Cloning of the genome of *Alcelaphine herpesvirus 1* as an infectious and pathogenic bacterial artificial chromosome


Department of Infectious and Parasitic Diseases, Immunology-Vaccinology (B43b), Faculty of Veterinary Medicine and Department of Pathology, Faculty of Medicine, University of Liège, B-4000 Liège, Belgium

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*Alcelaphine herpesvirus 1* (AlHV-1), carried asymptomatically by wildebeest, causes malignant catarrhal fever (MCF) following cross-species transmission to a variety of susceptible species of the order Artiodactyla. The study of MCF pathogenesis has been impeded by an inability to produce recombinant virus, mainly due to the fact that AlHV-1 becomes attenuated during passage in culture. In this study, these difficulties were overcome by cloning the entire AlHV-1 genome as a stable, infectious and pathogenic bacterial artificial chromosome (BAC). A modified *loxP-*flanked BAC cassette was inserted in one of the two large non-coding regions of the AlHV-1 genome. This insertion allowed the production of an AlHV-1 BAC clone stably maintained in bacteria and able to regenerate virions when transfected into permissive cells. The *loxP*-flanked BAC cassette was excised from the genome of reconstituted virions by growing them in permissive cells stably expressing Cre recombinase. Importantly, BAC-derived AlHV-1 virions replicated comparably to the virulent (low-passage) AlHV-1 parental strain and induced MCF in rabbits that was indistinguishable from that of the virulent parental strain. The availability of the AlHV-1 BAC is an important advance for the study of MCF that will allow the identification of viral genes involved in MCF pathogenesis, as well as the production of attenuated recombinant candidate vaccines.

**INTRODUCTION**

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of a variety of species in the order Artiodactyla, which includes cattle (reviewed by Reid, 2000). The main causative agents of MCF are two closely related gamma-herpesviruses of the genus *Rhadinovirus*, *Ovine herpesvirus 2* (OvHV-2) and *Alcelaphine herpesvirus 1* (AlHV-1). These viruses cause no apparent disease in their natural host species. Sheep are infected naturally by OvHV-2, which is responsible for the sheep-associated form of MCF (Reid et al., 1984) following cross-species transmission to susceptible hosts such as cattle. Wildebeest (*Connochaetes taurinus*) carry AlHV-1, responsible for the wildebeest-derived form of MCF (WD-MCF) (Plowright et al., 1960). In sub-Saharan Africa, cross-species transmission of AlHV-1 to susceptible host species occurs throughout wildebeest-grazing areas and largely affects cattle. Two recent socio-epidemiological studies performed in Kenya and Tanzania assessed the high economic impact of AlHV-1 MCF on pastoralists (Bedelian, 2004; Cleaveland et al., 2001). In addition, AlHV-1 MCF has also been reported throughout the world in zoological collections where mixed artiodactyl species including wildebeest are kept (Castro & Heuschele, 1985; Castro et al., 1984). As there is currently no vaccine against AlHV-1, the only strategy to prevent WD-MCF in susceptible species is to limit their contact with carrier wildebeest (Barnard, 1990; Cleaveland et al., 2001). WD-MCF is a disease described as a combination of lymphoproliferation and degenerative lesions thought to be due to autoimmune tissue damage. Although varying in severity and localization among susceptible hosts, gross lesions are mainly characterized by severe lymph node enlargement, as well as epithelial necrosis and erosions (Plowright, 1990). Histopathologically, three essential lesions have been described: (i) the destruction of small lymphocytes in lymphopoietic tissues, (ii) infiltration of large proliferating lymphoblastoid cells in many organs, primarily in perivascular locations, and (iii) mural angiitis of arteries and veins (Plowright, 1990). The lymphoid cells present in lesions have been described as T lymphocytes (Burrells & Reid, 1991; Ellis et al., 1992; Nakajima et al., 1992, 1994; Wilkinson et al., 1992). Large granular lymphocytes (LGLs) isolated from
MCF-induced lesions can be cultivated in vitro without IL-2. These infected LGLs harbour an activated T-cell phenotype and reproduce MCF when injected into naive animals (Swa et al., 2001). Experimentally, WD-MCF can be induced in rabbits (Buxton & Reid, 1980). The lesions observed are very similar to those described above.

AlHV-1 has a B-type genome structure consisting of a long unique region (LUR or L-DNA) flanked by polyrepetitive DNA (H-DNA) (Ensser et al., 1997). Despite publication of the entire AlHV-1 genome sequence (Ensser et al., 1997), little is known about the role of individual AlHV-1 genes in WD-MCF pathogenesis and in the biology of the infection of the natural host. This lacuna is a consequence of the difficulty of generating recombinant virus using classical homologous recombination in eukaryotic cells. Two intrinsic features of AlHV-1 have been responsible for this failure. Firstly, while AlHV-1 can be cultivated in vitro, the virus is strictly cell-associated in its low-passaged form (Plowright, 1990). Secondly, prolonged virus cultivation in vitro leads to spontaneous attenuation of AlHV-1, the high-passaged form of the virus being unable to induce WD-MCF in natural or experimental susceptible species (Handley et al., 1995). Virus attenuation is associated with genome rearrangements frequently involving the ORF50 and A6 regions (Handley et al., 1995; Wright et al., 2003).

More recently, the manipulation of large herpesvirus genomes has been facilitated by using bacterial artificial chromosome (BAC) vectors (Gillett et al., 2005; Messere et al., 1997; Wagner et al., 2002). These vectors allow the maintenance and mutagenesis of the viral genome in Escherichia coli, followed by reconstitution of progeny virions by transfection of the BAC plasmid into permissive eukaryotic cells. The application of this technology should circumvent the intrinsic problems described above in generating AlHV-1 recombinants.

In the present study, we have described cloning of the AlHV-1 genome as a stable, infectious and pathogenic BAC. This goal was achieved by insertion of a modified loxp-flanked BAC cassette into one of the two large regions of AlHV-1 L-DNA that lack obvious coding regions and are thought not to be essential. This insertion led to the production of an AlHV-1 BAC clone that was stably maintained in bacteria and was able to regenerate virions when transfected into permissive cells. The loxp-flanked BAC cassette was excised from the genome of reconstituted virions by growing them on permissive cells stably expressing Cre recombinase. The resulting virions induced MCF in rabbits in a similar way to the low-passage AlHV-1 parental strain.

**METHODS**

**Cell lines and virus strain.** Embryonic bovine lung cells (EBL; DSMZ ACC 192) and bovine turbinate fibroblast cells (BT; ATCC CRL-1390) were cultured in Dulbecco’s modified essential medium (DMEM; Invitrogen) containing 10% fetal calf serum (FCS) (BioWhittaker). EBL cells stably expressing Cre recombinase fused to a nuclear localization signal (EBL NLS Cre) (Gillett et al., 2005) were cultured in DMEM containing G418 (final concentration 200 µg ml⁻¹; Invitrogen). The pathogenic AlHV-1 C500 strain isolated from an ox with MCF (Plowright et al., 1975) was used throughout this study. The virus was maintained by limited passage (fewer than five) in BT cells.

**BAC cloning of AlHV-1.** Firstly, a modified pBeloBAC vector (hereafter called pBeloBACModified-EGFPNeo) was produced based on the sequence of the vector pBeloBACModified (GenBankAY665170; Gillet et al., 2005). In this vector, the enhanced GFP (EGFP) expression cassette driven by the human cytomegalovirus (Human herpesvirus 5; HHV-5) immediate-early promoter was replaced by a cassette encoding EGFP fused at the C terminus to aminoglycoside 3'-phosphotransferase (Neo). Expression of this fusion protein conferred EGFP autofluorescence and G418 resistance to expressing cells. The pBeloBACModified-EGFPNeo vector was produced as follows. The Neo ORF was amplified by PCR using the forward primer 5'-GGTACCATGAGTAAACAAGATGGATTG-3', containing a KpnI site (italic) and the first 21 nt of the Neo ORF, and the reverse primer 5'-GGTACCTAGAGAAGACGTTCAAGGAG-3', with a KpnI site (italic) and the last 21 nt of the Neo ORF. The vector pEGFP-C1 (Clontech, BD Biosciences) was used as template. After digestion with KpnI, the PCR product was cloned into the KpnI site of the pEGFP-C1 vector. The resulting EGFP–Neo cassette was then excised by AflIII. After filling in both ends, the fragment was inserted into the XcmI and BstEII blunt-ended sites of the pBeloBACModified vector resulting in pBeloBACModified-EGFPNeo. Blunt-ended sites were generated with T4 DNA polymerase (New England Biolabs). Secondly, the modified BAC cassette of the pBeloBACModified-EGFPNeo vector was inserted into the NsiI restriction fragment Q of the AlHV-1 L-DNA, a region that lacks obvious coding regions and is thought not to be essential (Ensser et al., 1997) (Fig. 1). Briefly, the AlHV-1 NsiI restriction fragment Q was cloned into the Bluescribe M13+ plasmid (Stratagene) containing an ampicillin resistance cassette (ApAmp). Linearized pBS-NsiI Q BAC EGFPNeo using Lipofectamine Plus (Invitrogen). Ten days later, to select BAC-recombinant virions, infected cells were plated on uninfected confluent BT cells (10⁶ cells per 75 cm²) in the presence of G418 (final concentration 200 µg ml⁻¹). When the cytopathic effect (CPE) covered 90% of the cell monolayer, viral DNA was prepared as described previously (Morgan et al., 1990) and 1 µg DNA was electroporated (2250 V, 132 Ω, 40 μF) into E. coli DH10B cells (Smith & Enquist, 2000). Transformed bacteria were selected using chloramphenicol.

**Southern blotting.** Southern blot analysis was performed as described previously (Markine-Goriaynoff et al., 2003).

**Antibodies.** The mouse monoclonal antibody (mAb) 15-A (VMRD) raised against AlHV-1 glycoprotein complex gp115 was used in this study (Li et al., 1994).

**Indirect immunofluorescent staining.** Cells grown on glass coverslips were fixed in PBS containing 4% (w/v) paraformaldehyde (Merck) for 10 min on ice and then for 20 min at 20 °C. After washing with PBS, samples were permeabilized in PBS containing 0.1% (w/v) NP-40 (Fluka) at 37 °C for 10 min. Immunofluorescent labelling (incubation and washes) was performed in PBS containing 10% FCS. Samples were incubated at 37 °C for 45 min with mAb 15-A
0.5 µg ml⁻¹) used as the primary antibody. After three washes, samples were incubated at 37°C for 30 min with R-phycocerythrin (PE)-conjugated F(ab')₂ goat anti-mouse immunoglobulins (PE- GAMA; 5 µg ml⁻¹; Dako) as the secondary conjugate. After washing, samples were mounted as described elsewhere (Vanderplasschen et al., 2000).

**Growth curves.** Multi-step virus growth experiments were conducted to compare the growth kinetics of the AlHV-1 C500 BAC-excised strain with those of the parental virus. A series of triplicate cultures of EBL cells was infected at an m.o.i. of 10⁻³. After 1 h of adsorption, cells were washed and overlaid with DMEM containing 10% FCS. Infected cultures (cells and supernatant) were harvested at successive intervals after infection and stored at −80°C. The amount of infectious virus was determined by plaque assay on EBL cells as described previously (Gillet et al., 2005). Syncytia were revealed by indirect immunofluorescent staining using mAb 15-A as the primary antibody.

**Syncytium size.** EBL cells grown on coverslips were infected with the AlHV-1 C500 BAC-excised strain or with the parental AlHV-1 C500 strain and overlaid with DMEM containing 10% FCS and 0.6% (w/v) carboxymethylcellulose (CMC; Sigma) to obtain isolated syncytia as described elsewhere (Vanderplasschen et al., 1993). At successive intervals after infection, syncytia were stained by indirect immunofluorescent staining using mAb 15-A as the primary antibody as described above. Pictures of syncytia were captured with a CCD camera system (DC 300F, IM50, version V1.20; Leica). The area of the syncytium was determined using Image] 1.34 software (http://rsb.info.nih.gov/ij/docs/intro.html).

**Induction of MCF in rabbits.** Specific-pathogen-free New Zealand white rabbits were housed individually throughout this study. Three groups were used, each comprising three rabbits. Animals in the first group were inoculated intravenously with 10⁶ mock-infected EBL cells, while those in groups 2 and 3 were inoculated with 10⁶ EBL cells infected with the AlHV-1 C500 strain and the AlHV-1 C500 BAC-excised strain, respectively. Infected cells for inoculation were harvested from cultures in which CPE reached 90% or more. Rabbits were examined daily for clinical signs. According to biochemical rules, rabbits were euthanized when the rectal temperature remained higher than 40°C for 2 consecutive days. The animal study was accredited by the local ethics committee of the University of Liège (Belgium).

**Histological analysis.** Organ explants from mock-infected or infected rabbits were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections of 5 µm were stained with haematoxylin and eosin prior to microscopic analysis.

**Virus isolation from infected rabbits.** Single-cell suspensions were prepared from the spleens and popliteal lymph nodes of infected rabbits as follows. The organs were removed and the tissues finely chopped and passed through a stainless steel sieve. Preparation of spleen cells was followed by lysis of red blood cells in ammonium chloride lysis solution (0.15 M NH₄Cl, 10 mM NaHCO₃, 0.1 M disodium EDTA, pH 7.4) for 5 min at room temperature before centrifugation at 300 g for 10 min. Spleen and lymph node cells were then washed twice in ice-cold PBS. Cells (10⁵) were seeded on to subconfluent BT cell monolayers grown in six-well cluster dishes. Cultures were observed daily for the appearance of CPE. To amplify viral isolates, infected cells were incubated on to a fresh BT monolayer when the CPE reached at least 20%.

**Detection of the AlHV-1 genome by PCR.** DNA samples were extracted from tissues using the Easy-DNA kit (Invitrogen). PCR amplification specific for AlHV-1 was performed using a set of primers derived from the AlHV-1 ORF50 sequence (Ensser et al., 1997). The forward primer C500-1 (5'-TACGGGAGCCCTGACATT- TCTCCTCTTGTG-3') and reverse primer C500-2 (5'-ATAACTGTGTTGATGGACATGCATCT-3') used for this reaction have been described previously (Li et al., 2000). Additionally, a PCR allowing differentiation between AlHV-1 C500 and AlHV-1 C500 BAC-excised strains was also performed. The forward primer 246NG (5'-CATTAGTGGGACAGAC-3') and reverse primer 246ND (5'-AGGCGATAATCTACTATAG-3'), hybridizing downstream and upstream of the BAC cassette insertion site, respectively, were used for this reaction. PCR products of 719 and 905 bp were generated when AlHV-1 C500 and AlHV-1 C500 BAC-excised strain genomes were used as template, respectively.

**Southern dot-blot assay.** A Southern dot-blot hybridization assay was developed to semi-quantify the viral DNA in DNA extracted from infected rabbit organs. DNA was isolated from organ fragments using the Easy-DNA kit (Invitrogen). DNA was denatured by
the addition of 0.1 vols 1 M NaOH. After incubation for 5 min at 37 °C, an equal volume of 20 × SSC (20 × SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0) was added and samples were incubated on ice for 2 min. DNA was then applied to a Hybond-XL membrane (Amersham Biosciences) pre-soaked in 10 × SSC using a HYBRI-DOT Manifold apparatus (Life Technologies). Membranes were treated as described above for the Southern blot assay using the NsiI Q fragment of the AlHV-1 C500 genome as probe.

Microscopy analysis. Epifluorescent microscopy analysis was performed with a DMRBE microscope (Leica) equipped with a DC 300F CCD camera (Leica) as described previously (Vanderplasschen et al., 1993).

Statistical analysis. Statistical comparisons were assessed by analysis of variance. Fisher–Snedecor’s F-test was used to assess the significance of the results (P < 0.05).

RESULTS AND DISCUSSION

Cloning of the AlHV-1 genome in E. coli

The approach depicted in Fig. 1 was used to clone the entire genome of AlHV-1. The insertion site of the BAC cassette was selected in one of the two large regions of AlHV-1 L-DNA that lack obvious coding regions and are thought not to be essential (Fig. 1). Transfection of linearized plasmid pBS-NsiI Q BAC EGFPNeo into AlHV-1-infected BT cells generated the C500 BAC recombinant strain. The ability of the latter virus to grow in the presence of G418 allowed its selection from the parental C500 strain. Expression of EGFP autofluorescence by the C500 BAC recombinant strain was used to monitor progress of the selection process. After five passages of the virus in the presence of G418, approximately 90 % of syncytia expressed EGFP (data not shown). Circular intermediates of the AlHV-1 C500 BAC genome were then isolated from infected cells and electroporated into E. coli. BAC DNAs were prepared from single colonies and screened by BamHI restriction digestion to select clones corresponding to the expected restriction profile (Enser et al., 1997) (data not shown). Approximately 30 % of the clones tested exhibited the correct restriction profile. One clone was selected for further analysis. The molecular structure of this plasmid was characterized by a combined restriction endonuclease/Southern blot approach using three different restriction enzymes, HindIII, NsiI (data not shown) and SacI (Fig. 2a). Taken together, these analyses confirmed that the AlHV-1 C500 BAC plasmid had the correct molecular structure corresponding to a BAC clone of the entire AlHV-1 genome.

Stability of the AlHV-1 genome in E. coli

BAC plasmids are usually propagated in E. coli strain DH10B, which carries a recA mutation that minimizes recombination. However, relative instability of such BAC plasmids has been reported for several herpesviruses, such as Murid herpesvirus 4 (Adler et al., 2000). To assess the stability of the AlHV-1 genome as a BAC plasmid, E. coli DH10B containing the AlHV-1 C500 BAC was cultured serially for 20 consecutive days, each day representing approximately 36 generations. After various periods of culture, the BAC plasmid DNA was extracted and characterized by HindIII endonuclease digestion (Fig. 3). There were no detectable differences among plasmids grown for various periods of time, demonstrating a high stability of the AlHV-1 C500 BAC plasmid in E. coli.

Reconstitution of infectious virus from the AlHV-1 C500 BAC plasmid and excision of the BAC cassette from reconstituted viruses

An advantage of BAC cloning technology for the manipulation of large DNA viruses is that infectious virus can be reconstituted from the BAC plasmid. Consequently, we tested whether infectious virus could be produced by electroporation of the AlHV-1 C500 BAC plasmid into BT cells. A plasmid preparation produced from bacteria grown for 20 days was used for this experiment (Fig. 3). Five days after electroporation, viral syncytia expressing EGFP were detected (data not shown), as well as virus spread into the culture. SacI restriction analysis of the DNA of reconstituted virus revealed profiles identical to the patterns observed for AlHV-1 C500 BAC plasmid (Fig. 2a). These data demonstrated that infectious AlHV-1 virions could be reconstituted efficiently by transfection of the AlHV-1 C500 BAC plasmid into permissive cells and further supported the stability of the AlHV-1 genome in E. coli. This contrasts with previous experiences with some other herpesviruses. For example, the HHV-5 BAC plasmid (Borst et al., 1999) required the expression of a transactivating factor for efficient production of virions. BAC plasmids containing both Bovine herpesvirus 1 (Mahony et al., 2002) and Human herpesvirus 1 (Stavropoulos & Strathdee, 1998) required drug treatment promoting immediate-early gene expression.

To excise the BAC cassette, AlHV-1 BAC-reconstituted virus was propagated in EBL NLS Cre cells to generate the AlHV-1 C500 BAC-excised strain. Deletion of the BAC cassette was monitored by the disappearance of the expression of EGFP (Fig. 2b) and by a combined restriction endonuclease/Southern blot approach (Fig. 2a). Five days after infection, syncytia generated by AlHV-1 C500, AlHV-1 C500 BAC and AlHV-1 C500 BAC-excised strains were indistinguishable when revealed with mAb 15-A (Fig. 2b, compare ii, v and viii), while EGFP expression was restricted to the AlHV-1 C500 BAC strain (Fig. 2b, compare i, iv and vii). The Cre–loxP-mediated deletion of the BAC cassette left a sequence of approximately 180 bp at the site of insertion, thus increasing the size of the NsiI Q fragment of the C500 strain to 2.4 kbp (Fig. 2a). Finally, in order to investigate the putative effect of the recombination process described above (insertion/excision of the BAC cassette) on AlHV-1 growth in vitro, AlHV-1 C500 and AlHV-1 C500 BAC-excised strains were compared using the growth and syncytium size assays described in Methods (Fig. 2c and d). Both viruses exhibited similar growth curves and syncytium sizes (P < 0.05).
Fig. 2. Characterization of the AlHV-1 C500 BAC plasmid and derived strains. (a) Characterization of AlHV-1 C500 BAC plasmid and derived AlHV-1 strains by a combined restriction endonuclease/Southern blot approach. AlHV-1 C500 BAC plasmid and the genome of AlHV-1 C500 BAC and AlHV-1 C500 BAC-excised strains were analysed by SacI restriction (left panels) and further tested by Southern blotting using a probe corresponding to the BAC EGFP–Neo cassette (right panels). Open and filled arrowheads indicate restriction fragments containing the H-DNA and BAC cassette, respectively. An additional band due to excision of the BAC cassette is indicated by an asterisk. Marker sizes (MS) in kb are indicated on the left. (b) Characterization of AlHV-1 strains derived from AlHV-1 C500 BAC plasmid by epifluorescent analysis of viral syncytia. EBL cells grown on glass coverslips were infected with AlHV-1 C500 (i–iii), C500 BAC (iv–vi) or C500 BAC-excised (vii–ix) strains and overlaid with DMEM containing 5% FCS and 0.6% (w/v) CMC to obtain isolated syncytia. Ten days after infection, syncytia were revealed by indirect immunofluorescent staining using mAb 15-A and PE–GAM as the primary and secondary antibodies, respectively. The sets of three horizontal panels (i–iii, iv–vi and vii–ix) represent analyses of the same syncytium. Panels (i), (iv) and (vii) and panels (ii), (v) and (viii) were analyses for EGFP and PE fluorescent emissions, respectively. The merged EGFP and PE signals are shown in (iii), (vi) and (ix). Bar, 50 μm. (c) Replication kinetics of the AlHV-1 C500 BAC-excised strain (□, dashed line) were compared with those of the parental AlHV-1 C500 strain (●, solid line) as described in Methods. The data presented are means ± SD of triplicate measurements. (d) Effect of BAC insertion on AlHV-1 syncytium size. EBL cells grown on coverslips were infected with the AlHV-1 C500 (shaded bars) and AlHV-1 C500 BAC-excised (open bars) strains and then overlaid with DMEM containing CMC as described in Methods. At successive intervals after infection, syncytia were revealed by indirect immunofluorescent staining using mAb 15-A and Alexa 568–GAM as the primary and secondary antibodies, respectively. The data shown are the mean ± SD for the measurement of ten randomly selected syncytia.

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Pathogenicity of the AlHV-1 BAC-excised strain in rabbit

The AlHV-1 BAC clone described above opens up the possibility of exploiting prokaryotic technologies for the production of AlHV-1 recombinants. However, an essential prerequisite for the use of such recombinants is that the AlHV-1 C500 BAC-excised strain retains the ability to induce MCF in vivo. To test this, rabbits were inoculated with the AlHV-1 C500 and AlHV-1 C500 BAC-excised strains. Both AlHV-1 C500 and AlHV-1 C500 BAC-excised strains induced MCF in rabbits with an indistinguishable pathology and similar incubation time. Rabbits infected with the AlHV-1 C500 and AlHV-1 C500 BAC-excised strains developed MCF at 20±33±1.15 and 19±33±2.08 days post-infection, respectively (Fig. 4). Clinical signs were identical in the two groups of infected rabbits and included apathy, anorexia, adipsia, hyperthermia and severe hypertrophy of popliteal lymph nodes. At necropsy, examination of the organs of the animals revealed no differences in gross pathology between the two groups of infected rabbits. Characteristic MCF lesions were observed, including severe splenomegaly (the size of the spleens of infected rabbits was at least twice the size of those of mock-infected rabbits) and generalized lymph node hypertrophy. The latter was particularly marked for the popliteal lymph nodes (the popliteal lymph nodes of infected rabbits were at least twice the size of those of mock-infected rabbits). Congestion and enlargement of the liver with greyish punctiform areas surrounding lobules was detected in both groups of infected animals, as well as irregular foci of various sizes (1–5 mm diameter) present within the cortical areas of the kidneys. Histological findings were indistinguishable between groups infected with the AlHV-1 C500 strain and the AlHV-1 C500 BAC-excised strain (Fig. 5) and were characteristic of histopathological lesions reported for MCF (see Introduction). Fig. 5 illustrates MCF lesions in several organs characterized by infiltration of lymphoblastoid cells. These were observed primarily in perivascular locations. Vasculitis affecting arteries and veins was also observed in infected animal tissues. The three mock-infected rabbits survived for the course of the experiment with no disease symptoms.

To demonstrate that the two groups of rabbits were infected with the correct virus strain and to exclude any possibility of virus spread between the two groups, PCRs were performed on the spleen and the popliteal lymph nodes of each infected rabbit (Fig. 6a). PCRs performed with the C500-1 and C500-2 primers confirmed that the tissues contained the AlHV-1 genome, while reactions performed with the 246ND and 246NG primers excluded the possibility of virus spread between the two groups of infected rabbits. In order to investigate the putative effect of the recombination processes described above on viral replicative fitness in vivo, viral loads were compared in rabbits infected with the AlHV-1 C500 or AlHV-1 C500 BAC-excised strain (Fig. 6b). The semi-quantitative Southern dot-blot approach revealed that the two tested viruses replicated comparably in vivo. Finally, the AlHV-1 C500 BAC-excised strain recovered from inoculated rabbits that had developed MCF was compared with the initial inoculation virus by SacI endonuclease restriction (Fig. 6c). No difference was observed between the virus used for inoculation and that isolated from the animals with MCF.

Fig. 3. Stability of the AlHV-1 C500 BAC plasmid in E. coli. DH10B cells containing C500 BAC plasmid were passed every day at a ratio of 1:10³ (v/v) for 20 consecutive days. At the indicated days, BAC DNA was prepared from the culture. Finally, BAC DNAs collected at various intervals were compared with parental C500 strain DNA by HindIII digestion. Marker sizes (MS) in kb are indicated on the left.

Fig. 4. Cumulative incidence of survival of AlHV-1-infected rabbits. On day 0, three groups, each consisting of three rabbits, were inoculated intravenously with 10⁶ mock-infected EBL cells (○) or 10⁶ EBL cells infected with the AlHV-1 C500 (■) or AlHV-1 C500 BAC-excised (△) strain. Percentage survival is expressed according to time post-inoculation.
The results demonstrated that the AlHV-1 BAC clone generated in this study has several important, intrinsic qualities: (i) it is infectious, as demonstrated by its ability to generate infectious virions after transfection in permissive cells, (ii) it is stable when propagated into bacteria, even over long periods of culture corresponding to approximately 720 generations, (iii) the BAC cassette was excised from the genome of reconstituted virus and (iv) the AlHV-1 C500 BAC-excised strain induced MCF in rabbits comparable to that induced by the C500 parental strain. This was despite long-term propagation in bacteria and several passages in eukaryotic cells required for the excision of the BAC cassette and amplification of the virus. This demonstrated that the AlHV-1 BAC clone is pathogenic.

In conclusion, the AlHV-1 BAC clone produced in this study will be an invaluable resource for the production of AlHV-1 recombinants. The feasibility of producing AlHV-1 recombinants using the BAC clone reported in the present study and prokaryotic recombination technologies has already been demonstrated. Indeed, an EGFP recombinant strain that induced MCF in rabbits indistinguishable from

**Fig. 5.** Histopathological characterization of MCF induced by the AlHV-1 C500 and AlHV-1 C500 BAC-excised strains in rabbits. Kidney (a–c), liver (d–f) and lung (g–i) were explanted from mock-infected rabbits (a, d, g) or from rabbits infected with the AlHV-1 C500 (b, e, h) or AlHV-1 C500 BAC-excised (c, f, i) strain. Each panel represents haematoxylin and eosin-stained organ sections. Abbreviations used are as follows. (a–c) A, Arterioles; RC, renal corpuscles; T, uriniferous tubules; MR, medulla rays. (d–f) A, Hepatic arterioles; V, portal veins; Bi, small bile ducts; Hp, hepatocytes. (g–i) A, Arterioles; Br, intrapulmonary bronchi and bronchioles. Mononuclear cell infiltration areas are indicated by open arrows. The images in this figure are representative of the analysis of one selected rabbit per group. Similar results were observed among rabbits of the same group. Bar, 250 μm.
that of the virulent parental strain has been produced (data not shown). Until now, the production of AlHV-1 recombinants has not been achieved by classical recombination in eukaryotic cells due to spontaneous attenuation of the virus when grown for long periods of time. The production of AlHV-1 recombinants will facilitate our understanding of MCF and may allow the production of recombinant viral vaccines to control AlHV-1 MCF.

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Fig. 6. Detection and characterization of AlHV-1 genome recovered from infected rabbits. (a) PCR detection of AlHV-1 genome in DNA extracted from spleen (Sp) and popliteal lymph nodes (LN) from mock-infected rabbits or from rabbits infected with the AlHV-1 C500 or AlHV-1 C500 BAC-excised strain. PCRs were performed with the C500-1/C500-2 and 246NG/246ND pairs of primers as described in Methods. (b) Comparison of AlHV-1 DNA loads in organs of rabbits infected with the AlHV-1 C500 or AlHV-1 C500 BAC-excised strain. DNA was extracted from the spleen and popliteal lymph nodes of mock-infected and infected rabbits. The indicated amount of DNA was then hybridized with the NsiI Q fragment of the AlHV-1 C500 genome as described in Methods. The sensitivity of the assay was assessed by hybridization to decreasing amounts of purified AlHV-1 DNA. (c) Characterization of the AlHV-1 BAC-excised strain recovered from an MCF-affected rabbit. AlHV-1 BAC-excised strain recovered from an MCF-affected rabbit was compared with the AlHV-1 C500 and the inoculated AlHV-1 C500 BAC-excised strains by endonuclease SacI restriction digestion. An additional band due to excision of the BAC cassette is shown by an asterisk. Marker sizes (MS) in kb are indicated on the left.


