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

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1 **Abstract**

2 Sheep-associated malignant catarrhal fever (MCF), caused by Ovine herpesvirus 2
3 (OvHV-2), is a usually fatal disease of various ruminants and swine. A system for
4 propagation of OvHV-2 *in vitro* has not yet been identified, although persistently infected
5 cells have been derived from diseased animals and used to establish an animal model in
6 rabbits. OvHV-2 structural proteins have not been detected in diseased animals and the
7 pathogenesis of OvHV-2 infection is poorly understood.

8 Recently, the genomic sequence of OvHV-2 has been determined, which allowed to
9 predict the amino acid sequences of putative OvHV-2 structural proteins. Based on those
10 predictions, we have generated antisera against two putative structural proteins (ORF43
11 and ORF63) of OvHV-2 in order to detect sites of active virus replication in
12 experimentally OvHV-2-infected rabbits with signs of MCF. Although histological
13 lesions typical of MCF were detected in multiple tissues, those sera detected viral capsid
14 and tegument antigens exclusively in the appendix but not in other tissues of rabbits with
15 MCF. More specifically, those viral proteins were detected in epithelial cells as well as in
16 M-cells. However, *in situ* hybridization revealed that ORF63 mRNA was present in
17 epithelial cells of infected rabbits but not in M-cells. Our data suggest that active OvHV-
18 2 replication takes place in certain tissues of animals with MCF and that M-cells may
19 play a role in the pathogenesis of MCF.

20

21

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

23

1 **Introduction**

2 Malignant catarrhal fever (MCF) is a sporadic, usually fatal infectious disease of cattle,
3 other ruminant species, and swine (Albini et al., 2003; Coulter et al., 2001; Hussy et al.,
4 2002; Muller-Doblies et al., 2001a; Muller-Doblies et al., 2001b; Muller-Doblies et al.,
5 1998). There are two etiologically distinct forms of MCF: (i) a wildebeest-associated
6 form, caused by Alcelaphine herpesvirus 1 (AIHV-1), and (ii) a sheep-associated form
7 (SA-MCF), occurring worldwide and caused by Ovine herpesvirus 2 (OvHV-2). Based
8 on their genomic sequences, both AIHV-1 and OvHV-2 belong to the Rhadinoviruses
9 within the subfamily *Gammaherpesvirinae* (Bridgen and Reid, 1991; Ensser et al., 1997;
10 Hart et al., 2007; Taus et al., 2007). In contrast to AIHV-1, there is no permissive cell
11 culture system for OvHV-2, although OvHV-2-infected T lymphocytes can be cultured
12 from diseased animals (Coulter et al., 2001; Reid et al., 1989).

13 Sheep, the reservoir host of OvHV-2, remain healthy upon natural infection with OvHV-
14 2 (Ackermann, 2005, 2006; Hussy et al., 2002). Apparently, OvHV-2 establishes latency
15 in sheep peripheral blood mononuclear cells, since the OvHV-2 genome is usually
16 present in a circular conformation, while transcripts corresponding to productive cycle
17 genes are barely detected (Thonur et al., 2006). In contrast, cultured T cells from diseased
18 cattle contain a mixture of circular and linear genome configurations indicative of a
19 mixture of latently- and productively-infected cells. Therefore, such cells can be used for
20 the infection of experimental animals, such as rabbits and hamsters, which consequently
21 develop an MCF-like disease (Anderson et al., 2007; Coulter et al., 2001; Reid et al.,
22 1989; Thonur et al., 2006).

23 Between 5 and 23 days post inoculation, infected rabbits develop MCF symptoms, which
24 are most similar to those seen in cattle, i.e. fever, nasal and ocular discharge,
25 conjunctivitis and diarrhea (Anderson et al., 2007; Buxton and Reid, 1980; Muller-
26 Doblies et al., 2001a). Usually, they die within two to three days following the first
27 febrile reaction. Sacrificed animals show hyperplasia of the lymphoid organs and
28 lymphoid vasculitis in various organs (Anderson et al., 2007; Liggitt and DeMartini,
29 1980; Muller-Doblies et al., 2001a). OvHV-2 DNA can be detected and quantified in
30 white blood cells as well as in proliferating lymphocytes within affected organs (Albini et

1 al., 2003; Baxter et al., 1993; Hussy et al., 2002; Hussy et al., 2001; Reid et al., 1986,
2 1989; Schock and Reid, 1996). Interestingly, transcripts for the OvHV-2 major capsid
3 protein but not the corresponding proteins have recently been detected in several tissues
4 from cattle and bisons as well as rabbits with MCF (Cunha et al., 2008; Gailbreath et al.,
5 2008). The similarity of the clinical, pathological, histological, and molecular biological
6 findings in rabbits and bovines with MCF suggest that a comparable pathogenesis leads
7 to this disease, which makes the rabbit a valuable *in vivo* model for studying MCF. Since,
8 under natural circumstances, MCF is hardly transmitted from one diseased animal to the
9 other (cattle to cattle or rabbit to rabbit), the question arose, whether or not productive
10 viral replication takes place at all throughout the course of the disease in those animals.
11 According to the recently determined genomic sequence of OvHV-2 (Hart et al., 2007;
12 Taus et al., 2007), ORF43 encodes for an essential viral capsid protein, which has
13 homologues in other herpesviruses, such as AIHV-1 (Ensser et al., 1997) and herpes
14 simplex virus (HSV) (Newcomb et al., 2001). ORF63 matches to a tegument protein of
15 AIHV-1, which is related to UL37 of HSV (Desai et al., 2001; Schmitz et al., 1995;
16 Watanabe et al., 2000). To generate antisera for immunohistological detection of
17 structural OvHV-2 antigens, parts of both ORFs were expressed in *E. coli* as glutathione
18 S-transferase (GST) fusion proteins. The purified proteins were then used for the
19 immunization of mice to generate specific antisera. Here we report on the detection of
20 ORF43 and ORF63 proteins in the appendix of rabbits with MCF.

21 **Methods**

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

25 **PCR.** The sequences of ORF43 and ORF63 are available from Genbank (AY366191 and
26 AY366192). DNA sequences encoding for the ORF43 region H284-N435 and the ORF63
27 region N833-L944 were amplified from cosmid C33-63 using primers containing *EcoRI*
28 sites for cloning of the products into the pGEX-6P-1 vector (Tab. 1).

1 The entire coding sequences of ORF43 and ORF63 were amplified from the same cosmid
2 using primers containing *Bam*HI (ORF43) and *Eco*RI sites (ORF63) for cloning into a
3 HSV-1-based amplicon vector (pHSVPrPUC, provided by Dr. Howard Federoff).

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

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

11 **GST-fusion proteins.** The regions of choice were amplified by PCR and inserted into the
12 *Eco*RI site of vector pGEX-6P-1 (Amersham). *E. coli* BL21 (Amersham) were used for
13 expression of protein according to the protocols of the manufacturer. After induction with
14 IPTG, the bacteria were kept for 4 hours at 30°C before being harvested. The cell pellet
15 was suspended in a mixture of 20 ml STE buffer (50 mM NaCl, 50 mM Tris base pH 8.0,
16 5 mM EDTA), supplemented with a protease-inhibitor cocktail (Sigma, Buchs,
17 Switzerland) and lysozyme (100 mg/ml). The soluble lysate containing DTT and sarcosyl
18 was sonicated before Triton X-100 (1% final concentration) and STE-buffer were added
19 to a final volume of 40 ml. This solution was mixed with glutathione sepharose beads
20 (Amersham) in PBS-A (0.14 M NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, 8.1 mM Na₂HPO₄)
21 and incubated at 4°C for 4 hours before washing with PBS-A and elution in 0.5 ml 10
22 mM glutathione elution buffer. Similarly, GST was expressed from the original vector
23 without fusion partner and the protein was purified as described above. The resulting
24 protein concentrations were determined in a GeneQuant II Spectrophotometer
25 (Amersham) at 280 nm, based on the predicted molecular weights and calculated molar
26 extinction coefficients (Gill and von Hippel, 1989). The eluates were used for
27 immunizations.

28 **Production of labeled sense and antisense RNA.** The same PCR products as used for
29 cloning into GST-vectors were cloned into the *Eco*RI site of the transcription vector
30 pSPT19 (Roche, Reinach, Switzerland). Prior to the transcription and labeling reaction

1 using the DIG RNA labeling kit (Roche) according to the manufacturer's instructions, the
2 plasmids were linearized with *NaeI* to produce antisense RNA (complementary to
3 mRNA) and *PvuII* to produce sense RNA (corresponding to mRNA). Briefly, 2 µg of
4 linearized DNA were incubated with 2 µl 10x NTP labeling mixture, 2 µl 10x
5 transcription buffer, 1 µl RNase inhibitor and 2 µl RNA polymerase T7 or SP6,
6 respectively, and incubated for 2 hours at 37°C. After DNA digestion and ethanol
7 precipitation, the probe was resuspended in DEPC treated water and chemically reduced
8 in size to about 200 nucleotides by incubating with 50 µl carbonate buffer (80 mM
9 NaH₂CO₃, 120 mM Na₂CO₃, 60°C, 25 min). After a further ethanol precipitation, the
10 pellet was resuspended in 200 µl DEPC water.

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

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

21 **Infection of rabbits.** Six New Zealand white rabbits were infected intravenously with
22 cell-associated OvHV-2. One rabbit (MP02/1092) received 10⁸ infected cattle T cells
23 (BJ1035). Five rabbits (MP02/1445; MP04/1045; MP04/1046; MP04/1189; MP04/1190)
24 received 10⁸ infected rabbit T cells taken from BJ1035 passage 1. Rabbits with clinical
25 MCF were euthanized between day 9 and 16 post inoculation, when rectal temperatures
26 had risen to >40°C for two days. Various tissues that are affected by OvHV-2 MCF were
27 collected (appendix, lung, spleen, liver, mesenteric lymph nodes and kidney) and a
28 portion of each tissue fixed in either 10% formal saline or zinc fixative (zinc salts
29 consisting of 0.1M tris base, 0.05% Ca acetate, 0.5% Zinc chloride and 0.5% Zinc
30 acetate). The same tissues were taken from uninfected control rabbits. According to

1 histological analysis, the tissues from rabbits with MCF, but not those from healthy
2 rabbits, showed typical lesions for example areas with hyperplasia of lymphoid cells
3 within the appendix, mesenteric lymph node and spleen, separated from areas with
4 ongoing necrosis/apoptosis. For more details, see elsewhere (Anderson et al., 2007).

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

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

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

25 **In situ hybridization.**

26 In situ hybridization was done using the DIG RNA labeling and detection kit (Roche)
27 according to the manufacturer's instructions. Briefly, slides containing tissue sections
28 from OvHV-2-infected rabbits or uninfected controls were deparaffinized using Xylol
29 and descending ethanol series before being pretreated with DEPC water and PBS. After
30 HCl treatment using 200 mM HCl for 20 minutes, the sections were permeabilized with

1 0.02 mg/ml RNase-free Proteinase K. Sections were prehybridized for 2 hours at 50°C
2 with 0.25 mg/ml yeast RNA in 50 ml hybridization mix (50% deionized formamide, 4 x
3 SSC, 1 x Denhard's solution). Hybridization was performed at 55°C over night. Each
4 section was overlaid with 40 µl hybridization mix containing 10% dextran sulfate, 0.6
5 µg/µl yeast RNA and 0.1 µg/µl of DIG-labeled antisense probe or 0.1 µg/µl of DIG-
6 labeled sense probe.

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

16 **Results**

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

1 expected to specifically recognize their antigens, even in formalin-fixed and paraffin-
2 embedded tissue.

3
4 **Detection of OvHV-2 structural antigens in M-cells and epithelial cells in the**
5 **appendix of infected rabbits.**

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

26 Similarly, epithelial cells but not goblet cells in the appendix of rabbits with MCF were
27 shown to harbor OvHV-2 antigen by immunostaining with anti-ORF43 serum (Fig. 2). In
28 contrast, tissues from uninfected rabbits revealed no immunostaining. The same infected
29 rabbit that was negative for viral antigen in M-cells was also negative in epithelial cells.
30 These results demonstrated that in 5 of 6 MCF-affected rabbits, OvHV-2 structural
31 antigens were present in epithelial cells as well as in M-cells of the appendix. This makes

1 the link between cosmid cloned OvHV-2 nucleotide sequences, the encoded antigens,
2 antisera produced to these, and viral native antigens *in vivo*. However, the nature of the
3 infection in antigen-positive cells remained to be determined.

4 **Detection of ORF63 mRNA in epithelial cells of the appendix from infected rabbits.**

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

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

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

24 **Discussion**

25 The main obstacle to a better understanding of the pathogenesis of sheep-associated
26 malignant catarrhal fever (MCF) is a lack of tools to study virus replication and other
27 biological properties of ovine gammaherpesvirus type 2 (Ackermann, 2005). Production
28 and characterization of specific antibodies against this virus has been difficult because
29 OvHV-2 cannot be serially propagated as free virus in cell cultures. In the present study,

1 we have overcome some of these shortcomings and provide insight into some novel
2 aspects of OvHV-2 pathogenesis in rabbits with MCF.

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

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

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

22 Similar to previous reports, typical histological signs of MCF were detected in appendix,
23 lung, spleen, liver, and kidney of experimentally OvHV-2-infected rabbits (Anderson et
24 al., 2007; Gailbreath et al., 2008). However, in our experiments, detection of OvHV-2
25 capsid and tegument proteins was restricted to certain cell types in the appendix, i.e.
26 epithelial crypt cells and M-cells. Initially, the two cell types were discriminated on
27 staining patterns and location of positive cells within the tissue. However, in a double
28 staining experiment, the M-cells were also identified by using a monoclonal antibody
29 against vimentin, which is considered a reliable marker for rabbit M-cells (Gebert, 1995;
30 Gebert et al., 1995). When assayed by in situ hybridization, ORF63 mRNA was detected
31 in epithelial cells but not M-cells from the appendix of rabbits with MCF. Since

1 immunohistology does not correlate well with in situ hybridization, the epithelial cells
2 and M-cells were discriminated in this instance solely based on their location.

3 On one hand, the above observations are in good agreement with the presence of
4 infectious cells in the appendix (Anderson et al., 2007; Anderson et al., 2008; Buxton et
5 al., 1984; Reid et al., 1986). On the other hand, others have reported on transcripts for the
6 OvHV-2 major capsid protein in a variety of tissues from animals with MCF (Cunha et
7 al., 2008; Gailbreath et al., 2008). Although the corresponding viral proteins were not
8 assayed for in the latter case, this seeming contradiction provokes a debate on the
9 accuracy and sensitivity of different detection methods or, alternatively, opens the
10 possibility that biological differences may be responsible for the disagreement. On one
11 hand, it has to be considered that RT-PCR using tissue extracts as template is likely to be
12 more sensitive than either in situ hybridization or immunohistology. On the other hand,
13 the cell providing the corresponding signal can be identified by in situ techniques but not
14 when using whole tissue extracts. Furthermore, it has to be kept in mind that we used a
15 Scottish OvHV-2 isolate (Rosbottom et al., 2002) for inoculating the rabbits, whereas
16 Gailbreath and colleagues used an American strain (Gailbreath et al., 2008). Indeed,
17 differences between the genomic sequences of European and American OvHV-2 strains,
18 which may explain biological differences among the two, have been described (Hart et
19 al., 2007; Taus et al., 2007). Finally, in our work the rabbits had been inoculated with
20 cell-associated virus by the artificial, though widely accepted, intravenous route, whereas
21 others have successfully made use of the intranasal inoculation of rabbits with cell-free
22 virus (Gailbreath et al., 2008).

23 Despite of these reservations, detection of the OvHV-2 structural antigens in M-cells with
24 the simultaneous failure to detect the corresponding mRNAs is notable. M-cells are
25 specialized epithelial cells, located within mucosal surfaces lining the respiratory and
26 intestinal tracts and capable of antigen presentation (Bockman and Cooper, 1973; Kabok
27 et al., 1995; Owen et al., 1986; Sansonetti and Phalipon, 1999; Siebers and Finlay, 1996;
28 Tizard, 2000). They take up antigens from the lumen in order to present them directly to
29 lymphocytes. Interestingly, antigens that enter M-cells are likely to not be degraded but
30 rather passed on to other cells or to the intercellular space. Indeed, soluble
31 macromolecules, small particles, and even whole organisms are transported by M-cells.

1 Therefore, some bacteria and viruses can use M-cells as a portal to enter the body
2 (Fotopoulos et al., 2002; Fujimura et al., 2004; Siebers and Finlay, 1996). For example, a
3 lymphotropic HIV-1 strain had been shown to cross M-cell monolayers before infecting
4 underlying CD4(+) target cells (Fotopoulos et al., 2002).

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

21 An interesting observation came from one rabbit that fell sick with MCF following
22 infection and showed ample amounts of ORF63 mRNA in its appendix tissue but failed
23 to react positively with either one of the antisera against the predicted structural OvHV-2
24 proteins. Apart from purely technical explanations for this fact, it should be kept in mind
25 that not all individual animals undergo the same course of disease following natural or
26 experimental infection with OvHV-2 (Muller-Doblies et al., 2001b; Reid et al., 1986,
27 1989; Taus et al., 2005).

28 In conclusion, antisera raised against two predicted structural proteins of OvHV-2 were
29 able to establish the previously missing link between cloned DNA and viral antigens in
30 tissues of animals with MCF. Interestingly, both viral mRNA and the corresponding
31 proteins were found in epithelial cells of the appendix of diseased rabbits, whereas viral

1 protein but not the corresponding mRNA was found in M-cells of the same tissue. Our
2 data corroborate the notion that M-cells may play a role as a possible route of entry for
3 gammaherpesviruses (Faulkner et al., 2000).

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18

19

1 **Tables and Figure captions**

2

3 **Tab. 1.** Primers for amplification of OvHV-2 DNA

Oligonucleotide ^a	Sequence (5'-3') ^b	Annealing (°C)/ Extension (min.)	Product length
fragment 43F	GATATCGAATTCCACCTAGATGAGTGTAGT ^c	50 / 1	478 bp
fragment 43R	TAGAGAATTCCCTAGTTAACCTGGTTGGAT ^c		
fragment 63F	CTCCGAATTCAATCCCCAAATTTTCCTTGC ^c	52 / 1	356 bp
fragment 63R	ACGCGAATTCTTACAGCTCCTGCGTAGGCT ^c		
43F	GTCGGATCCAGGATAAAGCTCTTAGGTATG ^d	60 / 2.5	1735 bp
43R	AGCGGATCCC GGGCCGCTACTCAGTCG ^d		
63F	CCTCTCTGGAATTCATGATGGAGAACAAGC ^d	55 / 4.5	2886 bp
63R	TGGAATTCGGCTGTGAGGCCATGGCGCTACG ^d		

4 ^aF: forward primer, R: reverse primer

5 ^bRestriction sites underscored: ^cEcoRI, ^dBamHI, ^eXbaI

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

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

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

16

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Figure 1

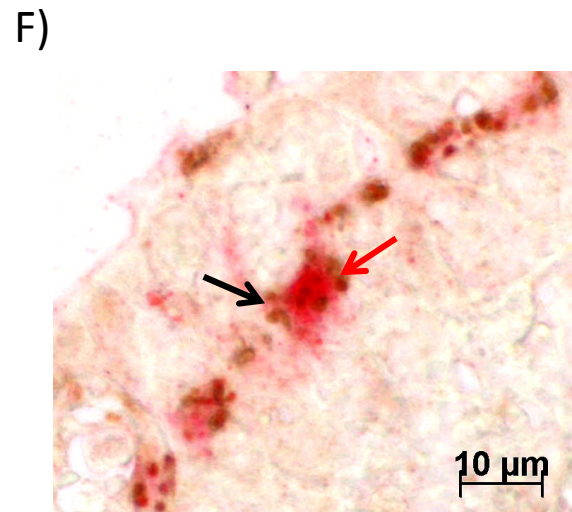
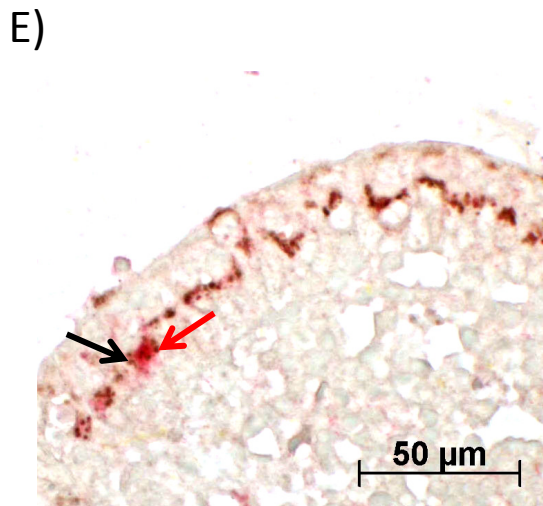
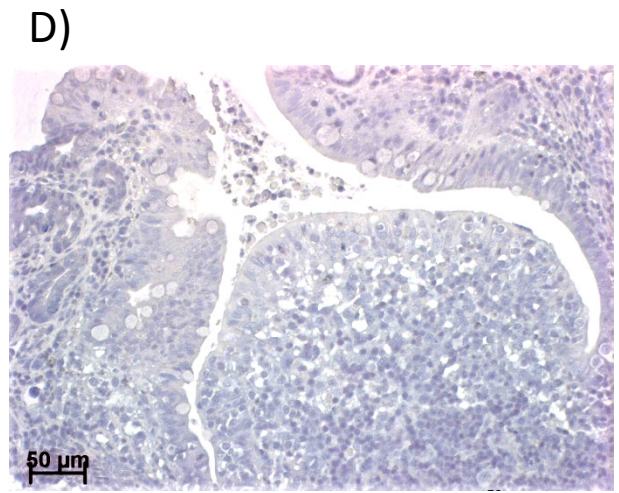
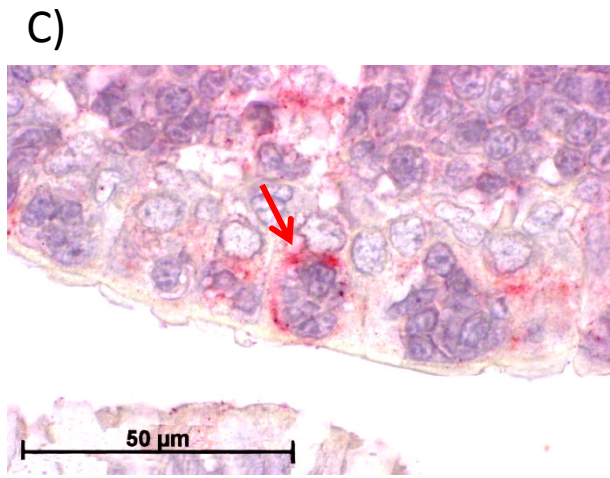
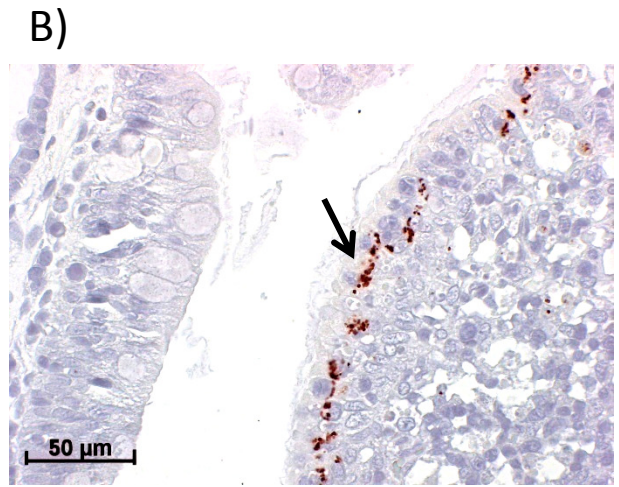
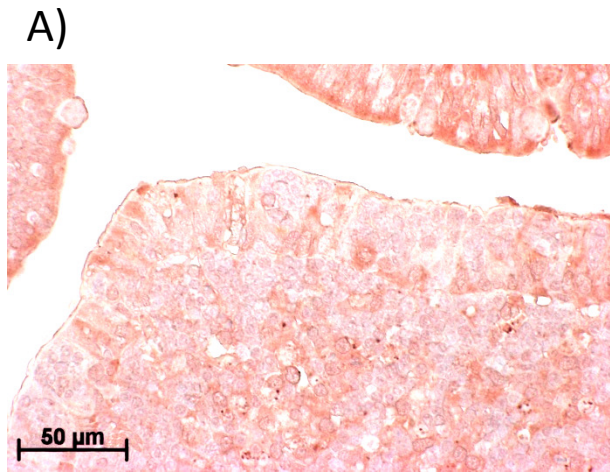
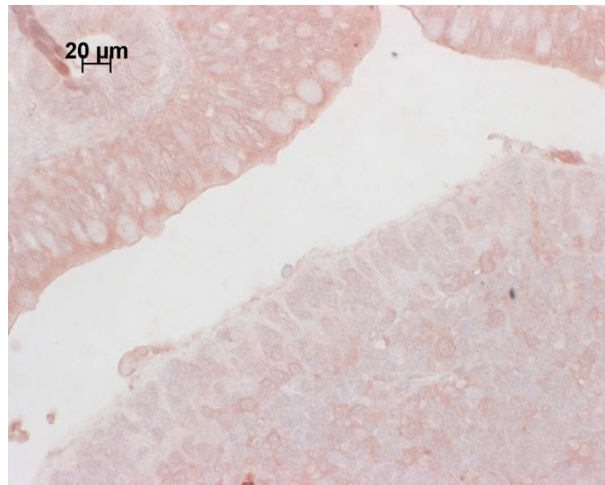
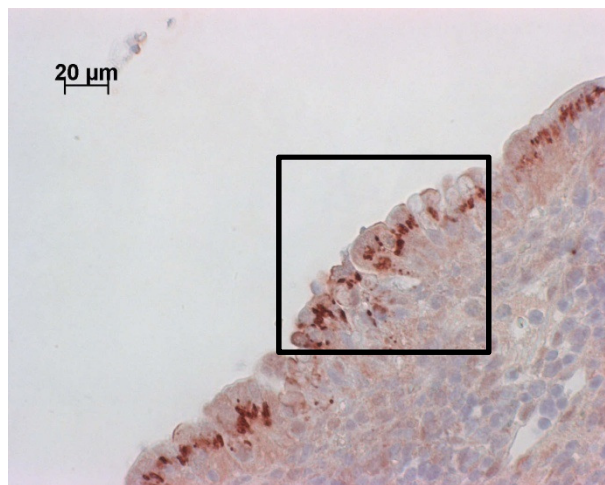


Fig. 1.

A)



B)



C)

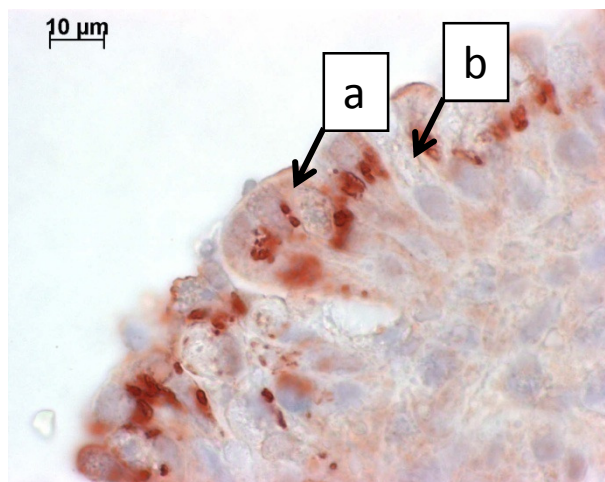
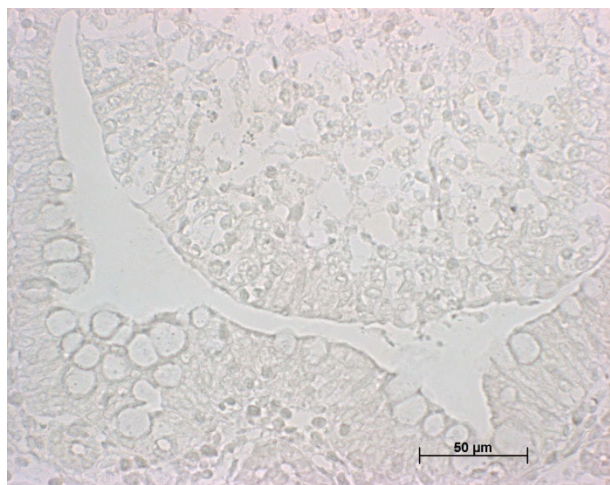


Fig. 2.

A)



B)



Fig. 3.