Partial characterization of a novel gammaherpesvirus isolated from a European badger (Meles meles)

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A herpesvirus causing a cytopathic effect was isolated from pulmonary fibroblast cultures established from a European badger (Meles meles). A study was undertaken to classify and to assess some in-vitro growth characteristics of this virus. From a panel of 27 mammalian cell lines, in-vitro replication of the badger herpesvirus (BadHV) was only demonstrated with a mink lung cell line, suggesting a high degree of host specificity. Using PCR with degenerate primers, three independent fragments of the BadHV genome were sequenced. The largest of these fragments comprised a 6.2 kb segment including the DNA polymerase and glycoprotein B genes. Phylogenetic analysis of these sequences demonstrated that the BadHV is novel and clearly grouped with members of the Gammaherpesvirinae. In view of the oncogenic and immunosuppressive potential of many related herpesviruses, it is possible that BadHV can impact on existing acute or chronic disease in badgers.

This report describes the partial characterization of a herpesvirus isolated from a European badger (Meles meles). The cytopathic effect (CPE) caused by this virus appeared in primary pulmonary fibroblast cultures established during the course of studies to evaluate a candidate DNA vaccine for Mycobacterium bovis (Chambers et al., 2001). These cultures were established from tissues collected as part of a statutory removal operation from a young adult female badger from Cornwall, South-West England, in 1996. At the time of presentation, this badger was in a poor nutritional state, weighing 6·0 kg. Post-mortem examination revealed a few scattered, 1 mm-diameter, white foci in the liver parenchyma and in the cortex of each kidney. Poorly defined 0·5 mm lesions were also observed in each lobe of the lungs. Histological examination of the lungs revealed multiple eosinophilic granulomas and diffuse inflammatory changes with eosinophils as the prominent cell type; the changes were of the type observed in verminous pneumonia. In the kidneys and liver, there were multifocal inflammatory mononuclear cell infiltrates in the interstitium. However, no inclusion bodies or other histological changes characteristic of virus infection were observed. Cultural, histopathological and immunological tests for the diagnosis of M. bovis infection proved negative.

After harvesting alveolar macrophages from lungs, a portion of the apical lobe was removed and comminuted and cells were dispersed using trypsin prior to seeding onto plastic culture flasks as described by Chambers et al. (2001). Once confluent, cultures were passaged by splitting at a ratio of 1:2. These cells were examined microscopically on alternate days. On the fourth passage, a CPE resembling that of a herpesvirus was observed. Examination of the infected cells by electron microscopy confirmed the presence of enveloped, herpesvirus-like particles with a capsid diameter of approximately 105 nm. Since there are no published reports of a herpesvirus in this species, a more detailed investigation into the phenotypic characteristics and molecular classification was undertaken.

The difficulty in obtaining known virus-free badger tissues for further culture indicated that propagation of the virus in an alternative, reproducible cell system would facilitate characterization of the virus. A computer search of the cell culture collections at ATCC and ECACC for cells derived from members of the Mustelidae revealed a single cell line, NBL-7 (ATCC CCL 64), an epithelial cell line derived from foetal mink lung (ML). The cells, each in two, 25 cm² plastic flasks, were inoculated within 48 h of seeding with 200 µl clarified, infected badger lung fibroblast supernatant. A further 25 cm² plastic flask of each cell type was sham-infected as a control. The medium in the flasks was replaced every 5 days. The cells were observed daily for 10 days before passaging by splitting at a ratio of 1:4. This passage was repeated twice, after which the flasks were frozen at −80 °C. The flasks were thawed and the

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The nucleotide sequence data reported in this paper have been submitted to GenBank under the accession numbers AF376034 (gB–Dpol fragment), AY050215 (BNRF1 gene-equivalent fragment) and AF275656 (terminase fragment).
**Fig. 1.** Alignment of the deduced amino acid sequences of the gB (a) and Dpol (b) ORFs of BadHV compared with representative gammaherpesviruses (GenBank accession numbers shown). Gaps (-) were inserted to maximize the alignment and dots (.) represent residues that are conserved with the BadHV sequence. Filled triangles (▼) indicate important conserved residues.
cysteine residues for gB, asterisks (*) show highly conserved amino acids involved in Dpol exonuclease catalysis and filled circles (●) show other invariant residues across all viruses shown. Potential N-glycosylation sites of gB are denoted by shaded bars above the alignment, the location of the proteolytic cleavage site is shown by a double box and putative transmembrane-spanning regions (TM1, TM2) are shown. The positions of important functional motifs in Dpol (3'-5' exonuclease, I–III; polymerase, A–C) are also shown (Knopf, 1998).
supernatant was clarified by centrifugation at 1000g for 10 min at 4°C. Early observations indicated that the virus could be propagated, albeit slowly, and could produce a visible CPE in the ML cells. This enabled the ML cell system to be used to confirm the presence or absence of replicating virus in an additional 26 mammalian cell types. Selection of these cell lines was based on a number of criteria including origin species with potential badger contact, embryonic, foetal or neonatal source, phylogenetically related species to origin species to the badger and of lung or lymphoid origin. In order to demonstrate the recovery of infectious virus, 1 ml of the clarified supernatant from each of the selected cell types was inoculated back onto ML monolayers in 25 cm² flasks. These back passages were observed for a further 10 days. Of the 26 cell types tested, propagation of the virus was successful only in the ML cells. Following further serial passage in the ML cells, the time period for appearance of a visible CPE was reduced from 21 days initially to 10 days post-inoculation of cell-free virus. By the tenth passage, an infective titre of 10⁵.5 p.f.u./ml was obtained in clarified supernatants of infected ML cells. In ML cells, the appearance of isolated foci of rounded cells with refractile borders marked the initial signs of the CPE. Affected cells then coalesced to form small to large syncytia with attendant gaps in the cell sheet and, by 13 days post-inoculation, the CPE was close to 100%. The failure to propagate the virus in any of the cell types screened other than the ML cells indicated that the badger herpesvirus (BadHV) has a restricted host range, suggestive of a beta- or gammaherpesvirus (Roizmann et al., 1992).

Cultures of ML cells exhibiting advanced CPE were chosen for PCR and subsequent sequencing studies. DNA was extracted from clarified-infected-cell supernatant as described previously (Banks, 1993). In order to generate sequence data, three sets of degenerate pan-herpesvirus primers were used (with approximate sizes of the amplicons): Dpol (300 bp; Rozenberg & Lebon, 1991), Dpol (1300 bp; King et al., 1998) and terminase (500 bp; Hargis et al., 1999). Amplicons were cloned (pGEM-T easy, Promega), after which three independent clones containing the PCR inserts were sequenced (Big-Dye, Perkin Elmer) and were assembled as overlapping fragments. The sequence of the Dpol gene was extended to encompass the full-length glycoprotein B (gB) and Dpol ORFs. This 6.2 kb fragment was sequenced using a combination of PCR with gB consensus primers [5' GAGCT(ACG)ATGCAGC- A(AG)AT(AC)AAAC 3' and 5' GTGTA(AG)TAGTT(AG)TAG- T(A)CT(TCCCT 3'] and gene-walking by using a Universal GenomeWalker kit (Clontech Labs). Both ORFs possess canonical polyadenylation signals (AATAAA: gB +101 nt, Dpol +179 nt) downstream of the termination codons. The overall G+C composition of this fragment (analysis of nucleotide sequence from ATG start of gB to stop codon of Dpol) was 37.8% compared with 57.2% and 66.1% for the respective comparable regions of Equid herpesvirus 2 (EHV-2) and Human herpesvirus 1 (HSV-1) (intervening UL28 and UL29 genes removed from HSV-1 for this analysis). Furthermore, the ratio of observed/expected frequencies of the dinucleotide CpG in this fragment was markedly suppressed, at 0.2, and was compensated for by a concomitant increase in CpA, this dinucleotide having an observed/expected ratio of 1.25. A CpG deficiency has been associated with methylation of the DNA and has been observed in beta- and gammaherpesvirus genomes (Honess et al., 1989; Karlin et al., 1994). The CpG deficiency of betaherpesviruses is restricted to transcriptional regulatory genes such as the immediate-early genes (Honess et al., 1989). Although less than a tenth of the BadHV genome sequence was determined in this study, the presence of a CpG deficiency in key structural and replicative genes provided preliminary evidence that the virus is most likely to be a gammaherpesvirus.

The putative proteins encoded by the gB (815 amino acids) and Dpol (996 amino acids) ORFs possess many of the features typically evident for other herpesviruses (Fig. 1). In particular, analysis of the gB ORF highlighted a single potential proteolytic cleavage site (R–R–K–R) located at residue 417 (Fig. 1a), collinear with members of the gammaherpesvirus subfamily (Holloway et al., 1999). The presence of a predicted signal peptide (aa 1–21; Nielsen et al., 1997) and at least two putative membrane-spanning regions (aa 680–702 and 707–726; Krogh et al., 2001) was also revealed. Other structural components were also evident, such as 10 conserved cysteine residues forming five critical disulphide bridges (Norais et al., 1996) and 15 potential N-linked glycosylation sites, of which only three were positionally conserved across members of the gammaherpesvirus subfamily. In common with gB, analysis of a multiple alignment containing the Dpol amino acid sequence of BadHV and other herpesviruses also revealed the presence of a number of highly conserved motifs (Fig. 1b). Many of these were shared among not only other herpesviruses, but also the DNA polymerase genes of eukaryotes and prokaryotes. In particular, there was conservation of important regions such as the 3′–5′ exonuclease motifs and putative polymerase catalytic sites that have been recognized for members of the family B of Dpol genes (Knopf, 1998).

Phylogenetic analysis was performed using the predicted translated amino acids of the ORFs of BadHV aligned (CLUSTAL) with homologous regions of other published herpesvirus sequences (Fig. 2). Trees were constructed using the PROTDIST (Dayhoff PAM matrix) and NEIGHBOR programs of PHYLIP (version 3.5c; Felsenstein, 1993) and were tested by SEQBOOT and CONSENSE programs to determine the degree of support for the particular tree nodes. Gammaherpesviruses are formally assigned to the subfamily Gammaherpesvirinae, which currently contains two genera, Rhadinovirus and Lympahocryptovirus, with herpesvirus saimiri (HVS; Saimiri herpesvirus 2) and Epstein–Barr virus (EBV; Human herpesvirus 4) as respective prototype members. Although not all species within the Gammaherpesvirinae are clearly resolved using the phylogenetic analysis, as shown by low (<70%) bootstrap values...
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Fig. 2. Consensus phylogram of 2000 neighbour-joining trees generated for translated amino acids of the BadHV gB (a) and Dpol (b) ORFs compared with equivalent genes of the following other gammaherpesviruses (with GenBank accession numbers in parentheses): EBV (Human herpesvirus 4) (V01555), PLHV, PLHV-1 and PLHV-2 [porcine lymphotropic herpesvirus (AY044427) and PLHV-1 (AF191042) and -2 (AF191043)], AlHV-1 (Alepheline herpesvirus 1; AF005370), OvHV-2 (Ovine herpesvirus 2; AF385439), EHV-2 and EHV-5 (Equid herpesvirus 2; U20824) and Equid herpesvirus 5 (AF050671), AtHV-3 (Ateline herpesvirus 3; AF083424), HVS (Saimiriine herpesvirus 2; X64346), BoHV-4 (Equid herpesvirus 4; AF318573), HHV-8 (Human herpesvirus 8; U75698), RRV (Rhesus rhadinovirus; AF29302), MRV (macaque rhadinovirus; AF210726 and AF005479), RhinoHV (rhinoceros herpesvirus; AF287948) and MuHV-4 (Murid herpesvirus 4; AF105037). HSV-1 (Human herpesvirus 1; D10879), a member of the alphaherpesvirus subfamily, was chosen as an outgroup for these analyses.

and short branch lengths of some of the bifurcations, BadHV is clearly rooted within the genus Rhadinovirus. Similar phylogenetic results were obtained after analysis of the terminase gene fragment of BadHV (data not shown). Partial Dpol gene sequences were also available for a number of additional gammaherpesviruses. Phylogenetic analysis performed using these limited data placed zebra herpesvirus (GenBank accession no. AF141889), wild ass herpesvirus (AF141888), tapir herpesvirus (AF141887), EHV-5 (AF141886) (Ehlers et al., 1999) and phocine herpesvirus 2 (T. Goldstein, personal communication) in the cluster of gammaherpesviruses closely related to BadHV (data not shown).

An additional fragment of BadHV DNA corresponding to 301 bp of the BNRFT gene-equivalent of EBV was amplified by PCR and sequenced direct from the DraI digest/link-adaptor library prepared for genome walking. This gene, encoding a membrane antigen, has only been identified in the genomes of members of the gammaherpesvirus subfamily (Baer et al., 1984) and was amplified using primer AP1 (Universal GenomeWalker kit, Clontech Labs) and a degenerate sense primer [5’-GA(AG)(AT)(GC)(ATCG)(AOG(ATCG)TG)(CT)(ATCG)-AA 3’]. This observation further supported the classification of BadHV as a gammaherpesvirus.

In summary, we believe that this is the first time that a herpesvirus has been isolated from a badger. We have tentatively assigned the virus the formal name Mustelid herpesvirus 1 (MusHV-1). The pathogenicity of this virus remains unknown, since herpesvirus infections are often latent and do not show lesions at the time of isolation. The parasitic lesions in the lung of this badger are frequent findings in badgers from Cornwall; they may have contributed to the poor bodily condition and may not be related to the virus infection. The non-specific kidney and liver inflammatory changes are of unknown origin. The prevalence of BadHV in the badger population of the UK is impossible to gauge accurately from the data presented here. Although relatively few virological surveys of the European badger have been carried out, with the exception of a rabies virus isolated from a badger in Poland (Serokowa, 1968) and evidence for canine distemper virus isolated in badgers (Armstrong & Anthony,
1942; van Moll et al., 1995), there are no other references to the isolation of a virus from this moderately common mammal. The data presented here are insufficient for any firm conclusions to be drawn in relation to the prevalence of this virus in the badger population of Britain. However, a second isolation of this virus was made from a badger collected in Shropshire, which may indicate that infection of the UK population of European badgers with this virus is not uncommon.

Many herpesvirus infections predispose to secondary bacterial infections and, although the badger from which the isolation was made was not infected with M. bovis, a limited serological survey using archived sera from badgers with known tuberculosis (TB) status may provide some clues to the seroprevalence of this virus in relation to TB infection. It may also be important to determine whether BadHV has an immunosuppressive effect that may impact on badger populations and which, in particular, could compromise the response to vaccination against TB.

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References


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