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Ovine herpesvirus-2 structural proteins in epithelial cells and M-cells of the appendix in rabbits with malignant catarrhal fever

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Abstract

Sheep-associated malignant catarrhal fever (MCF), caused by Ovine herpesvirus 2 (OvHV-2), is a usually fatal disease of various ruminants and swine. A system for propagation of OvHV-2 in vitro has not yet been identified, although persistently infected cells have been derived from diseased animals and used to establish an animal model in rabbits. OvHV-2 structural proteins have not been detected in diseased animals and the pathogenesis of OvHV-2 infection is poorly understood. Recently, the genomic sequence of OvHV-2 has been determined, which allowed to predict the amino acid sequences of putative OvHV-2 structural proteins. Based on those predictions, we have generated antisera against two putative structural proteins (ORF43 and ORF63) of OvHV-2 in order to detect sites of active virus replication in experimentally OvHV-2-infected rabbits with signs of MCF. Although histological lesions typical of MCF were detected in multiple tissues, those sera detected viral capsid and tegument antigens exclusively in the appendix but not in other tissues of rabbits with MCF. More specifically, those viral proteins were detected in epithelial cells as well as in M-cells. However, in situ hybridization revealed that ORF63 mRNA was present in epithelial cells of infected rabbits but not in M-cells. Our data suggest that active OvHV-2 replication takes place in certain tissues of animals with MCF and that M-cells may play a role in the pathogenesis of MCF.

Key words: Ovine herpesvirus 2, Malignant Catarrhal Fever, structural protein, rabbit
Introduction

Malignant catarrhal fever (MCF) is a sporadic, usually fatal infectious disease of cattle, other ruminant species, and swine (Albini et al., 2003; Coulter et al., 2001; Hussy et al., 2002; Muller-Doblies et al., 2001a; Muller-Doblies et al., 2001b; Muller-Doblies et al., 1998). There are two etiologically distinct forms of MCF: (i) a wildebeest-associated form, caused by Alcelaphine herpesvirus 1 (AlHV-1), and (ii) a sheep-associated form (SA-MCF), occurring worldwide and caused by Ovine herpesvirus 2 (OvHV-2). Based on their genomic sequences, both AlHV-1 and OvHV-2 belong to the Rhadinoviruses within the subfamily Gammaherpesvirinae (Bridgen and Reid, 1991; Ensser et al., 1997; Hart et al., 2007; Taus et al., 2007). In contrast to AlHV-1, there is no permissive cell culture system for OvHV-2, although OvHV-2-infected T lymphocytes can be cultured from diseased animals (Coulter et al., 2001; Reid et al., 1989).

Sheep, the reservoir host of OvHV-2, remain healthy upon natural infection with OvHV-2 (Ackermann, 2005, 2006; Hussy et al., 2002). Apparently, OvHV-2 establishes latency in sheep peripheral blood mononuclear cells, since the OvHV-2 genome is usually present in a circular conformation, while transcripts corresponding to productive cycle genes are barely detected (Thonur et al., 2006). In contrast, cultured T cells from diseased cattle contain a mixture of circular and linear genome configurations indicative of a mixture of latently- and productively-infected cells. Therefore, such cells can be used for the infection of experimental animals, such as rabbits and hamsters, which consequently develop an MCF-like disease (Anderson et al., 2007; Coulter et al., 2001; Reid et al., 1989; Thonur et al., 2006).

Between 5 and 23 days post inoculation, infected rabbits develop MCF symptoms, which are most similar to those seen in cattle, i.e. fever, nasal and ocular discharge, conjunctivitis and diarrhea (Anderson et al., 2007; Buxton and Reid, 1980; Muller-Doblies et al., 2001a). Usually, they die within two to three days following the first febrile reaction. Sacrificed animals show hyperplasia of the lymphoid organs and lymphoid vasculitis in various organs (Anderson et al., 2007; Liggitt and DeMartini, 1980; Muller-Doblies et al., 2001a). OvHV-2 DNA can be detected and quantified in white blood cells as well as in proliferating lymphocytes within affected organs (Albini et
Interestingly, transcripts for the OvHV-2 major capsid protein but not the corresponding proteins have recently been detected in several tissues from cattle and bison as well as rabbits with MCF (Cunha et al., 2008; Gailbreath et al., 2008). The similarity of the clinical, pathological, histological, and molecular biological findings in rabbits and bovines with MCF suggest that a comparable pathogenesis leads to this disease, which makes the rabbit a valuable in vivo model for studying MCF. Since, under natural circumstances, MCF is hardly transmitted from one diseased animal to the other (cattle to cattle or rabbit to rabbit), the question arose, whether or not productive viral replication takes place at all throughout the course of the disease in those animals. According to the recently determined genomic sequence of OvHV-2 (Hart et al., 2007; Taus et al., 2007), ORF43 encodes for an essential viral capsid protein, which has homologues in other herpesviruses, such as AlHV-1 (Ensser et al., 1997) and herpes simplex virus (HSV) (Newcomb et al., 2001). ORF63 matches to a tegument protein of AlHV-1, which is related to UL37 of HSV (Desai et al., 2001; Schmitz et al., 1995; Watanabe et al., 2000). To generate antisera for immunohistological detection of structural OvHV-2 antigens, parts of both ORFs were expressed in E. coli as glutathione S-transferase (GST) fusion proteins. The purified proteins were then used for the immunization of mice to generate specific antisera. Here we report on the detection of ORF43 and ORF63 proteins in the appendix of rabbits with MCF.

Methods

Infectious OvHV-2 (Rosbottom et al., 2002; Swa et al., 2001) was derived from T cells isolated from a cow that subsequently succumbed to SA-MCF (BJ1035, from frozen stock) and from the first passage of the same isolate in rabbits.

PCR. The sequences of ORF43 and ORF63 are available from Genbank (AY366191 and AY366192). DNA sequences encoding for the ORF43 region H284-N435 and the ORF63 region N833-L944 were amplified from cosmid C33-63 using primers containing EcoRI sites for cloning of the products into the pGEX-6P-1 vector (Tab. 1).
The entire coding sequences of ORF43 and ORF63 were amplified from the same cosmid using primers containing BamHI (ORF43) and EcoRI sites (ORF63) for cloning into a HSV-1-based amplicon vector (pHSVPrPUC, provided by Dr. Howard Federoff).

For PCR, template (2 ng C33-63 or 7.5 ng pEGFP-N3) was mixed with 2 µl of each primer (10 µM), 3 µl Pfu buffer (Stratagene, Amsterdam, The Netherlands), 1 µl Pfu polymerase (2.5 U/µl) (Stratagene), 1 µl dNTP (10 mM, Amersham Pharmacia, Biotech, Dübendorf, Switzerland), in a final volume of 30 µl.

Following denaturation at 95°C for 1 min, 35 cycles were completed at 95°C for 1 min, 1 min at the temperature specified for each product in Tab. 1, and 74°C for the time specified in Tab. 1.

**GST-fusion proteins.** The regions of choice were amplified by PCR and inserted into the EcoRI site of vector pGEX-6P-1 (Amersham). *E. coli* BL21 (Amersham) were used for expression of protein according to the protocols of the manufacturer. After induction with IPTG, the bacteria were kept for 4 hours at 30°C before being harvested. The cell pellet was suspended in a mixture of 20 ml STE buffer (50 mM NaCl, 50 mM Tris base pH 8.0, 5 mM EDTA), supplemented with a protease-inhibitor cocktail (Sigma, Buchs, Switzerland) and lysozyme (100 mg/ml). The soluble lysate containing DTT and sarcosyl was sonicated before Triton X-100 (1% final concentration) and STE-buffer were added to a final volume of 40 ml. This solution was mixed with glutathione sepharose beads (Amersham) in PBS-A (0.14 M NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, 8.1 mM Na₂HPO₄) and incubated at 4°C for 4 hours before washing with PBS-A and elution in 0.5 ml 10 mM glutathione elution buffer. Similarly, GST was expressed from the original vector without fusion partner and the protein was purified as described above. The resulting protein concentrations were determined in a GeneQuant II Spectrophotometer (Amersham) at 280 nm, based on the predicted molecular weights and calculated molar extinction coefficients (Gill and von Hippel, 1989). The eluates were used for immunizations.

**Production of labeled sense and antisense RNA.** The same PCR products as used for cloning into GST-vectors were cloned into the EcoRI site of the transcription vector pSPT19 (Roche, Reinach, Switzerland). Prior to the transcription and labeling reaction...
using the DIG RNA labeling kit (Roche) according to the manufacturer's instructions, the plasmids were linearized with NaeI to produce antisense RNA (complementary to mRNA) and PvuII to produce sense RNA (corresponding to mRNA). Briefly, 2 µg of linearized DNA were incubated with 2 µl 10x NTP labeling mixture, 2 µl 10x transcription buffer, 1 µl RNase inhibitor and 2 µl RNA polymerase T7 or SP6, respectively, and incubated for 2 hours at 37°C. After DNA digestion and ethanol precipitation, the probe was resuspended in DEPC treated water and chemically reduced in size to about 200 nucleotides by incubating with 50 µl carbonate buffer (80 mM NaH₂CO₃, 120 mM Na₂CO₃, 60°C, 25 min). After a further ethanol precipitation, the pellet was resuspended in 200 µl DEPC water.

**Antiseras against GST, GST-ORF43 protein, and GST-ORF63 protein.** Standard methods were used to immunize C57BL/6 mice with either purified GST alone or with purified GST-fusion proteins. Preimmune sera were collected prior to the first vaccination and the mice received two immunizations at three week intervals. Mouse sera were collected two weeks after the second immunization and stored at -20°C.

**Monoclonal antibodies and conjugates.** Mouse anti-Vimentin monoclonal antibody and anti-mouse-EnVision horseradish peroxidase conjugate as well as alkaline phosphatase-labeled anti-mouse polymer for double staining assays were obtained from DAKO (DAKO, Zug, Switzerland). Fluorescent Cy3 labeled conjugate was obtained from Stehelin AG (Basel, Switzerland).

**Infection of rabbits.** Six New Zealand white rabbits were infected intravenously with cell-associated OvHV-2. One rabbit (MP02/1092) received 10⁸ infected cattle T cells (BJ1035). Five rabbits (MP02/1445; MP04/1045; MP04/1046; MP04/1189; MP04/1190) received 10⁸ infected rabbit T cells taken from BJ1035 passage 1. Rabbits with clinical MCF were euthanized between day 9 and 16 post inoculation, when rectal temperatures had risen to >40°C for two days. Various tissues that are affected by OvHV-2 MCF were collected (appendix, lung, spleen, liver, mesenteric lymph nodes and kidney) and a portion of each tissue fixed in either 10% formal saline or zinc fixative (zinc salts consisting of 0.1M tris base, 0.05% Ca acetate, 0.5% Zinc chloride and 0.5% Zinc acetate). The same tissues were taken from uninfected control rabbits. According to
histological analysis, the tissues from rabbits with MCF, but not those from healthy
rabbits, showed typical lesions for example areas with hyperplasia of lymphoid cells
within the appendix, mesenteric lymph node and spleen, separated from areas with
ongoing necrosis/apoptosis. For more details, see elsewhere (Anderson et al., 2007).

**Immunohistochemistry.** Slides were deparaffinized in xylol and afterwards with a
descending alcohol series and counter-stained with haemalaun. Endogenous peroxidase
was blocked with water supplemented with 3% H₂O₂ and 0.2% NaN₃. To minimize
unspecific reactions, the slides were incubated for 10 minutes with DAKO Protein Block
Serum Free Solution (DAKO).

After washing with PBS, the slides were incubated over night with 100 µl of primary
antiserum (1:500). The slides were washed and incubated for 30 minutes with peroxidase
conjugate, anti-mouse-EnVision (DAKO, EnVision System with labeled polymer HRP
anti-mouse, K4001). After washing, the slides were developed for 10-15 minutes with
amino-ethyl carbazole (AEC) substrate (Stehelin).

For double staining, the same DAKO EnVision System with HRP anti-mouse was used in
combination with DAKO EnVision Doublestain System (DAKO, K1395). First, the
slides were treated as described above (primary staining). After development of the
primary staining, the slides were incubated with DAKO Double Staining block solution
and DAKO Protein Block Serum Free. The double staining block provides blocking of
resident immunoglobulin Fc receptors, which are present due to the primary
immunostaining. Then, mouse-anti-Vimentin monoclonal antibody was added for 30
minutes, followed by 60 minutes incubation with alkaline phosphatase-labeled anti-
mouse polymer. The secondary staining was visualized using Fast red phosphatase
chromogen solution (DAKO).

**In situ hybridization.**

In situ hybridization was done using the DIG RNA labeling and detection kit (Roche)
according to the manufacturer's instructions. Briefly, slides containing tissue sections
from OvHV-2-infected rabbits or uninfected controls were deparaffinized using Xylol
and descending ethanol series before being pretreated with DEPC water and PBS. After
HCl treatment using 200 mM HCl for 20 minutes, the sections were permeabilized with
0.02 mg/ml RNase-free Proteinase K. Sections were prehybridized for 2 hours at 50°C with 0.25 mg/ml yeast RNA in 50 ml hybridization mix (50% deionized formamide, 4 x SSC, 1 x Denhard’s solution). Hybridization was performed at 55°C over night. Each section was overlaid with 40 µl hybridization mix containing 10% dextran sulfate, 0.6 µg/µl yeast RNA and 0.1 µg/µl of DIG-labeled antisense probe or 0.1 µg/µl of DIG-labeled sense probe.

To remove unbound RNA probe, the sections were washed first in RNase solution (500 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8) containing 0.5 mg/ml RNase (DNase free) and 100’000 U/ml RNase T1 (Roche), then at 55°C 2 times in 2x SSC and 2 times in 0.2 SSC, before being incubated for 2x15 minutes at room temperature in blocking solution. For immunological detection, the sections were incubated for 1 hour with 1500 mU/ml anti-digoxigenin-AP Fab fragments (Roche) in blocking solution. After two washings, the reaction was visualized using nitroblue tetrazolium solution (NBT) and 5-brom-4-chloro-3-indolyl-phosphate (X-phosphate) substrate. Finally, the slides were mounted with glycergel before analysis under the light microscope.

Results

Production and preliminary characterization of antisera against ORF43 and ORF63 proteins. In order to generate antisera against putative structural proteins of OvHV-2, antigenic parts of the ORF43 and ORF63 encoded proteins were expressed as GST-fusion proteins and purified. Similarly, GST without fusion partner was produced. These proteins were then used for immunization. The specificity of the newly generated antisera was first tested on Western immunoblots as well as in transiently with amplicon particles transduced cell cultures (Heister et al., 2004; Steinmann et al., 1998), which provided the desired products as well as appropriate controls. While the sera did not react with cells transduced with the unrelated construct, specific signals were observed in cells transduced with ORF43 amplicon and immune stained with the anti-ORF43 serum. Similarly, ORF63 protein was detected by the anti ORF63 antiserum. A certain loss in sensitivity was observed in samples that had previously been fixed in formaldehyde and embedded in paraffin (data not shown). However, the produced antisera could be
expected to specifically recognize their antigens, even in formalin-fixed and paraffin-embedded tissue.

Detection of OvHV-2 structural antigens in M-cells and epithelial cells in the appendix of infected rabbits.

Previously, Rosbottom and others (Rosbottom et al., 2002) reported that rabbit T cell lines supported productive OvHV-2 replication. In addition, infectious material had been harvested from gut-associated lymphoid tissue such as mesenteric lymph nodes and appendix (Schock and Reid, 1996). It was hypothesized that structural viral proteins might be detected in such tissues of infected rabbits. When sections from tissues (see Materials and Methods) of OvHV-2-infected or non-infected rabbits were analyzed by immunohistology with mouse antisera against the putative tegument protein 63, specific labeling was detected exclusively in sections from the appendix of OvHV-2-infected animals. A strong signal was visible in the top layer of the dome epithelium of 5 of 6 infected (Fig. 1B) animals but not in uninfected animals (Fig. 1A). The signal was absent when preimmune sera (Fig. 1D) or sera against GST without fusion partner (not shown) were used. This staining pattern and the location of positive cells suggested that cells positive for OvHV-2 tegument protein 63 might be M-cells. Since vimentin is considered as a marker for rabbit M-cells (Gebert, 1995; Gebert et al., 1995), the same tissues from infected or uninfected rabbits were subjected to a consecutive staining with a monoclonal antibody against vimentin. While, tegument protein 63 was not detected in tissue from uninfected rabbits, M-cells were labeled in the same section (Fig. 1C). In contrast, double positive staining was detected in appendices from infected rabbits, mostly at the periphery of the domes (Fig. 1E,F). These observations suggested that structural protein of OvHV-2 were present in M-cells.

Similarly, epithelial cells but not goblet cells in the appendix of rabbits with MCF were shown to harbor OvHV-2 antigen by immunostaining with anti-ORF43 serum (Fig. 2). In contrast, tissues from uninfected rabbits revealed no immunostaining. The same infected rabbit that was negative for viral antigen in M-cells was also negative in epithelial cells. These results demonstrated that in 5 of 6 MCF-affected rabbits, OvHV-2 structural antigens were present in epithelial cells as well as in M-cells of the appendix. This makes
the link between cosmid cloned OvHV-2 nucleotide sequences, the encoded antigens, 
an antisera produced to these, and viral native antigens in vivo. However, the nature of the 
infection in antigen-positive cells remained to be determined.

Detection of ORF63 mRNA in epithelial cells of the appendix from infected rabbits.
Having detected viral proteins in cells from the appendix of OvHV-2-infected rabbits but not in other tissues that reacted positively for viral DNA by real-time PCR, it was of interest to further analyze the state of the infection not only in various tissues but also at the cell level. Preliminary testing by RT-PCR (not shown) indicated that ORF63 mRNA was detectable in extracts from appendix but not lung tissue of infected rabbits.

Therefore, various tissues (mesenteric lymphnodes, lung, liver, kidney, spleen, and appendix) of rabbits with MCF were tested by in situ hybridization for ORF63 mRNA by using a probe anti-sense to the target. Representative results are shown in Fig. 3. ORF63 mRNA-expressing cells were detected exclusively with the anti-sense probe in the appendix of all 6 infected rabbits but not in other tissues. Specificity controls in this experiment included the use of a sense probe, which was unable to hybridize to ORF63 mRNA, omission of the probe, and hybridization on tissues from uninfected rabbits.

Most interestingly, ORF63 mRNA was detected solely in epithelial cells, but not in M-cells. The fact that the viral mRNA was detected also in appendix epithelial cells from the one infected rabbit that had not given positive signals by immunohistology suggested that absence of detectable viral mRNA in M-cells was not merely attributable to a lack of sensitivity in the technique. Rather, a biological explanation had to be considered.

Discussion
The main obstacle to a better understanding of the pathogenesis of sheep-associated malignant catarrhal fever (MCF) is a lack of tools to study virus replication and other biological properties of ovine gammaherpesvirus type 2 (Ackermann, 2005). Production and characterization of specific antibodies against this virus has been difficult because OvHV-2 cannot be serially propagated as free virus in cell cultures. In the present study,
we have overcome some of these shortcomings and provide insight into some novel aspects of OvHV-2 pathogenesis in rabbits with MCF.

The salient features of our work are as follows: i) Antisera raised against two predicted structural proteins of OvHV-2 were able to establish the previously missing link between cloned DNA and viral antigens in tissues of animals with MCF. ii) Both viral mRNA and the corresponding proteins were found in epithelial cells of the appendix of diseased rabbits. iii) Viral protein but not the corresponding mRNA was found in M-cells of the same tissue. This supports the hypothesis that M-cells may play an important role in gammaherpesvirus pathogenesis.

Based on the genomic nucleotide sequence of OvHV-2, two genes encoding putative structural proteins were selected for the present study, i.e. ORF43 and ORF63. According to its predicted amino acid sequence, the ORF43 protein is a homolog of herpes simplex virus (HSV) UL6, which forms the portal for entry of the viral DNA into preformed capsids (Newcomb et al., 2001). Similarly, ORF63 has been predicted to encode for a tegument protein with homology to the UL37 protein of HSV (Watanabe et al., 2000). Therefore, detection of these two proteins can be considered as good indicators for ongoing viral replication in positive cells.

To generate antisera against those proteins, fragments thereof were expressed prokaryotically as GST-fusion proteins, which were purified and used to immunize mice. The newly generated antisera were carefully characterized (data not shown) before being used for immunohistology in various tissues of rabbits with or without MCF.

Similar to previous reports, typical histological signs of MCF were detected in appendix, lung, spleen, liver, and kidney of experimentally OvHV-2-infected rabbits (Anderson et al., 2007; Gailbreath et al., 2008). However, in our experiments, detection of OvHV-2 capsid and tegument proteins was restricted to certain cell types in the appendix, i.e. epithelial crypt cells and M-cells. Initially, the two cell types were discriminated on staining patterns and location of positive cells within the tissue. However, in a double staining experiment, the M-cells were also identified by using a monoclonal antibody against vimentin, which is considered a reliable marker for rabbit M-cells (Gebert, 1995; Gebert et al., 1995). When assayed by in situ hybridization, ORF63 mRNA was detected in epithelial cells but not M-cells from the appendix of rabbits with MCF. Since
immunohistology does not correlate well with in situ hybridization, the epithelial cells and M-cells were discriminated in this instance solely based on their location. On one hand, the above observations are in good agreement with the presence of infectious cells in the appendix (Anderson et al., 2007; Anderson et al., 2008; Buxton et al., 1984; Reid et al., 1986). On the other hand, others have reported on transcripts for the OvHV-2 major capsid protein in a variety of tissues from animals with MCF (Cunha et al., 2008; Gailbreath et al., 2008). Although the corresponding viral proteins were not assayed for in the latter case, this seeming contradiction provokes a debate on the accuracy and sensitivity of different detection methods or, alternatively, opens the possibility that biological differences may be responsible for the disagreement. On one hand, it has to be considered that RT-PCR using tissue extracts as template is likely to be more sensitive than either in situ hybridization or immunohistology. On the other hand, the cell providing the corresponding signal can be identified by in situ techniques but not when using whole tissue extracts. Furthermore, it has to be kept in mind that we used a Scottish OvHV-2 isolate (Rosbottom et al., 2002) for inoculating the rabbits, whereas Gailbreath and colleagues used an American strain (Gailbreath et al., 2008). Indeed, differences between the genomic sequences of European and American OvHV-2 strains, which may explain biological differences among the two, have been described (Hart et al., 2007; Taus et al., 2007). Finally, in our work the rabbits had been inoculated with cell-associated virus by the artificial, though widely accepted, intravenous route, whereas others have successfully made use of the intranasal inoculation of rabbits with cell-free virus (Gailbreath et al., 2008).

Despite of these reservations, detection of the OvHV-2 structural antigens in M-cells with the simultaneous failure to detect the corresponding mRNAs is notable. M-cells are specialized epithelial cells, located within mucosal surfaces lining the respiratory and intestinal tracts and capable of antigen presentation (Bockman and Cooper, 1973; Kabok et al., 1995; Owen et al., 1986; Sansonetti and Phalipon, 1999; Siebers and Finlay, 1996; Tizard, 2000). They take up antigens from the lumen in order to present them directly to lymphocytes. Interestingly, antigens that enter M-cells are likely to not be degraded but rather passed on to other cells or to the intercellular space. Indeed, soluble macromolecules, small particles, and even whole organisms are transported by M-cells.
Therefore, some bacteria and viruses can use M-cells as a portal to enter the body (Fotopoulos et al., 2002; Fujimura et al., 2004; Siebers and Finlay, 1996). For example, a lymphotrophic HIV-1 strain had been shown to cross M-cell monolayers before infecting underlying CD4(+) target cells (Fotopoulos et al., 2002).

The literature describes at least two possibilities for maintaining gammaherpesvirus infections in a specific organism. In the case of EBV, the EBV nuclear antigen 1 (EBNA-1) is able to efficiently tether the viral DNA to the host chromosome, both of which are duplicated throughout mitosis and provided to both daughter cells (Hu et al., 2002; Oehmig et al., 2004). Thus, EBV remains mostly latent in an infected organism. In contrast, the latency-associated nuclear antigen (LANA; homolog to EBNA-1) of human herpesvirus 8 (HHV-8) is much less effective in the same task. Therefore, maintenance of HHV-8 requires constantly ongoing viral replication, at least in some cells (Parravicini et al., 2000). It is possible that OvHV-2 in rabbits with MCF has a similar need for constant viral replication. Based on our results, we suggest a model according to which the epithelial cells in the appendix are productively infected, releasing infectious virus to the lumen. In a second step, M-cells would take up the virus from the lumen and transport it back into the organism for transferring it to lymphocytes, with which they interact closely. Such a circle would help to maintain the infection within the organism.

Furthermore, this model could explain for the presence of structural viral antigens and the simultaneous lack of viral mRNA in M-cells.

An interesting observation came from one rabbit that fell sick with MCF following infection and showed ample amounts of ORF63 mRNA in its appendix tissue but failed to react positively with either one of the antisera against the predicted structural OvHV-2 proteins. Apart from purely technical explanations for this fact, it should be kept in mind that not all individual animals undergo the same course of disease following natural or experimental infection with OvHV-2 (Muller-Doblies et al., 2001b; Reid et al., 1986, 1989; Taus et al., 2005).

In conclusion, antisera raised against two predicted structural proteins of OvHV-2 were able to establish the previously missing link between cloned DNA and viral antigens in tissues of animals with MCF. Interestingly, both viral mRNA and the corresponding proteins were found in epithelial cells of the appendix of diseased rabbits, whereas viral
protein but not the corresponding mRNA was found in M-cells of the same tissue. Our
data corroborate the notion that M-cells may play a role as a possible route of entry for
gammaherpesviruses (Faulkner et al., 2000).

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### Tables and Figure captions

#### Tab. 1. Primers for amplification of OvHV-2 DNA

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<sup>a</sup>F: forward primer, R: reverse primer

<sup>b</sup>Restriction sites underscored: EcoRI, BamHI, XbaI

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**Fig. 1. ORF63 protein in M-cells from OvHV-2-infected rabbits.** Histological sections from appendix of OvHV-2 infected (B,D,E,F) or uninfected (A,C) rabbits were subjected to immunohistology. A) uninfected rabbit, antiserum against tegument protein 63. B) infected rabbit, antiserum against ORF63 protein. C) uninfected rabbit, double staining with antiserum against ORF63 protein and antibody against vimentin. D) infected rabbit, preimmune serum. E) infected rabbit, double staining with antiserum against ORF63 protein and antibody against vimentin. F) same as E) with higher magnification. Black arrow: viral antigen stained with anti-tegument protein 63 serum; red arrow: M-cells, stained with antibody against vimentin.

**Fig. 2. ORF43 protein in epithelial cells from OvHV-2-infected rabbits.** Gut tissues from the appendix were treated for immunohistology with anti-ORF43 serum. A) Noninfected rabbit. B) Section through epithelial cells in the crypt. C) Section from B) at higher magnification. Arrows: a) viral antigen in epithelial cells; b) goblet cell.

**Fig. 3. In situ hybridization for ORF63 mRNA.** Samples from the same infected rabbit are shown. (A) Sense RNA probe (same orientation as mRNA), (B) Antisense probe (complementary to mRNA). The arrow points to an epithelial crypt cell containing ORF63 mRNA.
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Fig. 2.
Fig. 3.