High levels of retroperitoneal fibromatosis (RF)-associated herpesvirus in RF lesions in macaques are associated with ORF73 LANA expression in spindleoid tumour cells

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INTRODUCTION
An unusual multifocal fibroproliferative syndrome in macaques was first recognized at the University of Washington National Primate Research Center (WaNPRC) in 1976 (Giddens et al., 1985) and was subsequently identified in other primate centres across the USA (Marx & Lowenstine, 1987). Based on the anatomical location of the lesions, they were designated retroperitoneal (RF) or subcutaneous (SF) fibromatosis, collectively referred to herein as the RF syndrome (Giddens et al., 1985; Tsai, 1993). This fibroproliferative syndrome was associated with a form of simian acquired immunodeficiency syndrome (SAIDS) caused by infection with simian D-type retrovirus-2 (SRV-2) (Shiigi et al., 1986; Tsai, 1993). Morphological and histochemical analysis of the characteristic spindleoid tumour cells in RF lesions revealed many similarities with Kaposi’s sarcoma (KS), a multifocal vascular proliferative disease often associated with human immunodeficiency virus (HIV) infection and AIDS in humans (London et al., 1983; Tsai et al., 1985). The multifocal nature of both lesions, the common occurrence of proliferating spindle-shaped mesenchymal cells and the association with an acquired immune deficiency syndrome caused by retroviral infection suggested a common aetiology (Marx & Lowenstine, 1987). Although similar, notable differences between RF and KS include the increased vascularity and haemorrhagic nature of KS lesions and the substantial fibrosis seen in RF lesions (Boshoff & Weiss, 2001; Tsai, 1993).

In 1994, the KS-associated herpesvirus (KSHV; Human herpesvirus 8) was first identified in KS lesions in humans and subsequently identified in RF lesions in macaques (Bruce et al., 2002). This similarity in aetiology led to the hypothesis that RFHV (retroperitoneal fibromatosis herpesvirus) could be responsible for the RF syndrome. However, recent studies have questioned the role of RFHV in the pathogenesis of RF, with some findings suggesting that RFHV is not a major contributor to RF pathogenesis (Gaynor et al., 2013; Xia et al., 2014). This study aimed to investigate the role of RFHV in RF lesions and to determine if RFHV is a major contributor to the pathogenesis of RF.

Two distinct lineages of rhadinoviruses related to Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV; Human herpesvirus 8), the causative agent of KS, have been identified. In macaques, the RV1 lineage is represented by retroperitoneal fibromatosis (RF) herpesvirus (RFHV), the homologue of KSHV, whilst the RV2 lineage is represented by rhesus rhadinovirus (RRV), a more distantly related virus. Real-time quantitative PCR was used to estimate the loads of RV1 and RV2 rhadinoviruses in simian acquired immunodeficiency syndrome-associated RF (SAIDS-RF), a neoplasm of macaques with similarities to AIDS-associated KS. Both RV1 and RV2 rhadinoviruses were detected in macaques with RF. The RV1 loads were 220- to 4300-fold higher in RF tumours than in spleen, showing a strong tumour association (mean loads of 1 800 000 vs 2900 copies per 10^6 cells in tumours and spleen, respectively). In contrast, RV2 loads in the RF tumours were 100-fold lower than RV1 loads and showed similar levels in tumours and spleen (mean loads of 16 000 vs 24 000 copies per 10^6 cells, respectively). Immunostaining with antibodies reactive against RFHV ORF73 latency-associated nuclear antigen (LANA) showed intense nuclear staining of the spindleoid RF tumour cells. Correlation of viral load and the number of LANA-positive cells indicated that RF tumour cells contained multiple copies of the RFHV genome per cell. This pattern of infectivity is similar to that seen in KS tumours latently infected with KSHV. Our study demonstrates similarities in the biology of KSHV and RFHV and supports a role for RFHV in the aetiology of SAIDS-RF.
HIV-infected individuals with AIDS (Chang et al., 1994). KSHV DNA was detected in essentially all of the spindleoid-shaped tumour cells of endothelial origin, which are characteristic of KS lesions (Boshoff et al., 1995). The vast majority of these cells were latently infected with KSHV, with <1% of cells undergoing lytic activation and virus replication. KSHV latency was associated with the expression of a restricted set of latency-associated genes (Kedes et al., 1997), including ORF73, the major latency-associated nuclear antigen (LANA) (Rainbow et al., 1997). Epidemiological studies have demonstrated that KSHV is implicated strongly in the aetiology of two other AIDS-associated diseases, multicentric Castleman’s disease and is implicated strongly in the aetiology of two other AIDS-associated diseases, multicentric Castleman’s disease and is implicated strongly in the aetiology of two other diseases (et al., 1995).

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We previously reported the presence of RFHV DNA in archival RF lesions from SRV-2-infected macaques, suggesting a role for RFHV in the aetiology of SAIDS-associated RF (SAIDS-RF) (Rose et al., 1997). We recently cloned and sequenced the ORF73 LANA of RFHV and determined that the LN53 monoclonal antibody used to detect KSHV ORF73 LANA in KS tumour cells also reacts with RFHV LANA (Burnside et al., 2006). This antibody is strongly reactive with recombinant RFHV LANA in Western blot and immunofluorescence analyses and does not cross-react with recombinant ORF73 LANA homologues of the related RV2 rhadinoviruses, RRV and MneRV2. In the present study, we used real-time quantitative PCR (QPCR) assays for differential quantitation of the loads of macaque RV1 and RV2 rhadinoviruses in tumour and non-tumour tissues from a number of cases of macaques diagnosed with RF or SF. Our studies revealed high viral loads and a strong RF tumour association with the macaque RV1 rhadinovirus, RFHV. Immunohistochemical analysis using the LN53 antibody revealed that the majority of RF spindleoid tumour cells were latently infected with RFHV and expressed the RFHV ORF73 LANA. These findings mirror the prevalence and expression of KSHV in KS tumours.

**METHODS**

**Macaque RF cases.** The characteristics of the macaques are summarized in Table 1. One group of pig-tailed macaques (M. nemestrina) included RF1, RF2, RF3 and RF6 and had been diagnosed with SF and/or RF at the WaNPRC in the 1970s and 1980s. RF1 was a 2.8-year-old colony-born female that developed multiple fibromatous nodules in the subcutaneous tissues on the toes of the left foot and leg, but had no detectable tumour masses in the visceral cavities [animal 1 of Tsai et al. (1985)]. RF2 was a 5.8-year-old colony-born female breeder that developed multiple fibromatous nodules in subcutaneous tissues in the thigh and abdominal wall and non-fibrotic, carcinoma-like neoplasms in the liver, kidney and lung [animal 2 of Tsai et al. (1985)]. RF3 was a 3.7-year-old colony-born male that developed subcutaneous fibromatous nodules in the lower back, neck, groin, abdomen and chest. RF6 was a 2.2-year-old colony-born male that developed subcutaneous and retroperitoneal fibromatosis. Routine serological and co-culture assays had revealed the presence of SRV-2 infection in all of these animals. Archived frozen or formalin-fixed, paraffin-embedded RF tumour and non-tumour spleen tissues from these macaques were utilized in this study, as indicated in Table 1.

Another RF case was obtained from R. Shibata while at the National Institutes of Health, Bethesda, MD, USA. This pig-tailed macaque, RF4, had been infected experimentally in 1996 with a pathogenic simian immunodeficiency virus (SIV)-HIV chimeric virus (SHIV), strain, MD14-RQ. Although RF4 was asymptomatic for some time after SHIV inoculation, it rapidly developed disseminated nodular fibromatosis between weeks 23 and 24 post-inoculation and was euthanized during week 24 (Shibata et al., 1997). Fresh-frozen RF tumour and non-tumour spleen, brain, lung and bone marrow tissue from this macaque were obtained for analysis.

Also included in the present study was a recent case of an SRV-2-negative rhesus macaque, RF5, which had been infected experimentally with a lentivirus, strain SIVsmE660. Although RF5 remained clinically healthy for 3 years following SIV challenge, persistent viraemia and declining peripheral blood CD4+ T cells were seen as SAIDS-defining. Upon development of acute, severe disease symptoms due to intestinal neoplasms, euthanasia was elected. Necropsy revealed the presence of numerous stromal tumours in the submucosa of the distal colon, which were diagnosed histopathologically as RF-like (Bielefeldt-Ohmann et al., 2003). Formalin-fixed, paraffin-embedded RF tumour and fresh-frozen non-tumour spleen tissue from this animal were obtained courtesy of N. Letvin and D. Barouch (Harvard Medical School, MA, USA).
To obtain the ORF7/8 junctional region of MneRV2, PCR primers were derived from the 3’ end of the ORF7 gene and the 5’ end of the ORF8 gene of RRV strain 17577 (GenBank accession no. NC_003401). These primers, DAPLa and EELSb (Table 2), were used in PCR amplifications of DNA template from spleen tissue of Mne442N. A PCR product was obtained from RF tumour tissue of Mne442N (M. nemestrina) and MmuYN91-224 (M. mulatta), which contained RFHV M and RFHV Mm, respectively (essentially as described by Bruce et al., 2005). Specific PCR fragments were obtained and sequenced. A BLAST search of the NCBI DNA database showed a close similarity between these sequences and the ORF7/8 junctional region of the KSHV genome, indicating that the sequences were derived from the macaque RV1 rhadinoviruses RFHV M and RFHV Mm.

Real-time QPCR. Viral loads were determined using real-time QPCR with TaqMan primers and probes designed with Primer Express software (Applied Biosystems). The RV1 assay was designed to amplify a 116 bp amplicon from the ORF7/8 junctional region of macaque viruses belonging to the RV1 lineage using consensus primers derived from alignments of the ORF7/8 junctional region obtained from RFHV M and RFHV Mm: RV1a and RV1b with a TaqMan probe, RV1-FAM (Table 2; Fig. 1). The RV2 assay amplified a 71 bp amplicon using consensus primers RV2a and RV2b with a TaqMan probe, RV2-FAM (Table 2; Fig. 1), as described previously (Bruce et al., 2005). Viral copy number per cell was determined using a real-time QPCR assay targeting oncostatin M (OSM), a single-copy cellular gene. The OSM assay amplified a 76 bp ampli- cation from exon 3 of the macaque OSM gene using primers OSMa and OSMb with a TaqMan probe, OSM-FAM (Table 2), as described previously (Bruce et al., 2005).

Reactions (50 μl) contained approximately 250 ng template DNA, 1 μM forward and reverse primers, 100 nM probe, 200 μM each dNTP, 20 mM Tris/HCl (pH 8.4), 50 mM KCl and 2.5 U Platinum Taq polymerase (Invitrogen). Magnesium chloride concentrations of 2.0 mM (OSM), 1.75 mM (RV1) and 4.0 mM (RV2) were optimized for each of the three assays to give 100% efficiency (R > 0.98) at an annealing temperature of 62°C to allow all of the assays to be

A small, formalin-fixed, paraffin-embedded RF tumour sample from an SRV-2-positive, male pig-tailed macaque, RF7, was also available in the archives in amounts sufficient for immunohistochemical analysis. This macaque was a 2-year-old male with a large tumour mass and multiple nodules associated with a variety of internal tissues.

DNA extraction. Formalin-fixed, paraffin-embedded tissue was treated with xylene to remove the paraffin, followed by extensive ethanol washes. DNA was extracted from embedded and frozen tissue using standard proteinase K digestion and phenol/chloroform extraction and concentrated by ethanol precipitation.

Amplification of the ORF7/8 junctional region of the macaque RV1 and RV2 rhadinoviruses. To obtain the ORF7/8 junctional regions within the macaque RV1 rhadinoviruses RFHV M and RFHV Mm, a consensus degenerate hybrid oligonucleotide primer (CODEHOP) was designed to the amino acid motif NSKY within the C-terminal region of the KSHV ORF7, which is conserved among the ORF7 homologues of other gammaherpesviruses, essentially as described in Rose et al. (1998). An additional CODEHOP PCR primer was designed to the amino acid motif TVNC within the N-terminal region of the KSHV ORF8, which is conserved among the ORF8 homologues of other gammaherpesviruses. These primers, NSKYa and TVNCb (see Table 2), were used in PCR amplifications with DNA template obtained from RF tumour tissue of Mne442N (M. nemestrina) and MmuYN91-224 (M. mulatta), which contained RFHV M and RFHV Mm, respectively (essentially as described by Bruce et al., 2005). Specific PCR fragments were obtained and sequenced. A BLAST search of the NCBI DNA database showed a close similarity between these sequences and the ORF7/8 junctional region of the KSHV genome, indicating that the sequences were derived from the macaque RV1 rhadinoviruses RFHV M and RFHV Mm.

### Table 1. Summary of animal data

<table>
<thead>
<tr>
<th>RF</th>
<th>Animal #</th>
<th>Location</th>
<th>Species*</th>
<th>Sex</th>
<th>Age</th>
<th>Year</th>
<th>Clinical findings</th>
<th>SRV-2</th>
<th>Experimental reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF1</td>
<td>T80015</td>
<td>WaNPRC</td>
<td>Mn</td>
<td>F</td>
<td>2</td>
<td>1982</td>
<td>Subcutaneous fibrous nodules in the neck, no visceral component</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>RF2</td>
<td>M78114</td>
<td>WaNPRC</td>
<td>Mn</td>
<td>F</td>
<td>5</td>
<td>1984</td>
<td>Subcutaneous fibrous nodules on thigh and abdominal wall</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RF3</td>
<td>T81273</td>
<td>WaNPRC</td>
<td>Mn</td>
<td>F</td>
<td>2.5</td>
<td>1984</td>
<td>Subcutaneous fibrous nodules in subcutaneous tissues and in muscle and nerves</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>RF4</td>
<td>442N</td>
<td>NIH</td>
<td>Mn</td>
<td>?</td>
<td>2</td>
<td>1996</td>
<td>Multiple fibrous nodules in subcutaneous tissues and in muscle and nerves</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RF5</td>
<td>A01111</td>
<td>NENPRC/Mm</td>
<td>M</td>
<td>7</td>
<td>2001</td>
<td>Multiple fibrous nodules in mesentery, diaphragm and serosal surface of small and large intestines</td>
<td>+</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>RF6</td>
<td>T82422</td>
<td>WaNPRC</td>
<td>Mn</td>
<td>M</td>
<td>2</td>
<td>1984</td>
<td>Multiple fibrous nodules in the ileocaecal junction, omentum, mesentery, diaphragm and serosal surface of small and large intestines</td>
<td>+</td>
<td>NA</td>
</tr>
</tbody>
</table>

*M, M. nemestrina (pig-tailed macaque); Mm, M. mulatta (rhesus macaque).

NA, Not applicable.
performed simultaneously on the same plate (data not shown). After activation of the polymerase by incubation for 1 min at 95 °C, amplification was performed on a Bio-Rad iCycler equipped with an optical module for 45 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s. The copy number for each assay was calculated from the cycle threshold (Ct) using Bio-Rad software. Viral load was determined as a cellular genome copy equivalent using the formula: viral load (genome equivalent copies) = viral copy number/diploid OSM copy number. Samples were assayed in duplicate and the means determined. Standard deviations were calculated using the sum of the errors of the viral and OSM copy number determinations.

**PCR detection of SRV-2.** DNA samples from spleen and tumour tissue were tested for the presence of SRV-2 using the forward primer SRV-2-1 (5'-TCTCAATGTCATCGTGTC ACT-3') and reverse primer SRV-2-2 (5'-GATTAGAGCCAAGAATGAGT-3') to

### Table 2. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Gene target</th>
<th>Sequence (5'-3')†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV1 QPCR assay</td>
<td>RV1 ORF7</td>
<td>TTAAAGACATCTAGGCCCTCC</td>
</tr>
<tr>
<td>RV1α</td>
<td>RV1 ORF8</td>
<td>GCCACCGAGCGAGGAGCC</td>
</tr>
<tr>
<td>RV1-FAM‡</td>
<td>RV1 ORF7/8 junction</td>
<td>(FAM)-TCACCTTACGCTGGCGACCACAAT-(BHQ-1)</td>
</tr>
<tr>
<td>RV2 QPCR assay</td>
<td>RV2 ORF 60</td>
<td>TCTGAATATGTCACTCCGTTCTCA</td>
</tr>
<tr>
<td>RV2α</td>
<td>RV2 ORF 59/60 intergenic</td>
<td>GCCCGGAAATAGTAAGA</td>
</tr>
<tr>
<td>RV2-FAM‡</td>
<td>RV2 ORF 60 and 59/60 intergenic</td>
<td>(FAM)-TGATCTGACCTCCCATGTCG-(BHQ-1)</td>
</tr>
<tr>
<td>OSM QPCR assay</td>
<td>OSM exon 3 OSM</td>
<td>CCTCGGGCTCGGAACAC</td>
</tr>
<tr>
<td>OSMα</td>
<td>OSM exon 3 OSM</td>
<td>GCCCTGCTGGGCTCAG</td>
</tr>
<tr>
<td>OSM-FAM‡</td>
<td>OSM exon 3 OSM</td>
<td>(FAM)-TACTGCACTCGGAGCAGCAGACAG-(BHQ-1)</td>
</tr>
<tr>
<td>ORF 7/8 CODEHOP primers</td>
<td>Gammaherpesvirus ORF7</td>
<td>CGGTTGAAATATCNAARTAYHAA</td>
</tr>
<tr>
<td>NSKYa</td>
<td>Gammaherpesvirus ORF8</td>
<td>AACATGTCTAAATCTCGATTTNATNGT</td>
</tr>
<tr>
<td>TVNCb</td>
<td>OSM exon 3 OSM</td>
<td>CCTCGGGCGACAC</td>
</tr>
<tr>
<td>Gene-specific primer</td>
<td>RRV ORF7</td>
<td>AGCAAGACGCGGCGCTCGTTC</td>
</tr>
<tr>
<td>EELsβ</td>
<td>RRV ORF8</td>
<td>GAGAGCTCCTCCAGCAC</td>
</tr>
</tbody>
</table>

*Sense and antisense primers are indicated by ‘α’ and ‘β’, respectively.
†IUB code for ambiguous nucleotides: R = A or G; Y = C or T; H = A or C or T (not G); N = A, C, G or T.
‡The probe was a TaqMan dual-labelled probe with the fluorescent dye 6-carboxyfluorescein (6-FAM) at the 5' end and the 'black hole quencher (BHQ) dye at the 3' end.

Fig. 1. Primer location and specificity of the macaque RV1 QPCR assay. Corresponding sequences from the ORF7/8 junctional region of various primate rhadinoviruses were aligned. Rhadinovirus species and lineages are indicated. The primer set and probe were designed from the RFHVMn and RFHVMm sequences. The RV1α primer and RV1-FAM probe were designed from the sense strand, as shown, whilst the RV1b primer was derived from the antisense strand. The alignment shows the mismatches between the primer and probe sequences and the aligned RV1 and RV2 rhadinovirus sequences. Dots represent residues identical to those in the RFHVMn sequence and highlight the similarity of the primer sequences within the macaque RV1 rhadinoviruses and the dissimilarity with members of the RV2 lineage of rhadinoviruses.

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produce a 798 bp amplicon (Wang & Thouless, 1996). Reactions (50 μl) contained approximately 250 ng template DNA, 1 μM forward and reverse primers, 200 μM each dNTP, 20 mM Tris/HCl (pH 8-4), 50 mM KCl and 2-5 U Platinum Taq polymerase (Invitrogen). After activation of the polymerase by incubation for 1 min at 95°C, reactions were run for 45 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s. Products were analysed by electrophoresis on a 2 % agarose/TAE gel and stained with ethidium bromide.

**Immunohistochemistry.** Tissues fixed in 10 % neutral-buffered formaldehyde and embedded in paraffin were used after storage for 1–5–18 years. For immunolabelling of viral antigens, deparaffinized sections of the formaldehyde-fixed tissues were subjected to antigen-retrieval by boiling in 0-1 mM EDTA (pH 8-0) for 15 min, as described previously (Bielefeldt-Ohmann et al., 2005). Following a blocking step in Tris-buffered saline containing 0-05 % Tween 20, 1 % BSA and 1 % normal goat and horse serum, sections were incubated for 1–2 h at room temperature with a 1:800 dilution of a rat monoclonal antibody raised against the ORF73 LANA of KSHV (clone LN53; Advanced Biotechnologies). Bound antibody was visualized by reaction with biotinylated anti-rat antibody, followed by incubation with preformed avidin–biotin–horseradish peroxidase conjugates (Vector Laboratories) and finally diaminobenzidine or 3-amino-9-ethyl-carbazole as chromogen. Sections were counter-stained with Harry’s haematoxylin and examined in a Nikon Eclipse E600 microscope. Microphotographs were taken using a Nikon Coolpix 5300 camera and prepared for publication using Adobe Photoshop (CS).

**RESULTS**

**Development of real-time QPCR assays for differential quantitation of viral loads of macaque rhadinoviruses**

The similarities between SAIDS-RF syndrome in macaques and AIDS-associated KS (AIDS-KS) in humans suggest that the KSHV-related macaque RV1 and RV2 rhadinoviruses may play a role in the aetiology of the macaque tumour. TaqMan QPCR assays were developed to quantitate the levels of the macaque RV1 and RV2 rhadinoviruses. The macaque RV1 assay was designed to identify RFHV variants from different macaque species, including RFHVMen and RFHVMMm. Sequences of the ORF7/8 junctional region of macaque RV1 (RFHVMen and RFHVMMm) and RV2 (MneRV2 and RV2) rhadinoviruses were obtained as described in Methods. Multiple alignments of the sequences revealed conserved regions of strong sequence similarity between the macaque RV1 sequences. Consensus PCR amplification primers (RV1a and RV1b) and a TaqMan real-time fluorescent probe (RV1-FAM) were derived from sequences within the regions conserved between the macaque RV1 sequences that were not conserved in the macaque RV2 sequences using Primer Express software (Fig. 1). The resulting PCR assay produced a 116 bp amplicon.

We previously developed an RV2 QPCR assay to identify RRV, MneRV2 and RV2 rhadinoviruses from other macaque species using consensus primers derived from the 3′ end of the ORF60 gene and the ORF59/60 intergenic region (Bruce et al., 2005). The primer and probe sequences for this assay were chosen from regions that were conserved within the macaque RV2 rhadinoviruses and not conserved with the macaque RV1 rhadinovirus sequences. We also developed a TaqMan QPCR assay for the cellular OSM gene to determine cell number in primate tissue (Bruce et al., 2005). The conditions for amplification in the RV1 QPCR assay were optimized to allow simultaneous amplification of the RV1, RV2 and OSM QPCR assays.

A standard curve was obtained from DNA of an RF tumour sample (RF2) that contained high amounts of RFHVMen DNA (approx. two viral genomes per cell). A series of 4-fold dilutions of the tumour DNA was prepared using cellular DNA from a non-infected source to keep the DNA concentration constant. As seen in Fig. 2, the assay was linear across a range of dilutions from less than 50 to more than 10 000 copies of RFHVMen, with a slope of −3·321 (100 % efficiency) and $r^2=0.99$. Similar efficiencies and $r^2$ values were obtained for the RV2 and OSM QPCR assays (Bruce et al., 2005). To ensure that the RV1 assay did not detect RV2 rhadinoviruses, the assay was performed on DNA from the RRV ORF7/8 junctional region. No cross-reaction was detected, even in the presence of $10^{11}$ copies of an RRV PCR product from the ORF7/ORF8 region. Due to nucleotide differences between the ORF7/8 junctional regions of RHIV and KSHV, no cross-reaction was detected using the macaque RV1 QPCR assay on DNA obtained from KSHV-infected BCBL (body cavity-based lymphoma) cells. The RV1 QPCR assay, like the RV2 and OSM assays, was designed to target a small gene fragment to allow sensitive quantification, even with archived, formalin-fixed tissue. This allowed the viral load to be determined in different types of tissue samples with varying amounts and quality of DNA.

![Fig. 2. Standard curve obtained for the macaque RV1 rhadinovirus assay. A standard RV1 assay was performed on a series of 4-fold dilutions of DNA from the RF tumour sample of macaque RF2 diluted in DNA from a non-infected macaque (Table 1) over the range of 10 copies to $10^6$ copies of RV1 DNA (slope = $-3·321$, 100 % efficiency; $r^2=0.99$).](http://vir.sgmjournals.org)
Real-time QPCR of macaque rhadinoviruses

In a search of the tissue archives of the WaNPRC, six macaques with SAIDS-RF/SF were identified in which both normal spleen and RF tumour tissues were available (Table 1). These samples were either formalin-fixed, paraffin-embedded tissue blocks or fresh-frozen tissue that had been stored for up to 20 years. DNA was extracted from these samples and analysed by QPCR for the presence of RV1 and RV2 rhadinoviruses. Viral load (viral genome copies per 10^6 cells) was determined by comparison of the virus and OSM levels in duplicate samples.

Using the RV1 QPCR assay, DNA from the RV1 rhadinovirus RFHV was detected in the RF lesions of all six SAIDS-RF macaques (Table 3 and Fig. 3a). In each macaque, the RV1 load in the RF tumour was significantly higher than in the spleen tissue. The levels of RV1 DNA in the spleen tissue varied from undetectable (RF6) to 8700 (RF4) copies per 10^6 cells, with a mean of 2900 (Fig. 3a). The levels of RV1 DNA in the tumour-bearing RF tissue ranged from 700 (RF6) to 3,400,000 (RF2) copies per 10^6 cells, with a mean of 1,800,000. The RV1 loads were 220- to 4,400-fold higher in the tumour tissue than in the paired normal spleen tissue, with a mean of 1200, showing a strong tumour association (Fig. 4). Several non-tumour tissue samples, in addition to spleen, were available for analysis, including brain, lung and bone marrow for RF4, and brain, lung and muscle for RF6. The levels of RV1 in these normal tissues were all below the limit of detection of the QPCR assay (Table 3).

Using the RV2 QPCR assay, RV2 DNA was also detected in all six macaques (Fig. 3b). In contrast to RV1, however, the RV2 loads were all higher in the spleen than in the paired RF tumour-bearing tissue, with the exception of RF2, which had a very low viral load in the spleen (Fig. 4). The loads of RV2 DNA in the spleen ranged from 1900 (RF2) to 66,000 (RF4) copies per 10^6 cells with a mean of 24,000 (Table 3 and Fig. 3). In the tumour-bearing RF tissue, the RV2 DNA levels ranged from 91 (RF6) to 43,000 (RF4) copies per 10^6 cells, with a mean of 16,000. Quantification of RV2 rhadinovirus in the additional non-affected normal tissues from macaques RF4 and RF6, including lung, brain, muscle and bone marrow, yielded low viral loads ranging from 120 (bone marrow) to 26,000 (lung) copies per 10^6 cells, similar to that seen in the spleen and tumour tissues (Table 3).

A comparison of the RV1 and RV2 viral loads showed that the RV1 loads were significantly higher than the RV2 loads in all of the RF tumour tissues tested. The RV1 loads were on average 1120-fold higher than the RV2 levels in the RF tumour tissue with a range of 8- (RF6) to 4700 (RF3)-fold higher (Table 3). The opposite was true in the normal spleen tissue where the RV1 load averaged only one-third of the RV2 load, with the RV1/RV2 ratios ranging from <0.0002 (RF6) to 1.7 (RF2) (Table 3). This translated into a mean 3400-fold increase in the ratio of RV1 to RV2 in the tumour tissue when compared with the non-tumour spleen tissue.

Table 3. Quantification of viral load

Results for RF tumours are shown in bold. Virus loads in parentheses indicate that the QPCR was negative and the limit of detection of the assay is given. Virus ratios in parentheses were calculated using the limit of detection when the QPCR was negative.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sample type</th>
<th>Tissue</th>
<th>Virus load (genome equivalent copies per 10^6 cells)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>RV1</td>
</tr>
<tr>
<td>RF1</td>
<td>Formalin/paraffin</td>
<td>RF tumour</td>
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<tr>
<td>RF2</td>
<td>Frozen</td>
<td>RF tumour</td>
<td>3,400,000</td>
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<tr>
<td>RF3</td>
<td>Formalin/paraffin</td>
<td>Spleen</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Formalin/paraffin</td>
<td>RF tumour</td>
<td>840,000</td>
</tr>
<tr>
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<td>Frozen</td>
<td>Lung</td>
<td>(&lt;520)</td>
</tr>
<tr>
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<td>RF tumour</td>
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<td>RF tumour</td>
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<tr>
<td></td>
<td>Frozen</td>
<td>Muscle</td>
<td>(&lt;41)</td>
</tr>
</tbody>
</table>
SRV-2 PCR analysis

As previous studies during the RF epidemic in the 1970s and 1980s identified a strong correlation between SRV-2 infection and the occurrence of RF (Benveniste et al., 1986; Marx et al., 1985), we determined the SRV-2 status of the SAIDS-RF macaques in this study by PCR. As shown in Table 1, macaques RF1, RF2, RF3, RF6 and RF7 were positive for SRV-2 DNA in the tissue samples, confirming previous serological analysis. Macaque RF4, which had been experimentally infected with the SHIV (MD14RQ) chimera, also contained SRV-2 viral DNA in tumour and spleen tissue, demonstrating the existence of a previously undetected infection in this animal. Macaque RF5, which had been experimentally infected with SIV, was negative in this and previous PCR assays (Bielefeldt-Ohmann et al., 2005). Thus, each macaque RF case was associated with a retroviral and/or lentiviral co-infection.

Immunohistochemical localization of RFHV LANA in RF lesions

As high loads of RV1 rhadinovirus RFHV were detected in the RF tumour lesions by QPCR, we examined the RF lesions for the presence of RFHV antigens by immunohistochemistry. We employed a rat monoclonal antibody specific to a glutamic acid-rich motif that is repeated 15 times within the ORF73 LANA of KSHV (Kellam et al., 1999). We have shown that this monoclonal antibody is strongly reactive with the ORF73 LANA homologue of RFHV and is unreactive with the ORF73 LANA homologues of the macaque RV2 rhadinoviruses RRV and MneRV2. This has been determined in both Western blot and immunofluorescence analyses of recombinantly expressed protein (Burnside et al., 2006). Tissue sections from different types of RF and SF tumour lesions were subjected to immunostaining using the LN53 anti-LANA monoclonal antibody. Strong nuclear staining was detected in the vast majority of the spindleoid tumour cells in all of the lesions tested. Fig. 5(a) shows LANA staining in the spindleoid tumour cells of an RF lesion on the surface of the diaphragm of RF7, whilst cells within the normal muscle layers of the diaphragm were negative. Distinctive nuclear LANA staining was also detected in the spindleoid tumour cells within the SF skin lesion of RF2 (Fig. 5b). Multinucleated giant cells were consistently associated with LANA staining (Fig. 5b). Lymphocyte aggregates, also present in the RF lesions, were unreactive with the anti-LANA antibody, with the exception of an occasional cell. Staining in the nuclei of spindleoid tumour cells was also detected in the carcinoma-like neoplasms of liver and lung from RF2, which also had more typical fibromatosis lesions in the abdominal cavity and skin (data not shown).

Analysis of several sections of a submucosal tumour from RF5 revealed intense nuclear staining of RF tumour cells. Vascularization was sparse to moderate and LANA staining
of vascular endothelial cells was not evident (Fig. 5c, d, arrows). The mucosa overlying the tumour masses within the distal colon showed extensive necrosis. LANA staining was limited to the nuclei of spindleoid cells within the tumour mass and occasional cells within the lamina muscularis mucosa and lamina propria (Fig. 5c, e). Enterocytes within the adjacent crypts were not reactive with the anti-LANA antibody. Higher magnifications of cells within the RF lesions revealed a mixture of LANA-positive and -negative cells (Fig. 5d, f), with positive cells showing a distinct nuclear localization of the LANA antigen.

**DISCUSSION**

We developed a TaqMan QPCR assay to quantitate the load of the macaque RV1 rhadinovirus RFHV within RF tumour and non-tumour tissues in order to characterize the association between RFHV and the RF syndrome in macaques with SAIDS. We determined that RF tumour tissues contained significantly elevated levels of RFHV compared with non-tumour tissues, independent of macaque species, SRV-2/SIV status or tumour location. A mean load of 1 800 000 RFHV copies per 10^6 cells was detected in tumour lesions compared with 2900 copies per 10^6 cells in spleen tissue. When RV1 loads were compared between the tumour and spleen pairs from the different macaques, a mean 1200-fold increase in the RFHV load was detected in the RF lesions over that seen in spleen, indicating a strong association between the presence of virus and tumour. The absence of detectable RFHV in other non-involved tissue samples from the SAIDS-RF macaques, including brain, lung, bone marrow and muscle, further supports the tumour association.

Using the LN53 anti-KSHV LANA monoclonal antibody, we showed that RFHV ORF73 LANA was strongly expressed in the spindleoid tumour cells of a variety of different RF and SF lesions from SAIDS macaques. We have previously shown that this antibody reacts strongly with recombinant RFHV LANA in both Western blot and immunofluorescence analyses of transfected Cos-7 cells (Burnside et al., 2006). Tumor lesions from the subcutis, the serosa of abdominal organs and the submucosa of colon and rectum, as well as carcinoma-like neoplasms from liver and lung of macaques diagnosed with RF, all showed a characteristic intense nuclear staining with the anti-LANA antibody similar to the staining seen in KS lesions. The antibody reacted essentially with all of the spindle-shaped tumour cells present in the RF lesions, whilst staining was absent from the cytoplasm of the tumour cells and from the cells in the normal tissue surrounding the tumour lesions. In contrast to the reactivity detected in KS lesions, no staining was detected in vascular endothelial cells.

The high percentage of RF tumour cells stained by the anti-LANA antibody (>90%) correlates with our QPCR data, which detected on average approximately two RFHV genomes per cell in RF tumour-bearing tissue. As not all of the cells within the tumour lesions were spindleoid

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**Fig. 5.** Immunohistochemical localization of RFHV LANA in typical RF lesions. Formalin-fixed, paraffin-embedded tissue sections were stained with the LN53 rat anti-KSHV ORF73 LANA antibody. (a) Anti-LANA staining in spindleoid tumour cells in an early RF lesion (rf) on the diaphragm (d) of RF7. Bar, 240 μm. (b) Anti-LANA staining in spindleoid and multinucleated giant cells (arrow) in an SF lesion of RF2. la, Lymphocyte-aggregate within the lesion. Bar, 120 μm. (c–e) Anti-LANA staining of spindleoid cells in a colonic submucosal tumour of RF5. Arrows in (c) and (d) indicate lack of anti-LANA staining of blood vessel endothelial cells within and adjacent to RF lesions. With the exception of an occasional cell, anti-LANA staining was also lacking in fibroblasts and other cells in the lamina muscularis mucosa (lm) and in the enterocytes within the intestinal crypts (cr) seen in (e). Bars, 200 μm (c), 80 μm (d), 240 μm (e). (f) Higher magnification of anti-LANA staining showing obvious nuclear localization (arrows) within spindleoid tumour cells. Bar, 30 μm.
tumour cells, the load of RFHV within infected cells could be greater. We detected infrequent LANA staining within lymphocytes present in the different RF lesions. This correlates with the weak but positive LANA staining that we detected previously in a small percentage of lymphoid cells in the interfollicular areas of the ileocaecal lymph node of one of the RF macaques (Bielefeldt-Ohmann et al., 2005). These results suggest that the low levels of RFHV detected in spleen are due to the presence of latently infected B cells, as is seen with KSHV.

The anti-LANA staining detected in RF tumour cells was not due to the low levels of RV2 rhadinovirus detected in our QPCR assay. Sequence analysis of the LANA homologues of the RV2 rhadinoviruses RRV (Searles et al., 1999) and MneRV2 (Burnside et al., 2006) has shown that these proteins lack both the extended repetitive internal region and the repeated glutamic acid-rich motifs that form the epitope of the anti-KSHV LANA monoclonal antibody. We have produced recombinant MneRV2 and RRV ORF73 LANA proteins in transfected Cos-7 cells and shown that these proteins are unreactive with the anti-KSHV LANA antibody in Western blot and immunofluorescence analyses (Burnside et al., 2006). More importantly, the RV2 loads found in the RF lesions did not correlate with the number of cells stained with the LN53 antibody. RV2 loads of only 91–43 000 per 10⁶ cells were detected in the RF tumours, with three of the macaques having virus levels just above the level of detection. Even if each cell contained a single viral genome, only 0-009–4% of the cells within the tumour lesion could be infected with an RV2 rhadinovirus, whilst >90% of the tumour cells were LANA-positive. The RV2 load in the tumour could be due to infiltration of B lymphocytes, which are known to serve as a reservoir for latent RV2 rhadinovirus infection (Bergquam et al., 1999). Lymphocytes and lymphocyte aggregates are a common feature of RF lesions and constitute a large proportion of the cell population of the spleen. Thus, the RV2 load we detected in both the RF lesions and spleen tissue could be due to the presence of latently infected B lymphocytes. Confirmation of this awaits development of antibody reagents specific for RV2 LANA antigens.

KSHV is now recognized as the primary and necessary factor in the development of KS. Our results suggest that RFHV could play a similar role in the aetiology of RF. In both KS and RF, immunosuppression of the host is an important cofactor in the development and maintenance of the disease state. Immunosuppression caused by HIV lentivirus infection correlates with AIDS-KS and immunosuppression caused by infection with D-type simian retroviruses correlates with SAIDS-RF (Marx et al., 1985). In contrast, SIV lentivirus-associated immunodeficiency has not been associated with RF, despite the large numbers of macaques that have been infected with SIV/SHIV in AIDS studies at the various primate centres within the USA. Only two cases of SRV-2-negative, SIV-positive RF have been identified at the WaNPRC. One of these animals, RF5, developed RF-like lesions after a long-term (2.5 year) experimental infection with SIVsmE660 in the absence of a detectable infection with any D-type SRV variant (Bielefeldt-Ohmann et al., 2005). It is possible that the slow immunodeficiency progression in this animal, in combination with an underlying RFHV infection, was sufficient for development of RF. The lack of lentivirus-associated RF cases in other SIV cases may be due to a low prevalence of RFHV infection in the current primate colonies or to the normal rapid progression of disease with SIV. Further work is needed to dissect the role of lentiviruses, retroviruses and immunodeficiency in the induction and maintenance of SAIDS-RF.

Our study provides new evidence for similarities in the biology of the human and macaque RV1 rhadinoviruses KSHV and RFHV and supports the development of RFH-induced RF in immunocompromised macaques as an animal model for AIDS-KS.

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