Genetic and ultrastructural characterization of a European isolate of the fatal endotheliotropic elephant herpesvirus

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A male Asian elephant (Elephas maximus) died at the Berlin zoological gardens in August 1998 of systemic infection with the novel endotheliotropic elephant herpesvirus (ElHV-1). This virus causes a fatal haemorrhagic disease in Asian elephants, the so-called endothelial inclusion body disease, as reported from North American zoological gardens. In the present work, ElHV-1 was visualized ultrastructurally in affected organ material. Furthermore, a gene block comprising the complete glycoprotein B (gB) and DNA polymerase (DPOL) genes as well as two partial genes was amplified by PCR-based genome walking and sequenced. The gene content and arrangement were similar to those of members of the Betaherpesvirinae. However, phylogenetic analysis with gB and DPOL consistently revealed a very distant relationship to the betaherpesviruses. Therefore, ElHV-1 may be a member of a new genus or even a new herpesvirus subfamily. The sequence information generated was used to set up a nested-PCR assay for diagnosis of suspected cases of endothelial inclusion body disease. Furthermore, it will aid in the development of antibody-based detection methods and of vaccination strategies against this fatal herpesvirus infection in the endangered Asian elephant.

Introduction

An 11-year-old male Asian elephant (Elephas maximus) named Kiba died in the Berlin zoological garden in August 1998 of a systemic haemorrhagic disease, which progressed fatally within 24 h. Post-mortem inspection revealed signs of a herpesvirus infection (Burkhardt et al., 1999). Similar cases had already been reported in Asian elephants kept in American and European zoos and the syndrome was named endothelial inclusion body disease (Ossent et al., 1990; Richman et al., 1996). Recently, a novel elephant herpesvirus was identified and evidence was presented that it is associated aetiologically with the fatal endotheliotropic disease. The virus was named endotheliotropic elephant herpesvirus (Richman et al., 1999). In initial experiments on Kiba after his death, we attempted to detect the novel virus, for which we use the designation ElHV-1 (according to the rules of the ICTV; Murphy et al., 1995), with a PCR assay that targets the ElHV-1 terminase gene. Sequences were amplified from several organs and the blood that were very similar to those of the American cases (Burkhardt et al., 1999).

In the present study, we identified ElHV-1 in affected organ material from Kiba by ultrastructural analysis and partially characterized its genome. Genetic analysis was achieved by using (i) a pan-herpes consensus PCR to get access to the DNA polymerase (DPOL) gene and (ii) a PCR-based genome walking technique to amplify and sequence the complete DPOL gene as well as the complete glycoprotein B (gB) gene. Our goals were to elucidate firmly the evolutionary relatedness of ElHV-1 to other herpesviruses, to develop a sensitive nucleic acid-based detection method for herd control in zoological gardens and to identify a glycoprotein gene that can be used for the development of an ELISA and can serve as a basis for a vaccination approach.

Methods

- Transmission electron microscopy (TEM). Formalin-fixed and paraffinized liver tissue was deparaffinated, post-fixed with OsO4 and embedded in epoxy resin (re-embedding procedure). Ultrathin sections...
were contrasted with uranyl acetate/lead citrate and examined with an EM 10 transmission electron microscope (Zeiss).

**Sample collection and preparation of DNA.** Organ and serum samples were collected post-mortem from the male elephant Kiba, which died from endothelial disease in the zoological garden of Berlin in 1998. Serum samples from his offspring Plai Kiri and other members of the herd were collected in 2000. Total tissue DNA was prepared with the QIAamp DNA tissue kit. Serum was processed with the QIAamp DNA blood kit (Qiagen).

**Pan-herpes consensus PCR and genome walking.** Pan-herpes consensus PCR was performed as described previously (Ehlers et al., 1999a, b). Genome walking was used to extend the initial 178 bp sequence obtained by pan-herpes consensus PCR and was performed by Genexpress GmbH (Berlin), as described previously (Ulrich et al., 1999). Sequence analyses were done by Agowa GmbH (Berlin).

**ElHV-specific long-range PCR.** The primer combinations U4 (5’ GGGTCTATGTCAACGTCCTC 3’) plus D2 (5’ CTGCATGCAGGCTC- ATGCATCG 3’) and U4 plus D1 (5’ CGTAATTAGGCACACTCTTGCG 3’) were used by Agowa GmbH for amplification of 6839 and 6410 bp fragments of ElHV-1 DNA with the Expand High Fidelity PCR kit (Roche AG).

**ElHV-specific nested PCR.** PCR was carried out with 100 ng tissue DNA or 1 µl serum processed with the QIAamp DNA blood kit to an equal volume of eluate. In the second round of nested PCR, 1 µl of the first-round PCR was used as template. Reaction mixtures (50 µl) were set up with the HotStarTag Mastermix kit (Qiagen) according to the manufacturer’s instructions. For thermal cycling, Perkin-Elmer 2400 thermocyclers and 0.2 ml thin-wall tube strips were used. The primer combination ELDPOL..2s (sense; 5’ ACGCACACTTTATCAT- AGG 3’) plus ELDPOL...as (anti-sense; 5’ AGAACTGGAATCTGTCTTGTATGA 3’) was used in the first-round PCR (428 bp) and the primer combination ELDPOL...3s (sense; 5’ CTGGAGGGTCTCCGTTCAAAATT 3’) plus ELDPOL...as (antisense; 5’ CTGCTTTGATGAAAACGGGAGTT 3’) was used in the second round (316 bp). In the first-round PCR, the reaction mixtures were kept at 95 °C for 15 min and then cycled 40 times with 1 min denaturation at 94 °C, 1 min annealing at 63 °C and 1 min strand extension at 72 °C, followed by a final extension step at 72 °C for 5 min. In the second-round PCR, conditions were identical except that the annealing temperature was 65 °C.

**Nucleotide and protein sequence analysis.** Multiple sequence alignments were performed and protein pair distances calculated with the clustalW module of MacVector (version 6.01, Oxford Molecular Group). For phylogenetic analysis, gaps or insertions unique to a particular species were removed from the multiple sequence alignments and the remaining conserved regions were concatenated for each individual protein (McGeoch et al., 1995). Trees were then constructed with the PHYLIP package using the programs Protdist (Dayhoff PAM matrix) and Neighbor or, alternatively, the program Protpars with randomized input of sequences. The trees were evaluated statistically by bootstrap analysis (1000-fold resampling) by using the programs Seqboot and Consense (Felsenstein, 1985, 1993).

The following herpesvirus gB and DPOL genes were included in comparative analyses of amino acid and nucleotide sequences. *Alphaherpesvirinae*: AtHV1 (ateline herpesvirus-1) (gB, accession no. M97585); BoHV1 (bovine herpesvirus-1) (gB, accession no. M21474; DPOL, accession no. Z726205); BoHV2 (gB, accession no. M21628; DPOL, accession no. AF181249); CeHV1 (cercopithecine herpesvirus-1) (gB, accession no. U14664); EH1V (equine herpesvirus-1) (gB and DPOL, accession no. M86664); EH2V (gB and DPOL, accession no. AF300277); FeHV1 (feline herpesvirus-1) (gB, accession no. S49775; DPOL, accession no. AJ224971); GaHV1 (gallid herpesvirus-1 or infectious laryngotracheitis virus) (gB, accession no. X56093); GaHV2 (Marek’s disease virus) (gB, accession no. D13713; DPOL, accession no. L40431); HSV1 (herpes simplex virus type 1 or human herpesvirus-1) (gB and DPOL, accession no. X04771); HSV2 (human herpesvirus-2) (gB and DPOL, accession no. Z60099); HVP-2 (herpesvirus papio) (gB, accession no. U14662); MaHV1 (macropodid herpesvirus-1 or kangaroo herpesvirus) (gB, accession no. AF61754); PRV (pseudorabies virus or suid herpesvirus-1) (gB, accession no. M17321; DPOL, accession no. L24487); VZV (varicella-zoster virus or human herpesvirus-3) (gB and DPOL, accession no. X04370); PHV1 (phocid herpesvirus-1) (gB, accession no. Z68147) and SaHV1 (saimiri herpesvirus 1) (gB, accession no. M95785). *Betaherpesvirinae*: CaHV2 (caviid herpesvirus 2 or guinea pig cytomegalovirus) (gB and DPOL, accession no. L25706); CeHV8 (rhesus monkey cytomegalovirus) (gB, accession no. U76749; DPOL, accession no. Z68147).
Characterization of ElHV-1

Fig. 2. PCR amplification, ORF and amino acid sequence analysis of ElHV-1. (a) Fragments of the ElHV-1 genome spanning approximately 6–4 and 6–8 kbp were amplified by long-distance PCR (lanes 2 and 3). As markers, the 10, 8, 6 and 5 kbp fragments of a 1 kbp ladder (Fermentas GmbH) are shown (lane 1). (b) The 6839 bp sequence determined from the German ElHV-1 isolate is represented by a ruler. The result of the ORF analysis appears as a schematic diagram above the ruler. ORFs are represented as grey arrows with the direction of transcription indicated. The ORFs were found to be most closely related to the respective gene block (ORFs 40–37) of HHV6 and HHV7 (see Results), as indicated. (c) The gB and DPOL proteins are represented as black bars. In gB, the putative cleavage site, RRKR, and the transmembrane region, TM, are depicted. A von Heijne hydrophilicity plot of gB is shown below. In DPOL, the functional domains (motifs I, II, III, A, B and C) are indicated. Lines marked 1st and 2nd represent the regions amplified in the first and second rounds of the nested PCR. The line denoted ‘cons PCR’ represents the region of initial access to the DPOL gene from which the genome-walking process started.

Results

Detection of ElHV-1 by ultrastructural analysis

For ultrastructural visualization of the fatal endotheliotropic elephant herpesvirus, liver tissue from Kiba was examined post-mortem by TEM. Within the endothelial cells of the liver sinusoids, intranuclear inclusion bodies were detected that contained spherical virus capsids (about 80 nm in diameter) with lucent or electron-dense cores (about 60 nm) in an amorphous matrix (Fig. 1a). Conglomerates of nucleocapsids were also found, surrounded by an amorphous matrix, in the cytoplasm close to the nuclear membranes. In the marginal zone of such conglomerates, the formation of tegument occurred around the nucleocapsids, increasing the diameter of the virus particles to 110–120 nm (Fig. 1b). Envelope formation and the release of mature virus particles could not be observed clearly. The configuration, size and sites of replication

no. AF033184); HCMV (human cytomegalovirus or human herpesvirus-5) (gB and DPOL, accession no. X17403); HHV6 (human herpesvirus-6) (gB and DPOL, accession no. U68299); MuHV2 (murine herpesvirus-2 or rat cytomegalovirus) (gB and DPOL, accession no. U50550); TuHV1 (Tupaia herpesvirus-1) (gB, accession no. AF084543; DPOL, accession no. AF074327). Gammaherpesvirinae: AlHV1 (alcelaphine herpesvirus-1) (gB and DPOL, accession no. AF005370); BoHV4 (gB, accession no. Z15044; DPOL, AF271211); EBV (Epstein–Barr virus or HHV4) (gB and DPOL, accession no. X00784); EHV2 (gB and DPOL, accession no. U20824); EHV5 (gB, accession no. AF050671); HHV6 (gB and DPOL, accession no. U75698); HVS (herpesvirus saimiri or SaHV2) (gB and DPOL, accession no. X64346); HVA (herpesvirus atele or AtHV2) (gB and DPOL, accession no. AF083424). Herpesviridae: HHV8 (murine gamma-herpesvirus-68) (gB and DPOL, accession no. U97553); PLHV1 (porcine lymphotrophic herpesvirus-1) (DPOL, accession no. AF193042); PLHV2 (DPOL, accession no. AF193043); RRV (rhesus monkey rhadinovirus) (gB and DPOL, accession no. AF029302).
suggested herpesvirus particles. No virus replication was observed in the hepatocytes.

Detection of ElHV-1 by pan-herpes consensus PCR and genome walking

Pan-herpes PCR targetting the DPOL gene (Fig. 2c) was applied to tissue samples from Kiba, which contained ElHV-1, as revealed by TEM (this study) and terminase gene-specific PCR (Burkhardt et al., 1999). A partial DPOL sequence of 178 bp was identified that showed similarity to those of betaherpesviruses in a BLAST analysis of the GenBank database. Starting with this sequence, PCR-based genome walking was performed repeatedly. ElHV1-specific PCR primers were designed from the resulting sequences, which were scattered over a region of approximately 7 kbp. They were used to amplify 6.8 and 6.4 kbp fragments (Fig. 2a) and several shorter fragments (not shown). From these, a final consensus sequence of 6839 bp was generated.

ElHV-1 open reading frame (ORF) and nucleotide composition analyses

In an ORF analysis (Fig. 2b), the 6839 bp ElHV-1 sequence was found to span the 3' end (988 bp) of the gene encoding the putative processing and transport protein (PRTP), the complete gB gene (2535 bp), the complete DPOL gene (3123 bp) and the 3' end (166 bp) of an additional ORF (Fig. 2b).
Characterization of ElHV-1

Fig. 3. Phylogenetic analysis of gB and DPOL of ElHV-1. Phylogenetic trees were constructed by the neighbour-joining method, using multiple amino acid sequence alignments of conserved regions of the gB (a) and DPOL (b) proteins. The ElHV-1 gB and DPOL amino acid sequences were deduced from the nucleotide sequence determined in this study. DNA polymerases and gBs of other alpha-, beta- and gammaherpesviruses were obtained from GenBank (accession numbers given in Methods). The herpesvirus subfamilies are indicated. Bootstrap values are indicated at the branches of the tree.

Analysis of the gB gene

The ElHV-1 gB deduced from the gB ORF has a length of 845 amino acids. It shows the typical features of a membrane-bound glycoprotein. The N terminus contains a hydrophobic stretch (aa 24–38) that may constitute a signal sequence and, in the C-terminal part, there are other hydrophobic stretches (aa 700–750) that may function as transmembrane sequences (Fig. 2c). Seventeen cysteine residues are present, nine of which show positional conservation in the gBs of all other herpesviruses analysed. Furthermore, 11 potential N-glycosylation motifs and a putative endoproteolytic cleavage site (Arg–Arg–Lys–Arg\(^\text{133}\)) (Spaete et al., 1990; Wellington et al., 1996) were found in ElHV-1. The sequences CYSRP (aa 552–555),

6839 bp sequence exhibited a low G + C content of 40 mol%, but no marked suppression was found of the CpG dinucleotide frequency with concomitant increases of TpG and CpA, a property seen in several gammaherpesviruses with low G + C content (Albrecht et al., 1992; Ensser et al., 1997; Virgin et al., 1997).
GQLG (aa 573–576) and NPFG (aa 726–729) in ElHV-1 gB are well conserved amongst other herpesvirus gB genes.

In amino acid sequence comparisons, the ElHV-1 gB was most closely related to those of HHV6 and HHV7; however, the identity was only 32%. Other betaherpesviruses revealed 28–30% identity and alpha- and gammaherpesviruses respectively revealed 21–22% and 25–26% identity. In phylogenetic analysis with the neighbour-joining method (programs Prodist and Neighbor), ElHV-1 gB did not cluster with any of the herpesvirus subfamilies but instead formed a separate branch between the Betaherpesvirinae and the Gammaherpesvirinae (Fig. 3a). Also, in parsimony analysis (program Protpars), ElHV-1 gB branched close to the central point that segregates the three virus subfamilies (not shown).

Analysis of the DPOL gene and flanking ORFs

The DPOL gene was found, like gB, to be most closely related to those of the betaherpesviruses. The deduced protein has a length of 1041 amino acids and contains the functional domains of DNA-dependent DNA polymerases of eukaryotic viruses (Knopf, 1998; Blanco et al., 1991) (Fig. 2c). In amino acid sequence comparisons, the ElHV-1 DPOL was most closely related to those of HHV6 and HHV7, with identities of 39%. Other betaherpesviruses revealed 32–38% identity and alpha- and gammaherpesviruses respectively showed 27–32% and 34–36% identity. In phylogenetic analysis with the neighbour-joining and the parsimony methods, the ElHV-1 DPOL showed exactly the same branching as gB, i.e. between the Betaherpesvirinae and the Gammaherpesvirinae (Fig. 3b).

Two partial ORFs were identified, one located upstream of the gB gene and the other located downstream of the DPOL gene in the reverse orientation (Fig. 2b). The encoded proteins are also most closely related to the corresponding positional homologues of betaherpesviruses (ORFs 40 and 37 of HHV6; ORFs 56 and 53 of HCMV).

These genetic data show that a set of genes exists in ElHV-1 that are identical in arrangement with the respective gene blocks of the betaherpesviruses. However, the amino acid identity does not exceed 40%, indicating a comparatively distant relationship. This is supported by the phylogenetic trees (Fig. 3a, b).

ElHV-specific nested PCR

We set up an ElHV-specific nested PCR that is based on the DPOL gene and amplifies a 316 bp fragment in the second-round PCR. With this PCR system, heart and liver specimens and a serum sample taken from Kiba after his death in 1998 were analysed. Serum samples taken in 2000 from his offspring Plai Kiri, Plai Kiri’s mother Pang-Pha and the herd members Svea, Ayesha, Iyoti and Drumbo were also analysed. In the first and the second round of the nested PCR, the organ specimens and the serum from Kiba were strongly ElHV-1 positive. The

Discussion

In this study, we report the first ultrastructural identification of ElHV-1, in an Asian elephant that died from fatal endotheliotropic disease in a European zoo (Fig. 1). Furthermore, we present the first extended genetic characterization of this novel herpesvirus species (Figs 2 and 3). The observations that herpesvirus particles were abundant in the diseased liver of the elephant, Kiba (Fig. 1), and that ElHV-1 DPOL sequences were strongly amplified from liver, heart and serum samples (Fig. 4) as well as terminase sequences from additional organs (Burkhardt et al., 1999) provided compelling evidence that we had observed an acute herpesvirus infection and, therefore, that ElHV-1 was the aetiological agent in Kiba’s fatal endotheliotropic disease.

For genetic characterization of ElHV-1, the gB and DPOL genes as well as flanking genes were analysed. Although only moderate identity values (< 40%) were found in pairwise comparisons of gB and DPOL with those of other herpesviruses, the identification of conserved and functional sites in both gB and DPOL (Fig. 2c) unequivocally validated their identification. On the basis of the sequences of both genes, ElHV-1 was most closely related to the betaherpesviruses. The partial ORFs identified in the flanking regions were also found to be positional homologues of betaherpesvirus genes. Therefore, the identified gene block is betaherpesvirus-like. A low G+C content (40 mol%) was found, but no significant deviation from the expected CpG dinucleotide frequency was apparent (Honess et al., 1989). In this respect, ElHV-1 is also more similar to the betaherpesviruses than to the other two subfamilies. However, in phylogenetic analysis of gB and DPOL, ElHV-1 appeared to be distantly related to all three
herpesvirus subfamilies, with a long separate branch that was slightly closer to the Betaherpesvirinae than to the Gammaherpesvirinae (Fig. 3 a, b). Therefore, EIHV-1 might be classified as a member of the Betaherpesvirinae, but it would obviously constitute a new genus. Alternatively, EIHV-1 might be a member of a new herpesvirus subfamily; this can only be clarified by analysis of the complete EIHV-1 genome.

The sequence of the complete DPOL gene determined from the German EIHV-1 isolate in this study was compared with the 0·1 kbp DPOL sequences of EIHV-1 amplified from Asian elephants in North America (Richman et al., 1999). One silent base exchange was found (not shown). A similar observation was made in a comparison of terminase gene sequences (Burkhardt et al., 1999). This indicates that the German EIHV-1 isolate and the North American isolates have nearly identical genomes. In phylogenetic analysis with the neighbour-joining method, the American 0·1 kbp DPOL sequence also clustered close to the centre of the herpesvirus tree, but slightly closer to the Alphaherpesvirinae (Richman et al., 1999). This result is not in complete accord with our results, but this can be attributed to the shortness of the sequence. Further genetic characterization of the American isolates is needed for a more accurate comparison.

A sensitive nested-PCR assay was developed for the detection of EIHV-1. This assay can be used by zoological gardens to control suspected cases of endothelial inclusion body disease in their elephant herds, as demonstrated by the post-mortem analysis of Kiba and the analysis of serum from his offspring, Plai Kiri, and other Asian elephants at the Berlin zoo (Fig. 4). Detection of viraemia by PCR at an early stage of the disease could verify the clinical diagnosis of endothelial inclusion body disease and allow a timely attempt at treatment with an anti-herpesvirus drug, as was reported by Schmitt & Hardy (1998), who successfully used famiclovir in the treatment of a herpesvirus-infected, clinically ill Asian elephant. However, routine PCR analysis of blood of healthy elephants is probably not feasible, because detection of EIHV-1 in the blood might simply indicate latent virus in peripheral blood leukocytes (PBL) and not an acute disease process. Since the ability to establish a latent state is a general feature of herpesviruses of all three subfamilies (Stanberry, 1986; Stevens, 1989), this must also be assumed for EIHV-1. The nested-PCR assay presented in this study will help to identify such sites of EIHV latency. Preliminary analyses of PBL samples from the herd in Berlin (data not shown) revealed no evidence of EIHV-1 latency in PBL. However, this issue requires additional testing of Asian elephants from other locations.

Finally, the identification of the gB gene offers the opportunity to develop an EIHV-specific ELISA and other antibody-based detection methods that can aid in the diagnosis of the disease and detection of carrier animals. Furthermore, vaccination strategies by DNA immunization with a gB-recombinant expression vector or by infection with gB-expressing recombinant viruses can be envisaged, providing a potential tool for the protection of the endangered Asian elephant.

Note added in proof. Kiri, the offspring of Kiba, died on 28 December 2000, after an overnight peracute course of illness of only approximately 6 h. Pathological inspection showed multiple organ alterations very similar to those of Kiba. All organs analysed by nested PCR were strongly EIHV-1-positive, including liver, heart, kidney, intestine and blood. In addition, herpesvirus-like particles were found by electron microscopy.

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