Ovine herpesvirus 2 replicates initially in the lung of experimentally infected sheep

Hong Li, Cristina W. Cunha, Christopher J. Davies, Katherine L. Gaillbreath, Donald P. Knowles, J. Lindsay Oaks and Naomi S. Taus

Correspondence
Hong Li
hli@vetmed.wsu.edu

1Animal Diseases Research Unit, United States Department of Agriculture-Agriculture Research Service, Washington State University, Pullman, WA 99164, USA
2Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164, USA

Ovine herpesvirus 2 (OvHV-2), a rhadinovirus in the subfamily Gammaherpesvirinae, is the causative agent of sheep-associated malignant catarrhal fever (SA-MCF), a frequently fatal lymphoproliferative disease primarily of ruminants worldwide. Inability to propagate the virus in vitro has made it difficult to study OvHV-2 replication. Aerosol inoculation of sheep with OvHV-2 from nasal secretions collected from naturally infected sheep during shedding episodes results in infection of naive sheep, providing an excellent system to study OvHV-2 initial replication in the natural host. In this study, we showed that OvHV-2 delivered through the nasal route by nebulization resulted in infection in all lambs, but no infection was established in any lambs after intravenous or intraperitoneal injection. In nebulized lambs, while it was not detected initially in any other tissues, OvHV-2 DNA became detectable in the lung at 3 days post-infection (p.i.), increased to about 900 copies per 50 ng DNA at 5 days p.i., reached peak levels (~7500 copies) at 7 days p.i., and then declined to an average of 800 copies at 9 days p.i. Transcripts of OvHV-2 open reading frame 25 (coding for the capsid protein), an indicator of virus replication, were only detected in lung tissues, and were positively correlated with OvHV-2 DNA levels in the lungs. In addition, selected immune response genes were also highly expressed in the lung at 5 and 7 days p.i. The data indicate that lung is the primary replication site for OvHV-2 during initial infection in sheep and suggest that viral replication is promptly controlled by a host defence mechanism.

INTRODUCTION

Ovine herpesvirus 2 (OvHV-2) is a member of the subfamily Gammaherpesvirinae, which includes a number of important viruses that are associated with lymphoproliferative diseases and lymphoid tumours in humans and animals, such as Epstein–Barr virus (EBV), Kaposi’s sarcoma associated herpesvirus (KSHV) and murine herpesvirus 68 (MHV68) (Roizman, 2000). Domestic and wild sheep serve as reservoirs for the virus. Infection with OvHV-2 in the reservoir species is usually subclinical. However, infection often results in a fatal disease called sheep-associated malignant catarrhal fever (SA-MCF) when the virus is transmitted to other, less well-adapted hosts, primarily ruminant species, such as cattle, bison and deer (Plowright, 1990; Crawford et al., 1999). SA-MCF has been recognized as the cause of significant economic losses in several species, including farmed deer in New Zealand (Mackintosh, 1993), Bali cattle in Indonesia (Daniels et al., 1988), farmed bison in North America (Li et al., 2006; O’Toole et al., 2002; Schultheiss et al., 2000) and a wide variety of ruminants in zoological collections (Heuschele & Fletcher, 1984).

Increasing evidence has suggested that the pathobiology of OvHV-2 may be different from that of alcelaphine herpesvirus 1 (AlHV-1), a closely related MCF virus that is carried by wildebeest, although both viruses cause similar disease syndromes and lesions in clinically susceptible hosts (Plowright, 1990). Earlier studies showed that viral transmission and shedding patterns are different between OvHV-2 and AlHV-1 in their natural hosts: sheep shed the virus sporadically with a short-lived episode and new born lambs are not the source of infection (Li et al., 2004), while most newborn wildebeest calves are infected and shed virus...
continuously until 3–4 months of age and are the primary source of transmission (Mushi & Wafula, 1983). Recent studies revealed that the transcripts of the open reading frame (ORF) 25, a gene encoding a capsid protein, were present in virtually all tissues of cattle, bison (Cunha et al., 2007) and rabbits (Gailbreath et al., 2008) with OvHV-2-induced MCF, but not present in the tissues (spleen and lymph nodes) of rabbits with AIHV-1-induced MCF (Dewals et al., 2008), suggesting that virus lytic replication and latency are different in clinically susceptible hosts. While AIHV-1 readily grows in cell culture including several cell lines, OvHV-2 has not been successfully propagated in vitro, suggesting that their cell tropisms and/or in vitro culture requirements are also different.

The lack of a cell culture system to propagate OvHV-2 in vitro has constrained the ability to perform controlled transmission studies and studies of pathogenesis. However, much progress has been made in the past decade due to advances in molecular technologies. OvHV-2 epidemiology and transmission in its natural host, domestic sheep, were studied using more advanced molecular tools (Li et al., 1994, 2001; Baxter et al., 1993; Hussy et al., 2001). Although still controversial, most data show that the majority of lambs are not infected until after 2 months of age under natural flock conditions (Li et al., 1998). Placental transmission rarely occurs in sheep, and colostrum and milk from infected ewes have a very limited role in viral transmission, even though they contain virus-infected cells (Li et al., 1998, 1999). Newborn lambs are equally susceptible to infection as adults via aerosol transmission (Taus et al., 2005), and thus the lack of infection in the majority of lambs under 2 months of age is probably due to the relatively low levels of virus shed by adult sheep in the environment and inefficient infection rates (Li et al., 2002, 2000). Experimental infection of sheep has always been problematic, not only because of a lack of infectious virus, but also due to the lack of OvHV-2-free sheep. Recognition of the short window of time when lambs are free of infection during early life under natural flock conditions (Li et al., 1998) resolved the problem and provided an opportunity to develop a programme for production of OvHV-2-free sheep (Li et al., 1999). A study of OvHV-2 shedding kinetics in sheep using quantitative real-time PCR revealed that adolescent lambs between 6 and 9 months of age shed virus more frequently and intensively than adults (Li et al., 2004). These studies have made it possible to establish a source of infectious virus by collection of nasal secretions from sheep during shedding episodes. The virus collected by this method has been successfully used for experimental infection in carrier species (Taus et al., 2005), as well as in clinically susceptible species (Taus et al., 2006; O’Toole et al., 2007). OvHV-2 predominantly replicates in cells in nasal turbinate when naturally infected sheep experience intensive shedding episodes (Cunha et al., 2007), although the specific type(s) of cells that support lytic replication has not been determined. This replication appeared to be localized, which is consistent with the suggestion that this replication is limited possibly to a single cycle of replication (Li et al., 2004). This also suggests that virus shed from turbinate cells may be incapable of reinfecting turbinate cells. If true, OvHV-2 tropism may vary during the life cycle, with virus shed from turbinate cells infecting other type(s) of cells in the respiratory tract to establish infection in recipient sheep via nasal transmission. To investigate this possibility, we determined the tissue site(s) where OvHV-2 initial replication takes place when virus from sheep nasal secretions is transmitted by nebulization.

**METHODS**

**Animals and experimental groups.** Twenty-two lambs approximately 3 months old were obtained from an MCF virus-free flock that is maintained at Washington State University, Pullman, WA, USA in accordance with an approved animal care and use protocol. All lambs were seronegative for MCF viral antibody by competitive inhibition ELISA (cELISA) and no OvHV-2 DNA was detected in their peripheral blood leukocytes (PBL) using nested PCR. As shown in Table 1, the lambs were divided into four groups: group 1, consisting of 13 lambs (nos 1-1IN+ to 13-IN+), was nebulized with 2 ml pooled nasal secretions containing 108 OvHV-2 DNA copies; group 2 had five animals (nos 14-IN to 18-IN) nebulized with 2 ml nasal secretions collected from OvHV-2 uninfected sheep as a control group; groups 3 and 4 had two lambs each, which were inoculated intravenously (group 3, nos 19-IV and 20-IV) or intraperitoneally (group 4, nos 21-IP and 22-IP) with the same dose of virus used in group 1. The nebulization procedure was described previously (Li et al., 2004).

**Sheep nasal secretion inoculum.** Sheep nasal secretion inoculum for this study was collected using the same protocol as described previously (Taus et al., 2005). Briefly, nasal secretions were collected daily for 3 months from 15 OvHV-2-positive sheep that were 6 months of age at the beginning of the collection period. Nasal secretions were collected from the sheep using multiple swabs within 5 to 6 h of the initial sampling. High shedder sheep are defined as those sheep whose initial samples contained ≥100 000 copies of OvHV-2 DNA per μg total DNA. Diluted secretions were clarified, aliquoted and stored in liquid nitrogen, using 5% chicken ovalbumin. After confirming the presence of high viral DNA copy numbers, all preparations from individual shedding sheep were combined to make a pooled inoculum, and the inoculum was stored in liquid nitrogen. OvHV-2 DNA copies from the pooled inoculum were quantified by real-time PCR. The OvHV-2 infectivity of the pooled inoculum used in this study was quantified in sheep using a protocol described previously (Taus et al., 2005). The minimum infectious dose of this pooled inoculum for sheep was about 103 OvHV-2 DNA copies, which was comparable to the inoculum used in previous studies (Taus et al., 2005).

**Sample collection and preparation.** Lambs in group 1 nebulized with sheep nasal secretions containing infectious OvHV-2 were euthanized in pairs at 1, 3, 5, 7 and 9 days p.i., and the remaining three lambs in the group were euthanized at 56 days p.i. The lambs nebulized with nasal secretions from OvHV-2-negative sheep (group 2) were euthanized at 0 (nos 14-IN- and 15-IN-) and 56 (nos 16-IN-, 17-IN- and 18-IN-) days p.i., respectively. Both lambs in each group inoculated intravenously (19-IV+ and 20-IV+) or intraperitoneally (21-IP+ and 22-IP+) with virus were euthanized at 56 days p.i. Blood and nasal secretion samples were collected daily from each lamb for the first 9 days; only blood samples were collected weekly until the termination of the experiment. Multiple tissues (n=26) from
Table 1. Summary of the animal experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Designated lamb ID*</th>
<th>OvHV-2 inoculum</th>
<th>Route of inoculation</th>
<th>Days p.i. at termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-IN +</td>
<td>+ †</td>
<td>Intranasal</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>14-IN -</td>
<td>− §</td>
<td>Intranasal</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>19-IV +</td>
<td>+</td>
<td>Intravenous</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>21-IP +</td>
<td>+</td>
<td>Intraperitoneal</td>
<td>56</td>
</tr>
</tbody>
</table>

*IN +, Intranasal inoculation with sheep nasal secretions containing infectious OvHV-2 by nebulization; IN −, intranasal inoculation with the nasal secretions from OvHV-2-negative sheep; IV +, intravenous inoculation with sheep nasal secretions containing infectious OvHV-2; IP +, intraperitoneal inoculation with sheep nasal secretions containing infectious OvHV-2.

‡2 ml sheep nasal secretions containing 10⁷ copies of OvHV-2 DNA.

§2 ml nasal secretions collected from OvHV-2-negative sheep.

Various organs, especially those from the respiratory tract, were collected during necropsy. The tissues of the most interest included trigeminal ganglia, tonsil, pharynx, nasal mucosa, salivary gland, turbinates from six locations (both left and right caudal, middle and rostral), trachea from upper, middle and lower sections, and lung from caudal, middle and cranial lobes. Additional tissues collected were brain (periventricular and cortex), lymph nodes (retropharyngeal and mesenteric), spleen, liver, kidney, urinary bladder, large intestine and small intestine. All collected tissues were snap frozen and stored either at −80 °C or in liquid nitrogen for later use. The tissues used for histopathology were fixed in 10% neutral-buffered formalin and then embedded into paraffin blocks.

Plasma from EDTA-blood samples was collected for the MCF viral antibody assay and stored at −20 °C for later use. Total DNA for PCR assays was extracted from PBL samples, nasal secretion samples, and tissues using the FastDNA kit as described by the manufacturer (QBiogene). Total RNA for ORF25 RT-PCR was extracted from tissues using TRIzol Reagent as described by the manufacturer (QBiogene). Total RNA for ORF75 and ORF25 RT-PCR was extracted from PBL samples, nasal secretion samples, and tissues using the FastDNA kit as described by the manufacturer (QBiogene). The detailed procedure for the RT-PCR has been described in a previous report (Cunha et al., 2001).

A nested PCR and a real-time PCR were used to detect and quantify, respectively, OvHV-2 DNA. Both assays have been described previously (Li et al., 1995; Traul et al., 2007) and used different primer sets targeting the same region of OvHV-2 ORF75 (Baxter et al., 1993; Hussy et al., 2001).

An RT-PCR assay using primers targeting OvHV-2 ORF25, which codes for a major capsid protein, one of the OvHV-2 structural proteins expressed in the late stage of replication, was used to identify virus replication. RNA was reverse-transcribed and amplified in a single step using the OneStep RT-PCR kit as described by the manufacturer (Qiagen). The detailed procedure for the RT-PCR has been described in a previous report (Cunha et al., 2007). Briefly, RNA samples (100 ng) were added on ice to each 50 μl reaction [1 × Qiagen OneStep RT-PCR Buffer, 400 μM each dNTP, 1 × Q-Solution, 0.4 μM specific ORF25 forward primer (5′-ACTGCGG-ACTGCGGCTACTT-3′), reverse primer (5′-GTCCAGGAGGCGTGGTGTG-3′) and 2.0 μl Qiagen OneStep RT-PCR Enzyme Mix]. Negative RT control reactions, to confirm the absence of DNA contamination, were performed by replacing the One Step RT-PCR mix by a Hot Start Taq mix (Qiagen) in the reaction. The same RT-PCR cycling conditions were used except that the transcription step (50 °C for 30 min) was omitted. Amplification of cellular GAPDH was used to ensure that RNA was present and of adequate quality to be amplified.

cELISA. cELISA, which utilizes a monoclonal antibody against an epitope conserved among all MCF viruses examined to date (Li et al., 1994), was used for the detection of MCF viral antibody. The protocol for the cELISA was described previously (Li et al., 2001).

The cELISA, nested PCR, real-time PCR, RT-PCR and real-time RT-PCR. cELISA, which utilizes a monoclonal antibody against an epitope conserved among all MCF viruses examined to date (Li et al., 1994), was used for the detection of MCF viral antibody. The protocol for the cELISA was described previously (Li et al., 2001).
A real-time RT-PCR for analysis of the relative expression levels of selected sheep immune response genes was developed. Total RNA (1 µg) from each tissue sample was reverse-transcribed using oligo(dT) primers and the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s specifications. The selected immune response genes, forward and reverse primers, product lengths and GenBank accession numbers of the sequences used to design the primers are listed in Supplementary Table S1 (available in JGV Online). All primer pairs were designed to target areas with minimal secondary structure, to work at an annealing temperature of 60 °C and, where feasible, to span two exons. Real-time PCR was performed by using SYBR GreenER Master Mix (Invitrogen) in 25 µl reactions. Primers were used at a final concentration of 200 nM. Reactions were performed in an iCycler (Bio-Rad) with initial cycle (50 °C for 2 min, and 95 °C for 8 min and 30 s), followed by 40 amplification cycles (95 °C for 15 s and 60 °C for 1 min). A melt curve was performed for each reaction to confirm specificity of the amplicons; cycler parameters were 95 °C for 1 min, 55 °C for 1 min, followed by 80 cycles of increasing temperature in 0.5 °C increments (10 s each) beginning at 55 °C. All samples were run in duplicate. The data were analysed using the 2^(-ΔΔCt) method (Livak & Schmittgen, 2001). Each value was normalized to the values for the housekeeping genes, GAPDH and β-actin, obtained from the same sample and the relative expression levels (fold increase) were determined using the values from RNA samples collected at 0 day p.i. as the calibrator.

RESULTS

MCF viral antibody in plasma

As shown in Fig. 1, MCF viral antibody was detected by cELISA at 9 days p.i. and thereafter from the sheep that were nebulized with the nasal secretions containing infectious OvHV-2. MCF viral antibody was not detected in any of the control sheep that were nebulized with the nasal secretions collected from OvHV-2 uninfected sheep. Both sheep inoculated intravenously with the virus remained MCF viral antibody-negative until the termination of the experiment. However, one of two animals inoculated intraperitoneally with the virus became transiently seropositive from 12 to 17 days p.i., while the other remained seronegative (Fig. 1b).

OvHV-2 DNA in nasal secretions, PBL and tissues

OvHV-2 DNA was not detected by either real-time PCR or nested PCR in any samples collected from the lambs that were nebulized with nasal secretions from OvHV-2 uninfected sheep or from the animals inoculated either intravenously or intraperitoneally with virus. In the group of lambs nebulized with the nasal secretions containing infectious OvHV-2, viral DNA was detected by nested PCR in only a small percentage of tissues from 1 to 7 days p.i., ranging from 1.78 % (1 of 56) at 1 day p.i. to 17.87 % (10 of 56) at 7 days p.i., virtually all of which were lung tissue (Table 2). However, the majority of samples (69.64%, 39 of 56) had detectable OvHV-2 DNA at 9 days p.i. and all (100%, n=81) were positive by nested PCR at the termination of the experiment (56 days p.i.) (Table 2). Using real-time PCR, OvHV-2 DNA was only detected in the lung tissue from lambs at 3, 5, 7 and 9 days p.i., but not at 1 day p.i. At 3 days p.i., only four of six lung samples had detectable DNA copy number by real-time PCR, with a mean of 13 copies per 50 ng tissue DNA, ranging from 10 to 53 copies. At 5 days p.i., all six lung samples had detectable OvHV-2 DNA with a mean of 880 copies (ranging from 340 to 2540 copies), at 7 days p.i. and then dropped to a mean of 780 copies (ranging from 960 to 14 200 copies), at 9 days p.i. (Fig. 2). At the termination of the experiment (56 days p.i.), 46 of 78 tissues, including lung, had detectable OvHV-2 DNA by real-time PCR, ranging from...
## Table 2. OvHV-2 DNA in tissues of experimentally infected lambs by nested PCR

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0 day p.i.</th>
<th>1 day p.i.</th>
<th>3 days p.i.</th>
<th>5 days p.i.</th>
<th>7 days p.i.</th>
<th>9 days p.i.</th>
<th>56 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14-IN†</td>
<td>15-IN†</td>
<td>1-IN+</td>
<td>2-IN+</td>
<td>3-IN+</td>
<td>4-IN+</td>
<td>5-IN+</td>
</tr>
<tr>
<td>Brain perivent.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Brain cortex</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trigeminal</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tonsil</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pharynx</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate L. caudal</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate L. middle</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate L. rostral</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate R. caudal</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate R. middle</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Turbinate R. rostral</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trachea upper</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Trachea middle</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Trachea lower</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lung upper</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung middle</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung lower</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Retro. LN</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mes. LN</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spleen</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lg. intest.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sm. intest.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bladder</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nasal swab</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Positive (%)</td>
<td>0.60</td>
<td>1.78</td>
<td>7.14</td>
<td>14.20</td>
<td>17.86</td>
<td>69.64</td>
<td>100</td>
</tr>
</tbody>
</table>

*Lambs euthanized at 0 day p.i. were nebulized with the sheep nasal secretions collected from OvHV-2-negative sheep.

†Lamb ID number.
NT, Not tested.
14 to 12,800 copies per 50 ng tissue DNA (mean = 1,608 copies) (data not shown).

**OvHV-2 ORF25 transcripts in lung and other tissues**

As shown in Figs 2 and 3, at 1 day p.i. OvHV-2 ORF25 transcripts were detected by RT-PCR in only one of six lung samples (17%) representing three different lobes of lung from two lambs. Two lung samples (33%) were positive for the ORF25 transcripts from the lambs at 3 and 5 days p.i., respectively. The ORF25 transcripts were detected in all six lung samples (100%) at 7 days p.i., while only one sample (17%) from one lamb at 9 days p.i. was positive for the transcripts (Figs 2 and 3). ORF25 transcripts were not detected in any other tissues in the respiratory tract (pharynx, nasal mucosa, turbinates, trachea, tonsil or retropharyngeal lymph node) or in the spleen or mesenteric lymph nodes from the infected lambs at any time point (data not shown). GAPDH transcripts were detected in all RNA samples prepared from the tissues (data not shown). ORF25 transcripts were not detected in control reactions without reverse transcriptase or without template.

**Transcripts of selected sheep immune response genes in lung**

Twenty-two sheep immune response genes, comprising 17 cytokines [granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-23, alpha interferon (IFN-α), IFN-γ], transforming growth factor (TGF)-β and tumour necrosis factor (TNF)-α], three receptors (CD25, CD28 and CD152), one lytic protein (granulysin) and one transcription factor (GATA-3), were analysed for their expression levels in lung tissue from lambs nebulized with OvHV-2 at different days p.i. Eighteen of the immune response genes exhibited greater than an eightfold (three cycle) increase in expression, which is a conservative cut-off for a meaningful change in expression, compared with the calibration value for the lung samples from uninfected lambs euthanized at 0 day p.i. (Fig. 4). Among these 18 genes, six (IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-23, IFN-γ, GM-CSF, CD152, granulysin and GATA-3) had their highest expression levels at 7 days p.i. Only IL-2 had its maximal expression at 1 day p.i., with a
16.4-fold increase. Three additional genes (IL-12, TGF-b and CD25) had greater than a fourfold increase in expression. IFN-a was the only gene that did not exhibit an increase in expression. By 9 days p.i., the expression of all of the immune response genes had returned to approximately baseline levels (ranging from 27- to 1.9-fold increases).

**Histopathology**

Three sections of lung from each lamb from groups 1 and 2 were examined, including cranial, middle and caudal lobes. All lambs had a few mild to moderate peribronchial and peribronchiolar aggregates of mature lymphocytes and macrophages. One infected lamb in group 1 had rare focal areas of mild interstitial and alveolar inflammation with minimal necrosis and oedema. One control lamb in group 2 had a similar focus of interstitial and alveolar inflammation. Subtle differences between the two groups would be difficult to detect due to the small sample size, but based on the finding described here, there were no significant differences between infected animals and negative-control animals.

**DISCUSSION**

It has been suggested that the respiratory tract is a primary target for OvHV-2 during natural infection (Li et al., 2004). In this study, OvHV-2 replication was detected only in the lung tissue during initial infection by intranasal aerosolization, which mimics the natural nasal route of transmission. Whether this method of nebulization may be favourable to lung infection compared with a natural nasal route of transmission was not determined. However, no replication in any turbinate tissue was detected, suggesting that the virus does not replicate in turbinate tissues during the initial phase of infection. In contrast, OvHV-2 predominately replicates in the nasal turbinate of sheep during intensive shedding episodes, and nasal secretions contain infectious OvHV-2 virions (Cunha et al., 2007; Li et al., 2004). It is not surprising that the OvHV-2 collected from nasal secretions from shedding sheep may be incapable of reinfecting cells in the turbinate; this is consistent with the observation that OvHV-2 shedding from sheep is short-lived, usually lasting less than 24 h, implying a single cycle of replication (Li et al., 2004). The data herein further support that OvHV-2 in nasal secretions from productively infected turbinate cells may not be capable of reinfecting other turbinate cells, possibly due to a mechanism of cell tropism switching similar to that reported for Epstein–Barr virus (Borza & Hutt-Fletcher, 2002). The fact that OvHV-2 DNA, but not ORF25 transcripts, can be detected in the majority of tissues, and PBL, by 9 days p.i. indicates that the virus establishes latent infection in lymphocytes in the lung and then the latently infected lymphocytes circulate in the peripheral system.

It was unexpected that infection was not established when sheep were inoculated with the cell-free virus from nasal secretions intravenously or intraperitoneally, although a transient antibody response was observed in one lamb inoculated intraperitoneally. The transient response could result from a low level of viral antigen stimulation without viral replication or from a co-incident non-specific antibody response detected by cELISA, which was documented in a previous study (Li et al., 2001). Failure to
induce infection by intraperitoneal inoculation of the cell-free virus is consistent with findings on experimental infection of rabbits with OvHV-2 from sheep nasal secretions (K. L. Gailbreath and others, unpublished results). The reason for the failure is not clear. OvHV-2 could be immediately inactivated in the peripheral blood and body fluids after injection. However, it is unlikely since cell-free AlHV-1 from wildebeest nasal secretions can establish infection in rabbit or cattle after intravenous injection (Mushi & Wafula, 1983). A possible explanation for the failure of inducing infection by intravenous and intraperitoneal inoculation of the cell-free virus in sheep could be that the susceptibility of lymphocytes to OvHV-2 requires replication in the lung to switch its cell tropism. The data herein and from previous studies suggest that OvHV-2 productive replication sites in sheep are different for the entry and shedding events, and the virus probably changes its cell tropism at three different stages during the complete life cycle: turbinate (shedding), lung (entry) and lymphocytes (latency). The likely scenario is that certain type(s) of cells in the turbinate are susceptible and permissive to the virus from some of the latently infected lymphocytes that are switching to lytic infection in the turbinate. The lymphocyte-originated virus may be capable of infecting both lymphocytes (for latency) and specific cells in the turbinate that permit it to replicate, producing a sudden surge of infectious virus into the nasal secretions for shedding, while maintaining latency in lymphocytes. However, the turbinate-originated virus in nasal secretions may not be capable of infecting lymphocytes or reinfecting the cells in the turbinate. This is probably the reason why the shedding is so transient, lasting less than 24 h (Li et al., 2004). On the other hand, the turbinate-originated virus is capable of infecting certain cells in the lung, and lung-originated virus is capable of infecting lymphocytes. Therefore, we propose that cell-free virus infection and replication in the lung is required for OvHV-2 to establish infection in sheep. If this is the case, the phenomenon of cell tropism switching may explain why OvHV-2 has never been successfully grown in vitro despite many attempts.

Determining whether lung-originated virus is capable of reinfecting the cells in the lung is critical for the development of an in vitro propagation system for OvHV-2. The fact that the lambs nebulized with the virus had ORF25 transcripts detected in a lung sample at 1 day p.i. and all seroconverted at 9 days p.i. indicates that viral replication occurred immediately following nebulization. The rise in ORF25 transcript detection and viral DNA copy numbers up to 7 days p.i. suggests there were several cycles of viral replication in the lung. Since infectious OvHV-2 capable of infecting lung cells is available, identification of its target, permissive cell type(s) is the first logical step to establishing a cell culture system and is already in progress. Regardless of how much difference there is between the lung- and turbinate-originated virus, the virus generated from lung cell cultures may have great potential for use not only as virus stocks in animal experiments, but also for infecting lymphocytes in vitro, a system important for the study of virus–lymphocyte interactions, specifically in the latency stage.

The rapid drop of OvHV-2 DNA and ORF25 transcripts in the lung at 9 days p.i. suggest that OvHV-2 replication may be controlled by a host-defence mechanism. Preliminary analysis of the expression levels of selected immune response genes in the lungs of infected sheep showed that expression levels of certain genes were inversely correlated with the levels of viral DNA and transcripts in the lung, strongly suggesting that this may be the case. Based on the transcriptional profiles of the immune response genes, it seems that innate, cell-mediated and humoral responses were stimulated during early infection. Increased expression of IL-15, IL-6 and IL-8 at 1 day p.i. suggests that pulmonary macrophages and/or dendritic cells were being activated by an innate mechanism, probably involving a Toll-like receptor (Fig. 4a). Likewise, increased expression of IL-2, CD25 (the IL-2 receptor), CD28 (a lymphocyte activation receptor) and IL-13 at 1 day p.i. suggests activation of natural killer (NK) cells, NK T cells and/or γδ T cells (Fig. 4b). At 5 days p.i., the strong macrophage cytokine gene expression suggests activation of macrophages by T helper type 1 (Th1) lymphocytes with a maximal response occurring at 7 days p.i. (Fig. 4a). The cytokine expression profile also indicates that activated Th1 and Thelper 2 (Th2) cells were present in the lungs of the infected sheep from 5 to 7 days p.i. (Fig. 4b). Increased expression of CD28 and IL-17 at 5 days p.i. is indicative of an early Th1 response, while the effector phase of a Th1 response at 7 days p.i. is indicated by increased expression of the genes encoding IFN-γ, GM-CSF, granulysin and CD152. Increased expression of IFN-γ, GM-CSF and granulysin could also be associated with a cytotoxic T-cell response. Increased expression of IL-13 at 5 days p.i. suggests initiation of a Th2 response, while upregulation of the GATA-3 and IL-4 genes at 7 days p.i. indicates a definitive Th2 response also characterized by seroconversion (Fig. 1a). Presumably, the mature adaptive immune response at 7 days p.i. was responsible for the rapid decline in viral load that occurred between 7 and 9 days p.i., which in turn led to resolution of the pulmonary immune/inflammatory response by 9 days p.i.

Little has been reported about the host immune responses in control of OvHV-2 infections in either carrier sheep or clinically susceptible species, such as cattle and bison. Early studies of the immune response to AHV-1, a wildebeest-associated MCF virus, have reported only a humoral response. Although these studies demonstrated that neutralizing antibody responses developed in both experimentally infected cattle and rabbits with AHV-1 (Rossiter et al., 1977), the antibody response did not induce protection against clinical disease (Plowright et al., 1975). Similarly, studies of the humoral response to acute EBV infection reveal that infectious mononucleosis (IM) develops in the presence of strong neutralizing antibody (Callan, 2004). Mice with deficient humoral immunity (lacking the
CD28 co-stimulatory receptor) control initial lytic infection in the lung normally and establishment of latency in the spleen progresses normally (Lee et al., 2002). On the other hand, a cell-mediated immune response has been documented to play a critical role in the control of acute lytic replication in EBV and MHV68. Control of acute lytic replication is associated with strong, virus specific CD8⁺ T-cell responses during EBV-induced IM in humans (Hoshino et al., 1999) and in mice after initial MHV68 infection (Stevenson et al., 1999). A recent study revealed that MCF could be experimentally induced in sheep using a much higher dose of the virus than in cattle or bison (Li et al., 2005; Taus et al., 2005; O’Toole et al., 2007), suggesting that host immune responses probably played an important role in the control of initial replication of the virus, although other factors such as inherent host susceptibility may also be involved. Detailed characterization of how the immune response controls OvHV-2 lytic replication in the sheep lung will provide fundamental knowledge for the development of vaccine strategies, which are desperately needed by producers, to protect clinically susceptible hosts, especially North American bison, from SA-MCF.

ACKNOWLEDGEMENTS

This work was supported by USDA/ARS CWU 5348-32000-024-00D. The authors thank Janice Keller, Lori Fuller, Shirley Elias and Dan New for technical assistance, Emma Karel for animal care and handling, and Danielle Nelson for helpful discussions. We also thank Douglas Jasmer for critical review of the manuscript.

REFERENCES


