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ORIGINAL ARTICLE

Differential transcription of ovine herpesvirus 2 genes in lymphocytes from reservoir and susceptible species

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Abstract Ovine herpesvirus 2 (OvHV-2) is a lymphotropic gammaherpesvirus that asymptomatically infects most sheep, but causes malignant catarrhal fever in cattle, bison, pigs and deer. There is no permissive cell culture system but OvHV-2-infected T lymphocytes can be cultured from diseased animals. We showed that the OvHV-2 genome was in a circular conformation in sheep peripheral blood mononuclear cells and that the latency-associated ORF73 was transcribed, while expression of the productive cycle genes ORF9 (DNA polymerase) and ORF50 (R-transactivator) was barely detectable, suggestive of latency. Doxorubicin treatment of these cells induced the appearance of linear viral DNA and transcription of productive cycle genes along with several viral unique genes. In contrast, cultured T cells from diseased cattle and rabbits contained a mixture of circular and linear genome configurations indicative of a mixture of latently- and productively-infected cells. Most of the OvHV-2 unique genes were transcribed in these cells but ORF50 expression was only seen after doxorubicin treatment indicating a 'leaky' latent pattern of gene expression. 5-azacytidine treatment increased the proportion of circular DNA and inhibited the expression of most of the OvHV-2 unique genes except Ov2.5 (vIL-10) and Ov4.5 (Bcl-2 homologue) in the cattle cell line. These studies provide key insights into the differences in OvHV-2 gene expression in cells from reservoir and susceptible species and, for the first time, an *in vitro* system for studying the latent and productive phases of the OvHV-2 virus life cycle.

Keywords Malignant Catarrhal Fever (MCF) · Latency · Genome conformation · Gene expression · Doxorubicin · 5-azacytidine

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Introduction

Malignant Catarrhal Fever (MCF) is an often-fatal lymphoproliferative disease of susceptible ungulates that include cattle, deer, bison and pigs. The disease is systemic, usually acute, and characterized by lymphoproliferation and T cell infiltration into multiple tissues, associated with degenerative changes and vasculitis [1–3]. The known causative agents are either of two related γ-herpesviruses, ovine herpesvirus-2 (OvHV-2) and alcelaphine herpesvirus-1 (AlHV-1). The natural reservoir host for OvHV-2 is sheep [4] and for AlHV-1 is the wildebeest *(Connochaetes* species) [5]. MCF is dramatic, usually fatal and is currently a major problem in eastern and southern Africa, Indonesia and in bison herds in the USA.

A feature of MCF is that the disease appears to be associated with a low viral load. Virus-infected cells have been difficult to detect *in vivo* [6] although OvHV-2-infected T cells have been identified in MCF lesions in the brain of cattle and bison by *in situ* PCR [7]. Thus, to enable the study of the interaction of OvHV-2 with host cells, T-lymphoblastoid cell lines with the morphology of large granular lymphocytes (LGLs) have been established in culture from the tissues of MCF-affected animals [8–11]. These T cells contain OvHV-2 DNA and antigen [4, 11, 12] and can transmit MCF experimentally to rabbits and

hamsters [13, 14]. OvHV-2-positive T cell lines are constitutively and indiscriminately (non-MHC-restricted) cytotoxic and produce a range of cytokines, but not IL-2 [10, 15]. Our current hypothesis is that MCF is due to indiscriminate tissue damage caused by dysregulated cytotoxic T cells generated as a consequence of infection. The LGL T cells in culture represent the virus-infected cells *in vivo* and are invaluable for virus–cell interaction studies in MCF.

The genome of OvHV-2 has recently been fully sequenced (Stewart et al., unpublished; Accession number AY839756) allowing the molecular virology of OvHV-2 to be studied. The arrangement of OvHV-2 genes is co-linear with respect to other γ-herpesviruses. In addition there are 12 genes (termed Ov2–Ov10) that have no obvious sequence homologue in other γ-herpesviruses. Work with other γ-herpesviruses has shown that the functions of genes unique to each virus are often involved in host-specific pathogenesis. Thus, it seems likely that the OvHV-2 unique genes will be involved in the distinct pathology of MCF and are the focus of particular attention in this study.

In common with other herpesviruses, OvHV-2 was expected to infect cells productively and then establish latency [16, 17]. The lack of a productive culture system for OvHV-2 has meant that there has been no experimental means to study these stages of the virus life cycle. In a recent study it was suggested that OvHV-2-infected cattle T cells support predominantly latent infection whereas OvHV-2-infected rabbit T cells showed evidence of both productive and latent virus infection [18]. The ability to study the productive cycle in the absence of adequate culture systems was overcome with viruses such as Epstein–Barr virus (EBV) and Kaposi's Sarcoma herpesvirus (KSHV) by treatment of latently-infected lymphocytes with a range of virus-reactivating drugs. Effective inducing agents include the protein kinase C activator phorbol 12-myristate 13-acetate (TPA) [19], the DNA intercalating agent doxorubicin [20] and the DNA hypomethylating agent 5-azacytidine [21, 22].

The aim of this study was to analyze OvHV-2 gene expression and genome conformation in infected cells from the reservoir host (sheep) and from susceptible species (cattle, rabbit) and to study the effects on these cells of drugs used previously to reactivate latent EBV-infected cells.

Materials and methods

Cells and virus

OvHV-2-infected T cell lines were cultured from the tissue of MCF-affected animals using published techniques [11]. The BJ1035 line from an OvHV-2-infected cow has been described previously [11, 15] and was the source of virus DNA for the OvHV-2 genome sequence. BJ2586 was derived from a rabbit that was inoculated with BJ1035-associated OvHV-2 and which developed MCF. Both cell lines are virulent, producing clinical MCF in infected animals. These cell lines were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM glutamine (Invitrogen, Paisley, UK), 100 IU of Penicillin per ml, 100 μg of streptomycin per ml, 10% heat-inactivated foetal bovine serum and 350 IU of IL-2 per ml (PROLEUKIN, Chiron Therapeutics, Emeryville, CA).

Sheep peripheral blood mononuclear cells (PBMC) were obtained from the blood of an adult sheep that was PCR-positive for OvHV-2 DNA. Mononuclear cells were purified by centrifugation over lymphoprep (Nycomed, Oxford, UK), adjusted to 1×10^6 cells/ml in medium and maintained in the presence of 350 IU of IL2 per ml (that supports ovine T cell and B cell lymphoblast proliferation).

Preparation of DNA from OvHV-2 infected cells

Genomic DNA was isolated from OvHV-2-infected rabbit and cattle T cell lines (BJ2586 and BJ1035) using the DNeasy Tissue kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions and was used as the template for amplification of OvHV-2 gene fragments using the primer sets in Table 1.

OvHV-2 DNA probes

DNA fragments corresponding to OvHV-2 unique genes (Ov2, Ov2.5, Ov3, Ov3.5, Ov4.5, Ov5, Ov6, Ov7+Ov8, Ov8, Ov8.5 and Ov9) and selected conserved genes (ORF50, ORF73 and ORF9) were amplified using the primer sets described in Table 1. All PCR programmes comprised: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 1 min, specified annealing temperature (Table 1) for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. For the 3 kb PCR product of Ov7 plus Ov8, the Expand Long Template (Roche, Lewes, UK) PCR system was used. PCR products were gel-purified and labelled using the Rediprime II random prime labelling system (Amersham, Bucks, UK). Unincorporated nucleotides were removed using the QIAquick nucleotide removal kit (Qiagen, Crawley, UK).

Extraction of RNA from OvHV-2 infected cells

All cells were maintained in 10 ml medium in multiple T25 flasks. Total RNA from cells was extracted using the acid-phenol technique [23].

Table 1 PCR primers used in this study[a]

OvHV-2 genes		PCR primers	Annealing temperature (°C)	Product size (bp)
Ov2	Sense	5′-CAAGCTGTGTAACATATTCAACTC	52	651
	Antisense	5′-ATGTCCGATAATAAAAAGCCTG		
Ov2.5	Sense	5′-GCATTGGCCCACCAACTA	55	874
	Antisense	5′-TGACCCCAAAGTAGCTTTCC		
Ov3	Sense	5′-TTTTTAGAGCCATACTCCA	55	1367
	Antisense	5′-ACTCTAGGGGCACCTCGCT		
Ov3.5	Sense	5′-ATGGACAAGCTCTACACGC	52	489
	Antisense	5′-ACAGCTCAGGTGAGTCGGTG		
Ov4.5	Sense	5′-CCATGGCTACCACACAGTATGCG	52	631
	Antisense	5′-CTAGCCCATGGGTTGTGACACATTTCCACAAG		
Ov5	Sense	5′-CAACAGCACCCTGGTGGA	52	1244
	Antisense	5′-CATAGTTTAGATCCAGCACGCA		
Ov6	Sense	5′-ATGGCCAATAGCCCTGGT	52	952
	Antisense	5′-CTGCCTTGCAGCTTGCTT		
Ov7&8	Sense	5′-GGATCCATGAGGTTCCCTCACAAGCTCCT	55	2999
	Antisense	5′-TCAGCCCTAGGATCCCTCGGTTAAACACAGGACAATG		
Ov8	Sense	5′-TCCTGACGCGCCACC	55	2384
	Antisense	5′-TCAGCCCTAGGATCCCTCGGTTAAACACAGGACAATG		
Ov8.5	Sense	5′-CCATGGTGTCCCGGCTTCC	55	1165
	Antisense	5′-CCATGGATTTGGTGTGCTTCTTAG		
Ov9	Sense	5′-CCATGGTGGAAGCTGGCATACT	52	609
	Antisense	5′-CCATGGCGCGTCTCCATAACACAACCA		
ORF9	Sense	5′-CCTTCTGGAACCCCTACCTGAG	55	1580
	Antisense	5′-GGCAAGCAAACACCCTAATCTG		
ORF50	Sense	5′-ATGAGTGGCAAAAGACCCTC	55	1828
	Antisense	5′-TATGCACAGAGCCAGTGGAG		
ORF73	Sense	5′-CTAGTACTGACGGCGATGAAGATG	64	1426
	Antisense	5′-CTGTACTAATGTTAACCGCTCCTG		

[a] Primers were based on the sequence of the OvHV. -2 genome (J.P, Stewart et al., unpublished data)

Synthesis of labelled cDNA

Before cDNA synthesis, total RNA (5 μg) was treated with 5 U of RNase-free DNAse I (Invitrogen, Paisley, UK) at 20°C for 15 min to remove contaminating DNA. Radioactively-labelled cDNA, for hybridization analysis of OvHV-2-specific gene expression, was synthesized at 50°C with 200 U of reverse transcriptase (Superscript II, Invitrogen, Paisley, UK) in the presence of 100 pmoles of each required 3′ gene-specific primer and 100 nM dNTPs (Roche Applied Science, Lewes, UK) with dCTP replaced with 1.85 MBq of [α-^{32}P]dCTP (Amersham, Bucks, UK). Labelled cDNA was purified as described for DNA probes.

Southern blot analysis

OvHV-2 gene-specific PCR products (150 ng) were electrophoresed through 1% agarose gels and were transferred to nylon Hybond-N$^+$ membrane (Amersham, Bucks, UK) by a standard capillary blotting method, The DNA was fixed to the membrane by UV crosslinking. Hybridization to purified ^{32}P-labelled cDNA probes was done using the rapid-hyb buffer system (Amersham, Bucks, UK). Hybridization patterns were captured by 48 h exposure to a storage phosphor screen and read using a laser scanning imager (Molecular Imager; Bio-Rad, Hemel Hempstead, UK). Band intensity in the raw 16-bit image files was quantified with Bio-Rad Quantity One software using the volume analysis tools with local background setting. For image publication the intensity values corresponding to black and white pixels were optimized within the range 0–65535 and images were exported as 8-bit TIF files.

Gardella gel analysis of viral genome conformation

Gel analysis of viral DNA conformation was performed as described by Gardella and co-workers [24]. Briefly, a 25 cm × 15 cm horizontal agarose gel was poured so that the upper portion (3 cm), including sample wells, contained 2% SDS, 1 mg/ml proteinase K and 0.75% agarose in 1×TBE. The lower portion of the gel (22 cm) contained 0.75% agarose in TBE. The infected cells were washed twice with l×PBS, resuspended in loading dye (15% ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and 2×10^6 cells (50 μl) were then loaded into each well. Gels were run for 3 h at 45 V to allow cell lysis, then for 26–28 h at 120 V to separate circular from linear viral DNA. Southern blots of Gardella gels were probed

simultaneously with ^{32}P-labelled DNA fragments derived from the OvHV-2 Ov2, Ov4.5, Ov7, and Ov9 genes.

Regulation of the latent/productive (lytic) virus cycle

OvHV-2-infected cells were treated with 5-azacytidine (50 μM) or doxorubicin (0.02 μM). Preliminary dose-response experiments were carried out to determine the optimum concentration of each compound, i.e., the highest non-cytotoxic concentration (data not shown). In each case, cultured infected cell lines or PBMC were exposed to the compounds at the above concentrations for 3 days, after which the drugs were serially diluted by replacing two-thirds of the cell suspension with fresh medium for two days (doxorubicin) or every 3 days (5-azacytidine). 5-Azacytidine-treated cultures were maintained for 5 weeks as previously reported [25]. Doxorubicin-treated cultures were maintained for 5 days [20]. Total RNA was extracted from the treated and control cells as described above.

Results

OvHV-2 conformation, gene expression and reactivation in sheep lymphocytes

To analyze the state of the OvHV-2 genome in the natural reservoir species, peripheral blood mononuclear cells (PBMCs) were extracted from OvHV-2-positive sheep. Pilot experiments indicated that the number of infected cells was too low for direct analysis, so the lymphocytes were first cultured in the presence of IL-2 for 15–20 days. The OvHV-2 genome in these cells was then subjected to Gardella gel analysis. This technique discriminates linear from covalently-closed circular (CCC) viral genomes, which are characteristic of productive or latent herpesvirus infection respectively. A Southern blot of the Gardella gel was probed with multiple ^{32}P-labelled OvHV-2 fragments. As shown in Fig. 1a (−), sheep PBMCs appeared to contain entirely circular virus DNA despite the period in culture, suggesting that the vast majority of OvHV-2-positive cells were latently infected.

To confirm this observation, the expression of homologues of known γ-herpesvirus latency and lytic cycle genes (ORF 9, DNA polymerase; ORF 50, R-transactivator that induces the latent to productive cycle switch; ORF73, the latency-associated nuclear antigen gene) was analyzed along with that of a panel of OvHV-2 unique genes. To analyze the expression of multiple genes using limiting amounts of RNA, labelled OvHV-2-specific cDNAs were generated from total RNA extracted from sheep PBMCs. These cDNAs were then used to probe Southern blots of selected OvHV-2 gene fragments which were generated by PCR amplification using primers shown in Table 1. Variations of this technique have been used successfully in the past to analyze gene expression in EBV [26] and MHV-68 [27]. This technique made efficient use of the limited and labile RNA samples. In addition, we were careful to ensure that the specific activity of the probes was consistent and all blots were analyzed by 48 h exposure to the same storage phosphor screen. Storage phosphor detection of radioactive bands and analysis with the Bio-Rad Molecular Imager gave high sensitivity and a wide dynamic range, allowing relative quantification of gene expression in the absence of real-time PCR assays of OvHv-2 gene expression. It should be noted, however, that the images used in Figs. 1–3 have been optimized to allow best visualization of the hybridization bands present and therefore are not directly comparable. For each figure the intensity values set for black and white pixels in the image are given in the corresponding legend. This may allow relative expression levels to be estimated. Each experiment was repeated at least three times with consistent results.

Figure 1b showed that in untreated sheep PBMCs there is hybridization to Ov3.5 and ORF73 gene fragments and barely detectable hybridization to a few other genes. The expression of ORF73 and restricted expression of other genes support the view that sheep PBMCs exhibited a latent state. Thus, the expression of Ov3.5 identified it as a putative latency-associated gene in sheep lymphocytes. Alternatively, the hybridization to Ov3.5, and more faintly to other OvHV-2 genes (unique genes Ov7, Ov8.5, Ov9 and lytic cycle homologues ORF 9 and ORF 50), may be a consequence of a low level of virus reactivation (productive cycle) in culture.

To determine whether the restricted viral gene expression in sheep PBMCs was due to a predominantly latent state, we attempted to induce changes in gene expression and genome conformation associated with productive cycle infection in these cells. Preliminary experiments with agents known to induce virus reactivation from latency with other γ-herpesviruses (e.g., TPA) showed no effect. However, doxorubicin has recently been shown to induce the productive cycle in EBV-infected lymphocytes [20]. Sheep PBMCs were treated with this agent and OvHV-2 genome conformation and gene expression were analyzed as above.

Figure 1a shows that doxorubicin treatment (+) induced the presence of linear viral DNA which was absent prior to treatment (−). Analysis of viral gene expression post-treatment (Fig. 1c) showed significant transcription of the OvHV-2 productive cycle genes ORF9 and ORF50 along with ORF73, and that expression of all of the unique OvHV-2 genes tested was induced or increased. Thus, doxorubicin appeared to induce the productive cycle in a proportion of sheep PBMCs.

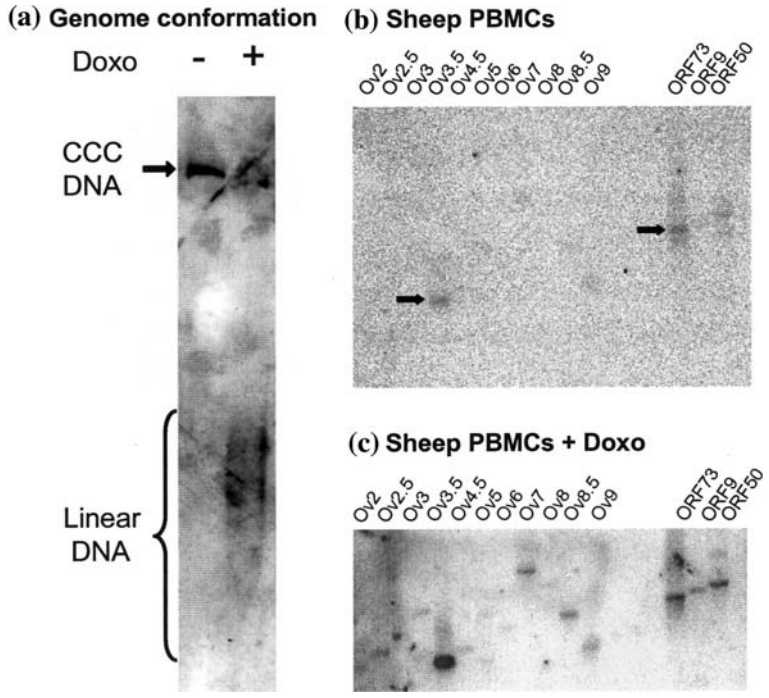

Fig. 1 Analysis of OvHV-2 genome conformation and gene expression in sheep PBMC before (−) and after (+) treatment with doxorubicin (Doxo; 0.2 μM). (a) Gardella gel analysis of sheep PBMCs. Cells were lysed *in situ* and extracts were electrophoresed through a 0.8% agarose-TBE gel. A Southern blot of the gel was then probed simultaneously with ^{32}P-labelled probes specific for OvHV-2 Ov2, Ov4.5, Ov7 and Ov9 followed by 48 h exposure to a storage phosphor screen. The positions of closed circular (CCC) DNA and linear viral DNA are indicated on the left. (b, c) Expression of OvHV-2 genes in sheep PBMC. Gel-purified PCR products corresponding to OvHV-2 genes were electrophoresed through 1% agarose gels. The lane positions of OvHV-2 unique gene fragments (Ov2–Ov9), ORF73, ORF9 and ORF50 are indicated above the panels. Southern blots were probed with labelled cDNAs derived from OvHV-2-positive sheep PMBCs cultured in the absence of treatment (b), and in the presence of doxorubicin (c). For display, the intensity values set for black were 700 (b) and 5,000 (c)

OvHV-2 conformation and gene expression in infected cattle and rabbit T cell lines

In the absence of an *in vitro* culture system for OvHV-2 expression, we next went on to study OvHV-2 infected cells from species which are susceptible to MCF. Two OvHV-2 infected T cell lines were used, one from a cow (BJl035) and one from a rabbit which had been infected with BJ1035 virus (BJ2586). Viral genome conformation was analyzed in these cells using Gardella gels. The OvHV-2-infected cattle T cells showed predominantly circular DNA with a relatively small amount of linear DNA (Fig. 2a(−)). In contrast, the rabbit T cells contained mainly linear DNA with a barely-detectable level of circular DNA (Fig. 3a(−)). This suggested, based on comparison with other γ-herpesviruses, that the majority of cattle T cells were latently-infected with a small proportion supporting the productive cycle, while the majority of the rabbit T cells supported a productive infection.

Viral gene transcription in infected cattle and rabbit T cells was studied in a similar manner to sheep PBMCs. Figs. 2b and 3b show that, in contrast to sheep PBMCs, most of the unique OvHV-2 genes were expressed in the cattle and rabbit T cell lines and that the degree of hybridization was far higher, with band intensities being an order of magnitude greater in both cases than those found in the sheep cells after doxorubicin treatment. However, the different cell lines had distinct patterns of OvHV-2 unique gene expression. In the cattle cell line, all the unique genes were expressed, with highest relative expression of the Ov8 and Ov8.5 transcripts (Fig. 2b). In contrast, mRNA for the Ov5 gene was not detected in the rabbit cell line, while Ov9, Ov7 and Ov4.5 gave the strongest signals (Fig. 3b). Of particular note is the observation that while mRNA for ORF50 could not be detected in BJ1035 or BJ2586 by blotting or by RT-PCR, a low level of ORF73 transcript was detected in BJ1035 by RT-PCR (Fig. 4).

Doxorubicin induces markers of the OvHV-2 productive cycle in infected T cell lines

We analyzed the ability of doxorubicin to induce changes in gene expression and genome conformation in BJ1035. The infected rabbit T cell line BJ2586 was not studied, as this contained predominantly linear viral genomes to start with. As shown in Fig. 2a(−), BJ1035 contained

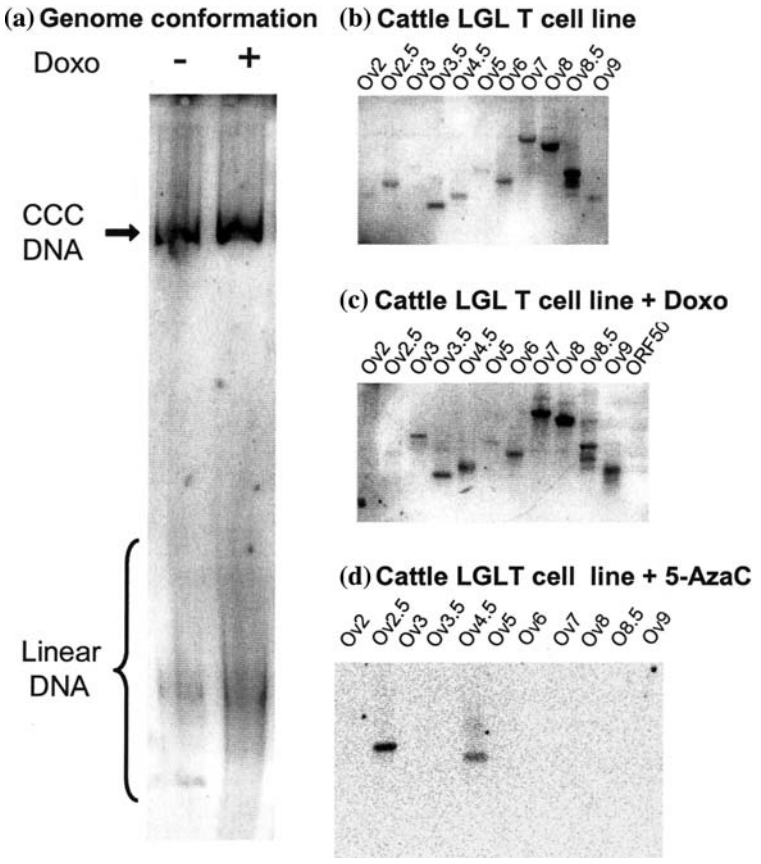

Fig. 2 Analysis of OvHV-2 genome conformation and gene expression in cattle LGL T cells before (−) and after (+) treatment with doxorubicin (Doxo, 0.2 μM) or 5-azacytidine (5-AzaC, 50 μM). (a). Gardella gel analysis. Cell extracts lysed *in situ* were electrophoresed through a 0.8% agarose-TBE gel and probed as described in Fig. 1. The positions of closed circular (CCC) DNA and linear viral DNA are indicated on the left. (b, c, d) Expression of OvHV-2 genes was assayed as described in Fig. 1, using labelled cDNA generated from cells grown in the absence of treatment (b), in the presence of doxorubicin (c) or in the presence of 5-azacytidine (d). For display, the intensity values set for black were 30,000 (b), 12,000 (c) and 2,800 (d)

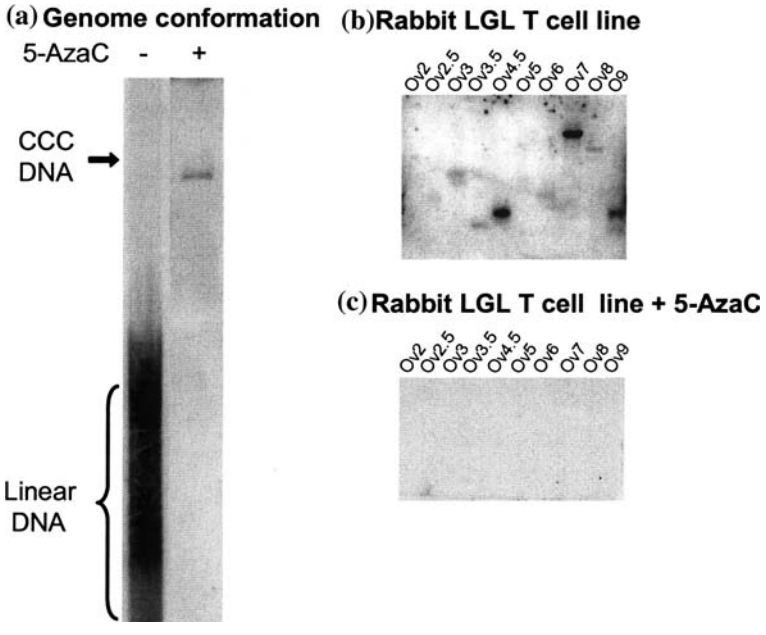

Fig. 3 Analysis of OvHV-2 genome conformation and gene expression in rabbit LGL T cells before (−) and after (+) treatment with 5-azacytidine (5-AzaC, 50 μM). (a) Gardella gel analysis. Cell extracts lysed *in situ* were electrophoresed through a 0.8% agarose-TBE gel and probed as described in Fig. 1. The positions of closed circular (CCC) DNA and linear viral DNA are indicated on the left. (b, c) Expression of OvHV-2 genes was assayed as described in Fig. 1, using labelled cDNA generated from cells grown in the absence of treatment (b) or in the presence of 5-azacytidine (c). For display, the intensity values set for black were 16,000 (b) and 1,000 (c)

predominantly closed circular viral genomes before treatment. After doxorubicin treatment a greater proportion of viral genomes was observed in the linear conformation with an increase in the ratio of linear to circular DNA of more than 7-fold (Fig. 2a). Furthermore, while the expression of most OvHV-2 unique genes did not change significantly after doxorubicin treatment of BJ1035, the expression of Ov3 was clearly increased and

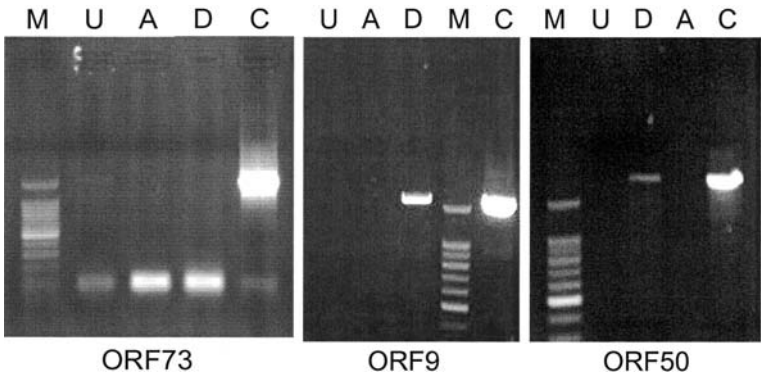

Fig. 4 RT-PCR detection of transcription from the ORF73, ORF9 and ORF50 genes. Total RNA samples from untreated BJ1035 cells (U) and BJ1035 treated with doxorubicin (D) or Azacytidine (A) were treated with RNAse-free DNAse, reverse-transcribed and subjected to RT-PCR using the primer sets in Table 1. PCR products from the cDNA samples or from positive control reactions (C) and a 100 base-pair ladder (Promega, Madison, WI) size markers (M) were electrophoresed in 1% agarose gels and stained with ethidium bromide

the ORF50 transcript was detected for the first time (Fig. 2c). The induction of expression of the productive cycle genes ORF9 and ORF50 by doxorubicin was further confirmed by RT-PCR (Fig. 4). Thus, as in sheep PBMCs, doxorubicin treatment of infected cattle T cells had effects on genome conformation and gene expression that indicated a shift towards the productive cycle.

5-Azacytidine restricts virus gene expression in infected T cell lines

In addition to doxorubicin, we investigated the effect of 5-azacytidine on the cattle and rabbit virus infected T cell lines, as this drug is known to have profound effects on EBV gene expression in latently-infected cell lines. After treatment of BJ1035 cells with 5-azacytidine, the cells were viable and sub-cultured similarly to untreated cultures indicating that their multiplication rate was similar to that of untreated cells. Gardella gel analysis showed that there was no significant increase in the amount of linear viral DNA (data not shown). However, there was a clear reduction in the transcription of the OvHV-2 genes under study. After 5-azacytidine treatment, only Ov2.5 and Ov4.5 mRNA could be detected and their hybridization signals were much lower than in untreated cells (Fig. 2d). In addition, the treatment did not induce the expression of key productive cycle genes such as ORF9 and ORF50 (Fig. 4). This demonstrated that 5-azacytidine did not induce changes in virus genome conformation or gene expression indicative of the productive virus life cycle in the infected cattle T cell line. Indeed, the highly restricted expression of only Ov2.5 and Ov4.5 mRNA in treated cells suggests that these might be transcripts specific to a restricted form of latency. However, expression of the LANA homologue ORF73 could not be detected by RT-PCR after 5-azacytidine treatment (Fig. 4).

In a final experiment, the rabbit cell line BJ2586 was treated with 5-azacytidine. Gardella gel analysis revealed a dramatic shift in the conformation of the viral genome with a clear shift from linear to closed circular viral genomes (Fig. 3a). Furthermore, transcription of all unique genes was reduced to undetectable levels in BJ2586 after 5-azacytidine treatment (Fig. 3c). A similar result was obtained with sheep PBMCs (not shown).

Discussion

Until recently, studies on the biology of OvHV-2 have been difficult due to the lack of sequence data and the lack of a tissue culture system for propagating virus. Our recent completion of the DNA sequence and ability to grow OvHV-2-positive T cells in culture has opened up the study of OvHV-2 molecular virology and pathogenesis. The pathogenesis of the disease *in vivo* is associated with the infection of T cells. The T cell lines grown *in vitro* from diseased animals represent these virus-infected T cells *in vivo* in MCF and are invaluable for determining the interaction of virus and host genes responsible for the T cell dysregulation that is likely to be a key event in the distinct pathogenesis of MCF. In addition, the study of other γ-herpesviruses such as KSHV and MHV-68 has shown that the encoded functions of virus ''unique'' genes with no obvious homologues in other γ-herpesviruses are responsible for aspects of specific pathogenesis in host species. The aim of this study was to analyze the virus life cycle and expression of selected OvHV-2 genes, including the unique genes, in lymphocytes of the natural reservoir host, sheep, and in infected T cells derived from MCF-affected susceptible cattle and rabbit species. We have now defined important aspects of the putative latent and productive OvHV-2 life cycle and differences between

infected cells of the reservoir and susceptible species. In sheep PBMCs the OvHV-2 genome was in a latent conformation and ORF73 and Ov3.5 were transcribed. Doxorubicin treatment resulted in the appearance of linear viral DNA and a significant increase in viral gene transcription, including productive cycle genes. This indicates the effectiveness of doxorubicin in driving the productive cycle in OvHV-2-infected ovine cells and strongly suggests that the virus was in a predominantly latent state in untreated cells. In contrast, cultured T cells from diseased cattle and rabbits supported both circular and linear genome conformations indicating a mixture of latent and productive cycle virus. Most of the OvHV-2 unique genes were transcribed in these cells but the productive cycle genes ORF50 and ORF9 were only expressed after doxorubicin treatment. Thus, the transcription pattern in these infected T cell lines was unusual and did not appear to fit that of a classical γ-herpesvirus latency or productive cycle. This was in spite of the suggestion from the Gardella analysis that there was a mixture of latently- and productively-infected cells in both lines. An unexpected finding was that 5-azacytidine treatment inhibited the expression of most of the OvHV-2 unique genes except Ov2.5 (vIL-10) and Ov4.5 (Bcl-2 homologue) in the cattle cell line.

Latency and reactivation are key features of herpesvirus biology in relation to pathogenesis. Gamma-herpesviruses characteristically become latent in lymphocytes and their reactivation has evolved to depend on cellular transcription factors that relate to those cell types. OvHV-2-associated disease is believed to be the result of a non-productive infection of T lymphocytes but spread from host to host involves virion production. In a way analogous to viruses such as EBV, the only *in vitro* sources for the study of OvHV-2 are cultured lymphocytes. The ability to study latent and productive infection thus depends on our ability to manipulate virus-positive T cells. Agents such as TPA which are known to reactivate EBV and KSHV were ineffective. Study of the OvHV-2 productive cycle was, however, achieved by identifying doxorubicin as a chemical stimulus for the induction of a linear viral genome conformation and gene expression characteristic of the productive virus cycle (ORF9 and ORF50). Doxorubicin is also effective in inducing EBV productive replication [20]. Although markers of the productive cycle (linear DNA and productive cycle gene expression) were induced in infected cattle and rabbit T cell lines, we were unable to detect virions in the cell-free supernates or intracellularly by either *in vitro* infection of target cells or by electron microscopy even after doxorubicin treatment (data not shown). This strongly implies that the productive cycle in these cells is partial, being largely abortive. Virus particles can be produced by these cells as they transmit disease to naïve animals [13, 14, 28] and capsids have also been seen [18] but these are rare.

We have demonstrated that 5-azacytidine inhibited the productive phase of the OvHV-2 life cycle and the expression of most of the unique OvHV-2 genes in the cattle and rabbit T cell lines. 5-Azacytidine is a DNA demethylating agent which has differing effects on herpesvirus gene expression. It has been shown to stimulate re-activation from latency in alphaherpesvirus saimiri (saimirine herpesvirus-1; [25]) and in a variety of EBV-infected epithelial cell lines, but not in EBV-infected lymphoblastoid cell lines [20–22, 29, 30]. It has also been shown to alter the pattern of EBV gene expression from the more restricted type I latency to type III in Burkitt's lymphoma cells lines by demethylation of the methylation sensitive Cp promoter element [31]. Hence, the effects of 5-azacytidine are not predictable, but in OvHV-2-infected LGL T cell lines it appeared to drive the virus life cycle towards latency, with a far more restricted pattern of gene expression being induced. This shift toward latency was particularly evident in the rabbit cell line, which also showed clear changes in the conformation of the viral genome after 5-azacytidine treatment.

These studies provide key insights into the differences in OvHV-2 gene expression in lymphocytes from reservoir and susceptible species and, for the first time, an *in vitro* system for detailed studies of the latent and productive phases of the OvHV-2 virus life cycle. Furthermore, we have shown that LGL T cells from MCF-affected animals expressed many of the OvHV-2 unique genes. Our hypothesis is that expression of OvHV-2 unique genes could be involved in driving the distinct pathogenesis of MCF. This study provides a platform for further research to identify the gene transcription patterns associated with OvHV-2 latency and reactivation *in vivo* and to determine their contribution to T cell transformation and the molecular pathogenesis of MCF in susceptible species.

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