 % of CD8+, 33 % of γδ cells, 23 % of MHC II+ cells and 66 % of CD5+ cells were GFP-positive (Fig. 3b).

Infection of PBMCs from at least three different animals showed a high variability in the distribution of infected cells, particularly in the CD2+ population. Thus, a mean of 35.6 % of infected cells, ranging from 7.1 to 67.2 %, were CD2+ (Fig. 3c). By contrast, the variability of infected CD5+ cells was much less visible, ranging from 45 to 56.5 % of the GFP-positive cells (Fig. 3c).

These results showed that all of the tested subsets were susceptible to SG33 infection, although the distribution of infected cells was quite variable among the animals.

MYXV infection of ovine PBMCs is abortive

As we showed above that activated ovine PBMCs could be infected by MYXV, we studied virus multiplication in resting and activated ovine PBMCs over a 48 h period. Rabbit and ovine cells were infected with SG33–GFP at an m.o.i. of 1, which is a compromise between infection rate and cell viability. Virus production was determined by virus titration on RK13 cells at 0, 4, 12, 24 and 48 h p.i. (Fig. 4). We observed virus production in rabbit PBMCs with an increase in virus titre measured over a 48 h period (Fig. 4). By contrast, no increase in virus titre could be detected in either resting or activated ovine PBMCs (Fig. 4). These data suggest that activation did not influence ovine PBMC permissivity to MYXV strain SG33.

*Fig. 3. MYXV cell subset targets in ovine PBMCs. (a) Activated cells were infected with SG33–GFP at an m.o.i. of 2 and analysed at 16 h p.i. for GFP expression on gated live cells. The numbers in quadrants indicate the percentage of cells. The results shown are representative of an individual experiment. (b) Distribution of infected cells. Open bars, cell type among infected cells; shaded bars, infected cells among cell type. The results shown are representative of an individual experiment. (c) Variability among infected cells was measured by the mean of at least ten independent samplings in three independent infection experiments. Results are shown as means ± SEM.*
Recombinant MYXV induces inflammatory and immune responses after i.d. injection in sheep

In order to evaluate the safety and immunogenicity of MYXV-based vaccines in small ruminants, two groups of sheep were inoculated with recombinant SG33–VP60 virus by the i.d. route. This recombinant virus allows the production of the major capsid protein (VP60) of RHDV, and its efficiency in simultaneously protecting rabbits against both myxomatosis and rabbit haemorrhagic disease virus has previously been reported (Bertagnoli et al., 1996). The VP60 gene is inserted in the same locus as GFP in the recombinant SG33–GFP.

Two sheep were inoculated i.d. with various doses (10^5–10^7 p.f.u.) of virus at multiple sites. Skin biopsies were taken from the injection sites from 0 to 72 h p.i. After fixation in 10% neutral formalin, samples were prepared for histological examination. As early as 6 h p.i., we observed a perivascular dermatitis, at all virus doses tested, with an inflammatory infiltrate predominantly comprised of neutrophils (Fig. 5a). From 48 h p.i., the inflammatory infiltrate appeared to be mostly composed of mononuclear cells, indicating the development of a local progressing inflammatory response. The intensity of the inflammatory response seemed to be proportional to the virus dose inoculated. Thus, for 10^5 p.f.u., the lesional intensity was most often light to moderate, whereas for 10^7 p.f.u. it frequently showed a marked intensity (Fig. 5b).

In order to study the outcome of the infection in situ, immunostaining for MYXV was also carried out. Expression of viral proteins was particularly detected from 6 h p.i. in cells closely resembling macrophages, based on the infected-cell morphology (Fig. 5c). This expression appeared to be stable during the course of the infection (Fig. 5c).

To assess the immunogenicity of recombinant MYXV in small ruminants, a group of sheep (n=10) was injected twice i.d. with 2x10^6 p.f.u. SG33–VP60 virus. I.d. injection did not result in any swelling or lesion at the injection sites or at distal sites, and all sheep remained healthy. In addition, nested PCR failed to detect MYXV in blood samples taken 1, 2, 3, 4 and 7 days after each injection.
(data not shown). Serum samples were taken at days 0, 21, 35 and 42 from each animal. Seroconversion to MYXV was observed following the booster injection (Fig. 6a) and an immune response to VP60 could be detected for eight of the ten vaccinated sheep by ELISA (Fig. 6b). In order to assess the biological relevance of the antibodies detected, a seroneutralization assay against MYXV was performed on the four strongest serum samples determined previously by ELISA (Fig. 6c). Neutralizing antibodies were detected in each serum, with titres ranging from 1:20 to 1:60, proportional to the ELISA titres and comparable to the titre measured for an equivalent control rabbit serum (Fig. 6c).

**DISCUSSION**

We demonstrated in this study that MYXV, a poxvirus commonly reported to be specific to rabbits, can be used as a non-replicative and safe viral vector to generate specific immune responses in ovine species.

After using two strains of MYXV, we focused on the attenuated SG33 vaccine strain. This strain, obtained after numerous passages in rabbit and embryonic chicken fibroblasts at 33 °C (Saurat et al., 1978), is the only MYXV strain used to vaccinate rabbits against myxomatosis in France (Chantal & Bertagnoli, 2004).

As we aimed to develop MYXV as vector for vaccination of sheep by the i.d. route, we investigated MYXV infection of primary ovine fibroblasts generated from the dermis. We demonstrated that primary ovine fibroblasts could be infected by both the wild-type T1 strain and the vaccine SG33 strain of MYXV, and that the infection was abortive. It has been shown previously that MYXV can productively infect non-host primary fibroblasts in vitro depending on cellular interferon (IFN) production (Wang et al., 2004; Johnston et al., 2005). In our work, primary fibroblasts were generated from adult ovine skin; even though they were cultured over only three passages, they should produce type I IFN. Low-passage fibroblasts from neonatal explants are permissive to MYXV infection, whereas high-passage human primary fibroblasts fail to support MYXV replication (Johnston et al., 2005). Moreover, when type I IFN responses are eliminated, the MYXV species barrier can be broken in primary murine fibroblasts and these cells can become fully permissive to infection (Wang et al., 2004). These findings show that cell permissivity for MYXV is dependent on the control signal-transduction pathways, particularly those conducting signals to the IFN responses.

Knowing that the ability to grow in lymphocytes is crucial to the pathogenicity and dissemination of MYXV in rabbits, the safety of MYXV as a vaccine vector for ovine species was investigated by characterizing the infection of ovine PBMCs. We first showed that both T1 and SG33 MYXV strains infected activated ovine PBMCs at a higher rate than resting PBMCs. As the difference between resting and activated cells could be observed as early as 6 h p.i., the restricted infection of resting cells might be due to an early event deficiency and could be linked to the expression of cellular factors induced following activation. This finding could be correlated with a recent observation indicating that activation of human T cells allows the production of a de novo receptor, enabling both susceptibility and permissivity of these cells for VACV (Chahroudi et al., 2005).

However, in our experiments, we could not determine whether low infectivity in resting ovine cells came from a deficiency in a binding and/or a post-binding event.

As PBMCs are composed of different cell subsets, mainly B and T cells, we next investigated the tropism of MYXV for ovine PBMC subsets. We only studied the interaction between a recombinant SG33 virus and PBMCs because, as

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**Fig. 6.** Immune responses against MYXV and VP60. (a, b) Anti-MYXV (a) and anti-VP60 (b) antibody responses using ELISA of ten sheep injected i.d. twice (on days 0 and 21, arrows) with recombinant SG33–VP60. The serological responses were evaluated at 0, 21, 35 and 42 days after the first immunization. Titres were determined by comparison with a hyperimmune serum as a reference. The higher point range was arbitrarily defined as 1000 units. The grey lines correspond to mean titre values. (c) Comparison of anti-MYXV antibody titres obtained by ELISA (shaded bars) and seroneutralization (filled bars) tests. In rabbit: --, negative standard rabbit serum; R1, hyperimmune standard rabbit serum; R2, rabbit serum with an anti-MYXV titre of 1000 measured by ELISA. In sheep: --, preimmune serum (day 0) of sheep number 4; 1–4, the four strongest ovine serum samples determined previously by ELISA.
a vaccine strain, SG33 is the most suitable candidate for the generation of a MYXV-based vaccine. Moreover, this virus has several virulence factors deleted, including the scrapin MV-Lap responsible for the downregulation of cellular markers such as CD4, MHC I and CD95 (Guérin et al., 2002; Mansouri et al., 2003; Collin et al., 2005), which might improve the immune response. We showed that B and T cells could be infected by MYXV. Moreover, under our experimental conditions, B cells were the predominant infected cell type. This finding is interesting, as B cells are antigen-presenting cells and they could present antigens during infection, allowing the development of a specific immune response. Non-specific activation increased infection rates in both B and T cells (CD2+, CD4+, CD8+ and γδ), indicating that activation allows the expression of non-subset-specific de novo factor(s) present in both B and T cells.

The infection level in activated ovine leukocytes was variable among the animals and within the population of infected subsets. This variability could be explained by the initial cell activation and maturation levels. Moreover, even if stimulation of ovine PBMCs greatly enhanced infection levels, we demonstrated that it did not allow cellular permisivity, as no increase in virus titre was detected at 48 h p.i. This is different from what is known about infection of rabbit leukocytes with MYXV. Indeed, in stimulated rabbit lymphocytes, MYXV replicates approximately tenfold more than in resting cells (Opgenorth et al., 1992). However, a previous study of RL-5 cell infection with MYXV containing a disruption of the M11L gene showed that virus yield was significantly reduced in comparison with the wild-type MYXV (Macen et al., 1996). This is consistent with infection of rabbit PBMCs with SG33–GFP, deleted for both the M11L and MGF genes. Indeed, our results revealed that this recombinant virus replicated with no significant difference in resting and activated cells, and the increase in virus production at 48 h p.i. in each case was approximately tenfold.

For further evaluation of our results, a MYXV-based vector in small ruminants was evaluated by in vivo experiments. I.d. injections were performed and did not induce side effects, either local or general. Histological analysis revealed a progressing inflammatory response at the inoculation site. Moreover, expression of viral antigens was observed mainly in cells with macrophage cell morphology. We failed to specifically characterize these infected cells using specific mAb labelling because of their low labelling efficiency. Nevertheless, our first observations of infected dermal cells indicated that antigen presentation to the immune system could be efficient. The observation that the number of infected cells in situ was stable and that only sparse infected cells were detected during the course of the infection suggested that there was no local replication and correlated with the lack of viraemia. I.d. inoculation of SG33–VP60 recombinant virus induced the development of a humoral response against the product of the transgene (i.e. RHDV VP60). Nevertheless, an important variability among animals was observed. Specific anti-VP60 antibodies could not be detected for two out of ten animals, a further two produced a good response and six developed a moderate response against VP60. The same variability and kinetics were observed for the anti-MYXV antibody response. As we could not test the ability of the anti-VP60 antibodies produced to neutralize RHDV, we performed a neutralization assay against MYXV, even though MYXV is not a pathogen for small ruminants. Comparison with a control rabbit serum revealed that the antibody response in sheep was as efficient as rabbit serum in neutralizing MYXV in vitro. Studies on the influence of the vaccination protocol on ovine humoral and cellular responses are currently in progress for further characterization of the vaccine response in sheep. It should be noted that the doses used in our study were far lower than those reported for ovine vaccination with a recombinant canarypox virus (two injections of >10⁷ p.f.u. per animal; Boone et al., 2007).

In this study, we showed that ovine primary fibroblasts can be infected by MYXV, even though these cells are not permissive for MYXV infection. The abortive infection of small-ruminant PBMCs makes it impossible for MYXV to disseminate via leukocytes in these species. Our study also shows for the first time that MYXV could be a safe viral vector to induce humoral immune responses with biological relevance in small ruminants. Nevertheless, further investigations are needed to improve our knowledge of the immune response induced following MYXV-based vector vaccination. To this aim, current studies are in progress in our laboratory to investigate antigenic presentation, in particular the capacity of MYXV to infect ovine dendritic cells.

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