Sequence analyses of human herpesvirus-8 strains from both African human immunodeficiency virus-negative and -positive childhood endemic Kaposi’s sarcoma show a close relationship with strains identified in febrile children and high variation in the K1 glycoprotein

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Human herpesvirus-8 (HHV-8) DNA sequences have been identified in all forms of Kaposi’s sarcoma (KS), a cancer found primarily in adult AIDS patients. We have identified HHV-8 strains in a rare human immunodeficiency virus (HIV)-negative form of KS, which is endemic in children in parts of sub-Saharan Africa. This was shown in Zambia, where we also had identified HHV-8 sequences in blood from HIV-negative febrile children without KS. In order to investigate the relationship of these Zambian strains to each other and to those from other forms of KS, we compared them to strains we have characterized from European AIDS KS (Denmark) and all published sequences from all forms of KS. Four distinct genomic regions were examined by PCR and sequencing: ORF26, ORF75, gH and K1. The results showed a distinct grouping of strains from both sets of Zambian children in all genomic regions studied, but which was most pronounced in the K1 glycoprotein gene. This gene was highly variable, encoding up to 25% amino acid sequence variation. In contrast, the Zambian groups were closely related to each other, with only 2% variation. Similar results were found in comparisons to the K1 sequences from HIV-positive febrile infants or KS children. The data raise the possibility that in areas where rare childhood endemic KS occurs, geographical variation in HHV-8 may relate to differences in virulence or transmission.

Introduction

Human herpesvirus-8 (HHV-8) DNA sequences were first identified in biopsy tissue from an AIDS-related Kaposi’s sarcoma (KS) (Chang et al., 1994). Subsequently, sequences have been identified in all forms of KS: classical, African endemic, AIDS-associated ‘epidemic’ (Chuck et al., 1996; Huang et al., 1995), post-transplant (Besnard et al., 1996), and other non-KS conditions like Castleman’s diseases (Soulier et al., 1995) and primary effusion lymphomas (PEL, or body cavity-based lymphoma, BCBL) (Cesarman et al., 1996). The complete genomic sequence has been derived from a strain, BC1, which has established a persistent infection within a B lymphocyte cell line derived from a BCBL (Russo et al., 1996). It has also been derived from an AIDS KS sample, termed here KS-FN (Neipel et al., 1997a, b). The genome encodes homologues of conserved proteins of the human herpesviruses and shows greatest similarity and gene order with the gamma-herpesviruses including Epstein–Barr virus (EBV; Russo et al., 1996), but more specifically a sub-group referred to as gamma-2 herpesviruses, which includes the monkey virus herpesvirus saimiri (HVS) and other animal herpesviruses with relations to various lymphoproliferative diseases (Albrecht et al., 1992; Efstratiou et al., 1990; Ensser et al., 1997; Gompels et al., 1988).

We have been investigating a rare form of KS which is found in HIV-negative children, termed childhood endemic KS (KS-CE). This condition occurs in regions of sub-Saharan Africa, including Zambia and Uganda. Our studies have centred on a Zambian paediatric population where we have identified HHV-8 by PCR analyses in both KS-CE as well as infants admitted to hospital who have encountered their first
febrile episode but do not show symptoms of KS (Kasolo et al., 1997). The occurrence of KS-CE within certain populations could be due to many factors. From the description of the association of an infectious agent, HHV-8, with the development of KS, one possibility that can be addressed is the contribution of strain variation to disease.

Here we have extended our Zambian study to investigate the nature of the HHV-8 strain variants present in these distinct paediatric patient groups. We used sequence analyses in order to examine the relationship of the strains present within the KS and non-KS children and also to compare to other strains now grouped within proposed classification schemes. In addition, we have analysed AIDS KS strains from a geographically distinct region (Denmark) in order to examine the relationship to other European strains, to compare with the level of variation between strains found in a different area and to outgroup our analyses from the Zambian strains. Furthermore, as the results showed that the Danish and Zambian strains were easily differentiated, these acted as internal controls for any laboratory PCR contamination.

Few reports are about the types and variability of circulating HHV-8 strains. Recent results have addressed the question of strain variability by comparing variation of limited regions of HHV-8 over the minor capsid region (233 bp fragment, ORF26) and the tegument region (ORF75) of HHV-8 DNA sequences from mostly adult KS patients (Zong et al., 1997). We have used these regions as well as two additional regions: one being a conserved region which shows strain variation in other herpesviruses, glycoprotein H (gH; Gompels et al., 1993), and another which is unique to HHV-8, the K1 glycoprotein gene (Russo et al., 1996), and which may have a role in HHV-8-specific biology. We show variation in all regions analysed. In particular, we demonstrate that the K1 glycoprotein is highly variable, whereas it is markedly conserved between both groups of Zambian children analysed here in the presence or absence of KS. These results are discussed in terms of K1 variation, virulence and transmission.

**Methods**

**Patients and DNA preparation.** DNA was prepared and analysed from 53 samples of whole blood (2-4 ml) from febrile infants (FI) and 10 biopsy tissue samples from children with endemic KS seen at the paediatric department of the University Teaching Hospital (UTH), Lusaka, Zambia, as described previously (Kasolo et al., 1997). The UTH is a 2000-bed hospital and is the largest general hospital in Lusaka, serving an estimated million people (10% of the population). In addition, it is the main national referral hospital and receives most KS referrals from across the country. Except where designated HIV-positive (see Fig. 6), all the infants and children in this study were HIV-seronegative (Figs 1-5) as determined by the commercially available ELISA (Welcozyme ELISA VK 57, Murex Diagnostics). All seronegative samples were also negative as determined by the commercially available ELISA (Welcozyme ELISA VK 57, Murex Diagnostics). All seronegative samples were also negative as determined by the commercially available ELISA (Welcozyme ELISA VK 57, Murex Diagnostics). All seronegative samples were also negative as determined by the commercially available ELISA (Welcozyme ELISA VK 57, Murex Diagnostics). All seronegative samples were also negative as determined by the commercially available ELISA (Welcozyme ELISA VK 57, Murex Diagnostics).

The thermostopy procedure was 94 °C for 4 min, then 50 cycles at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 120 s. Each run contained two negative controls (water and cellular DNA prepared from a human T lymphocyte cell line, JHhan). The PCR products were resolved and detected visually by 0.8% agarose gel electrophoresis with ethidium bromide staining. All samples were amplified independently at least twice and in all cases HHV-8 DNA was not detected in the PCR negative controls.

**DNA purification, cloning and sequencing.** The DNA PCR products generated by the gH and K1 primer sets with introduced BamHI restriction enzyme sites for cloning, PCR was done under standard conditions (Kasolo et al., 1997). The thermostopy procedure was 94 °C for 4 min, then 50 cycles at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 120 s. Each run contained two negative controls (water and cellular DNA prepared from a human T lymphocyte cell line, JHhan). The PCR products were resolved and detected visually by 0.8% agarose gel electrophoresis with ethidium bromide staining. All samples were amplified independently at least twice and in all cases HHV-8 DNA was not detected in the PCR negative controls.

**PCR amplification.** Sample DNA was amplified with five HHV-8 primer sets. One additional set was used as a cellular DNA-positive control (human prolactin gene) (Kasolo et al., 1997). The ‘KS233’ set is from ORF26, a 233 bp fragment of the minor capsid protein gene (Chang et al., 1994). The other primer sets were designed using the genomic sequence (Russo et al., 1996), including ORF75, a 423 bp fragment of a tegument protein gene; ORF22, 332 bp of the gH gene; and ORFK1, 221 bp encoding the N-terminal fragment (K1N) and 616 bp encoding the K1 external domain (K1EX).

**Primer sets:** (1) KS233, ORF26 (Chang et al., 1994), 5’ agcgcagaggtattcaccact 3’ and 5’ ctgcaagtcagcaacaggca 3’; (2) tegument, ORF75, 5’ agggaggcttccaggtctag 3’ and 5’ agatagcgccttgctgacggct 3’; (3) gH, ORF22, 5’ ctagaggctgatcagggctgtag 3’ and 5’ gtcgaggcttcagcatagca 3’; (4) K1 N terminus (K1N), 5’ agaggattcgtatgtcatctgacq 3’ and 5’ atgggatcgaattaalgcgtctgatq 3’; (5) K1 external domain (K1EX), 5’ gggtcctttcaggacttaa 3’ and 5’ taccagttcatgcaactat 3’.

**Primer sets 2-4 are modified to contain restriction enzyme sites for cloning.** PCR was done under standard conditions (Kasolo et al., 1997). The thermostopy procedure was 94 °C for 4 min, then 50 cycles at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 120 s. Each run contained two negative controls (water and cellular DNA prepared from a human T lymphocyte cell line, JHhan). The PCR products were resolved and detected visually by 0.8% agarose gel electrophoresis with ethidium bromide staining. All samples were amplified independently at least twice and in all cases HHV-8 DNA was not detected in the PCR negative controls.

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adults with all forms of KS including classic Mediterranean,
Zong et al, 1997). For these reasons, this region was first
analysed in the specimens collected here.

In our previous studies, the KS233 primer set was used to
identify and semi-quantify HHV-8 present in both blood from
febrile infant patients, all under 1 year, it was possible that
Given the high levels of HHV-8 DNA identified and the age of
febrile infants and childhood endemic KS
Chang et al. (1994) first identified HHV-8 sequences in an
AIDS KS. Analysis was extended to a 20 kb fragment and the
strain is termed here KSHV (Moore et al., 1996). The minor
capsid gene was described, together with a specific set of
primers for use in PCR-based detection of HHV-8. This is
‘KS233’, the 233 bp fragment within the KS330Bam clone,
marked in Fig. 1 as KSHV (Chang et al., 1994). With the
determination of the complete genome sequence from a PEL-
derived strain, BC1, this gene is now defined as ORF26 (Russo
et al., 1996). The KS233 primers for ORF26 are the most
widely used primer set for PCR detection of HHV-8 and
sequence analyses. It is a conserved, essential gene in all
herpesviruses and has been the basis of proposed sub-
classification schemes for HHV-8 strains (Huang et al., 1995;
Zong et al., 1997). For these reasons, this region was first
analysed in the specimens collected here.

Here we present the sequences of the complete fragments
identified in these Zambian children’s samples and compare
them to all the sequences in this region published to date,
which have been placed in a provisional classification scheme
(Zong et al., 1997). The published sequences are derived from
adults with all forms of KS including classic Mediterranean,
USA AIDS epidemic, African endemic (adult, from Uganda and
DRC, formerly Zaire) as well as the lymphomas, BCBL or PEL
(Chang et al., 1994; Huang et al., 1995; Neipel et al., 1997a;
Russo et al., 1996; Zong et al., 1997). We also analysed adult
AIDS KS from Denmark as a further distinct geographical
location in order to investigate the relationship to the Zambian
strains and also to compare to the level of variation found
between strains in a different location. The results showed that
the Danish HHV-8 sequences had greater variation between
strains and also were the most distinct from the Zambian
strains. Thus, the Danish sequences also served to outgroup the
Zambian phylogenetic analyses and acted as controls for
any laboratory PCR contamination. At the end of these
analyses all samples were re-tested blind in random mixtures of
DNA samples from the distinct locations. All primer sets were
used including those described in the following sections and
all the results were duplicated, with no evidence of PCR
contamination. This was also controlled by using negative
controls of multiple water samples and uninfected cellular
DNA, which all remained negative after repeated amplifica-
tions.

The results of the PCR and sequence analyses of ORF26
showed there was an overall 2% variation across this region at

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### Results

**KS233 analyses show a new group of HHV-8 in febrile
infants and childhood endemic KS**

Chang et al. (1994) first identified HHV-8 sequences in an
AIDS KS. Analysis was extended to a 20 kb fragment and the
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In our previous studies, the KS233 primer set was used to
identify and semi-quantify HHV-8 present in both blood from
febrile infant strains are indicated by an asterisk here and in all following figures.
The KS-CE are indicated by ‘A’ through to ‘J’ and correspond to patients
from reference (Kasolo et al., 1997) SS14461, SS16217, SS16233,
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SS16552. Danish AIDS KS samples analysed are indicated by DMKS (1 to
4). Prototype strains are BC1, KS-FN and KSHV derived from USA
BCBL/PEL, German AIDS KS and USA AIDS KS, respectively (Chang et al.,
1994; Neipel et al., 1997a; Russo et al., 1996). Percentage differences
are from calculated branch points.

![Phylogenic analysis of the KS233 fragment within ORF26, minor
capsid gene; nucleotide sequence. The strains from the febrile infants and
childhood endemic KS are indicated as A/KS-CE and group differently
from all other HHV-8 sequences analysed to date. These other strains are
from adults and are from all forms of KS plus BCBL. The compilation is
from Zong et al. (1997), showing three groups A, B or C. Types of the
different A, B or C strains are indicated as reference strains as RA1–3,
RB1–5 and RC1–2. Unclassified strains described are also included,
plus the prototype strains from the original HHV-8 KS sequence, termed
KSHV, as well as the genomic BC1 strain from a BCBL. Both of the
prototype strains group with the reference strains of group A here. Febrile
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**Fig. 1.** Phylogenic analysis of the KS233 fragment within ORF26, minor
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Fig. 2. (a) Phylogenetic analysis of the ORF75 gene (tegument protein) fragment covering KS631; nucleotide sequence. Same nomenclature as for Fig. 1. All reference strains described by Zong et al. (1997) are used for comparison (RA, RB, RC) and are the published sequence to date including all forms of KS as well as BCBL, again in adults. Note in this case that the prototype strains are diverging in their grouping, KSHV with reference strains A, and BC1 with reference strains C. (b) Phylogenetic analysis of ORF22, the gH gene fragment. Nucleotide sequence variation to 2%. Unusually, all the substitutions are coding changes, thus the same relationships are shown for both nucleotide and amino acid sequence phylogenies (see text). The amino acid sequence phylogeny shown here has greater variation, to 3%. Nucleotide changes are in Table 1. They encode the following amino acid changes at these BC1 genomic positions: 37315 Ala/Ser; 37412 Val/Ala; 37483 Lys/Gln; 37490 Gln/Lys; 37504 Phe/Val; 37520 Val/Ala.

10 sites. Few were coding changes (less than three). Most sites were represented in multiple strains indicative of virus variation rather than random errors introduced during PCR. The phylogenetic analysis showed that the adult Danish AIDS KS (DMKS) clustered with established classified groups, whereas the KS-CE (samples A to J) clustered with the FI (samples 13, 14 and 351) in a distinct grouping (Fig. 1). There was one exception, KS-CE sample A, which clustered with strains representing a B group classification (Zong et al., 1997). This could be interpreted as an exception to the KS-CE grouping. Alternative interpretations are that the virus is a recombinant or that the level of variation observed at this site is too low to make adequate groupings. In order to further investigate the relationships of these strains, additional sequences across the genome were examined.

Conserved gene analyses supports grouping

Two further regions of the HHV-8 genome were analysed for strain variation. Another gene was also one of the first HHV-8 sequences reported, KS631Bam, marked in Fig. 2 as KSHV strain (Chang et al., 1994). This sequence is in a gene encoding a tegument protein, ORF75, which is conserved amongst all gammaherpesviruses examined (Albrecht et al., 1992; Gompels et al., 1988; Russo et al., 1996). It has also been used together with KS233 for strain grouping (Zong et al., 1997). A new primer set was designed to amplify a 425 bp gene fragment. We also chose to examine a 332 bp fragment from the N-terminal region of the gH homologue, ORF22, a gene that encodes a glycoprotein conserved in all human herpesviruses which is important for infection (Gompels et al., 1995; Russo et al., 1996). Our previous studies of strain variation in other herpesviruses had shown that this is a region which showed sufficient variation for grouping strains (Gompels et al., 1993).

Sequences derived for both these regions, ORF75 and ORF22 (gH), for the DMKS, FI and KS-CE strains were compared to all published KS HHV-8 sequences. The phylogenetic analyses show again that the FI and KS-CE sequences cluster together in a new group (Fig. 2a, b). In both regions, up to 2% variation is observed at six sites in gH and 10 sites in ORF75. Similar levels of variation were encoded in the amino acid sequence, the most was in gH (3%) (Fig. 2b). Within ORF75, all FI and KS-CE strains cluster together in a separate group. This is also shown for gH, with minor exceptions for FI 13 and KS-CE sample A (Fig. 2).

Taken together, the analyses for ORF22 (gH), ORF26 (capsid) and ORF75 (tegument) support a new grouping for the FI together with the KS-CE strains.

Strains unclassifiable in comparison to previous grouping systems

Hayward and colleagues have proposed a system of classification of HHV-8 strains based on variation in the ORF26 and ORF75 genes (Zong et al., 1997). Using their
Table 1. HHV-8 sequence variation from childhood endemic KS and febrile infants compared to AIDS KS versus prototype and grouped reference strains

The reference and prototype strains are from adult KS or BCBL. Prototype strains KSHV genomic fragment, KS-FN and BC1 genomic sequences are from the USA, German AIDS KS and USA BCBL, respectively (Moore et al., 1996a; Neipel et al., 1997; Russo et al., 1996). Reference group strains from Zong et al. (1997): AKS1, USA AIDS KS; 431KAP, DRC (formerly Zaire) endemic KS; ST1 and ST2, Uganda AIDS KS. The study strains are from adult AIDS KS from Denmark (DM-KS) and childhood samples from Zambia which are 'FI' febrile infant strains from blood, and 'KS-CE', childhood endemic KS. Nucleotides in bold denote group (A, B or C) diagnostic sites for ORFs 26 and 75 as devised for the reference strains by Zong et al. (1997). Asterisks indicate nucleotides which do not fit in the reference grouped strains and contribute to the unclassified status (UN). These are: 1, T; 5, G; 6, C; 7, G or T; 9, C; 16, C; 17, C; 20, T; 21, C; 22, C; 24, C. Genomic positions for nucleotides 1–24 for the BC1 strain are: 1, T; 2, C; 3, C; 5, G; 6, C; 7, G or T; 8, C; 9, C; 16, C; 17, C; 20, T; 21, C; 22, C; 24, C.

<table>
<thead>
<tr>
<th>DNA (Group)</th>
<th>Reference</th>
<th>Prototypes</th>
<th>DM-KS</th>
<th>FI/KS-CE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ORF22 (gh)</td>
<td>ORF26 (mCP)</td>
<td>ORF75 (Tegument)</td>
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<tr>
<td>AKS1 (A)</td>
<td>-- -- -- -- --</td>
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<tr>
<td>431KAP (B)</td>
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<td>T G G C C A T G A</td>
<td>-- -- -- -- --</td>
<td>-- -- -- -- --</td>
</tr>
<tr>
<td>ST2 (B)</td>
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<td>G T G G T C G T G</td>
<td>-- -- -- -- --</td>
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<tr>
<td>ST1 (C)</td>
<td>C T G G T A A C G</td>
<td>T G A C C G T T G</td>
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<td>KSHV (A)</td>
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<tr>
<td>BC1 (A/C)</td>
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<td>G T A A C C G C T G</td>
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<td>Prototypes</td>
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<tr>
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<td>C T C G G T</td>
<td>G A A C G C C A</td>
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<tr>
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</tr>
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<td>KS-CE E (UN)</td>
<td>G T C G G G</td>
<td>C T C G G A</td>
<td>C A C T G G</td>
<td>C T C T</td>
</tr>
<tr>
<td>KS-CE F (UN)</td>
<td>G C C G G C</td>
<td>T C C G G A</td>
<td>C A C T G G</td>
<td>C T C T</td>
</tr>
<tr>
<td>KS-CE G (UN)</td>
<td>G C C G G C</td>
<td>T C C G G A</td>
<td>C A C T G G</td>
<td>C T C T</td>
</tr>
<tr>
<td>KS-CE I (UN)</td>
<td>G C C G G C</td>
<td>C C C G G C</td>
<td>C C C G G C</td>
<td>C C C G G C</td>
</tr>
</tbody>
</table>

–, Not known.

scheme and all the sequences they analysed, the data collected on the DMKS, FI and KS-CE samples were compared (Table 1). Prototype strains BC1, KSHV and KS-FN were included as well as reference strains from the A, B and C groups chosen from USA AIDS KS (AKS1, A group), African endemic KS (431KAP, group B) and African AIDS KS (ST2, group B; ST1, group C). Here none of the samples conform easily to any of the A, B or C classifications, although the DMKS appears more similar to European and American AIDS KS (groups A and B).

The FI and KS-CE strains clearly do not belong within the A grouping. They mostly seem to share some features with one of the other groups, or sometimes all three. In some cases samples have no diagnostic residues, in others they seem to be a combination, and in all of them there appear to be new diagnostic residues that do not conform to the A, B or C grouping. Moreover, compared to the gH sequences all the prototype strains are identical but different from the strains analysed here. The new diagnostic residues for the FI and KS-CE strains appear in up to 14 sites and appear to be more characteristic of this new grouping. Taken together, these analyses, summarized in Table 1, from three distinct parts of the genome show that the FI and KS-CE strains seem to fall into a new unclassifiable group consistent with the analyses shown by phylogeny (Figs 1 and 2).
**K1 analyses show high variation and support a new strain grouping in both HIV-negative and -positive patients**

Since the sequence analyses of ORF22, ORF26 and ORF75 pointed towards a new grouping of FL and KS-CE strains, examination of sequences with a higher level of variation would unambiguously define this new group. Preliminary analyses of the genomic strains suggested that the K1 gene may be such a site suited for strain grouping. The HHV-8-specific K1 gene encodes a putative glycoprotein and is located at the extreme left end of the genome, adjacent to the terminal repeats (Russo et al., 1996), a region within other herpesviruses which is often subjected to a higher rate of variation due to the mutagenic effects of the repetitive sequences (Gompels et al., 1995). The K1 region was chosen to further explore the differences observed in the FL/KS-CE grouping. In addition, comparisons to K1 sequences from another BCBL cell line (termed here BCBL) (Lagunoff & Ganem, 1997) showed 6% variation (not shown). Furthermore, at the end of the studies described here comparisons were made with KS-FN, the complete genomic sequence from an AIDS KS (Neipel et al., 1997a). This also showed high K1 variation. The most variation between these prototype strains was observed within the encoded extracellular domain, in particular at the N-terminal region (not shown).

Primers were designed to examine variation in the N-terminal variable part of K1, termed K1N. The level of variation shown here would be sufficient to test the grouping of the FL and KS-CE strains. K1 may also encode amino acid sequences diagnostic for this new grouping which could be indicative of biological differences. A variable 221 bp region encoding the N-terminal domain of the K1 glycoprotein was selected for PCR and sequence analyses. Sequencing of the FL, KS-CE and DMKS strains followed by phylogenetic analyses showed that both the nucleotide and amino acid sequence variation clearly grouped the FL and KS-CE strains distinctly from the adult samples. Up to 10% and 25% variation was observed between the nucleotide and amino acid sequences, respectively (Fig. 3). The European AIDS KS strains clustered separately from the BCBL and PEL strains. The most distinct grouping was between the FL and KS-CE, which were closely related as shown for the other regions, with only 1–2% variation in contrast with up to 25% variation observed in comparisons to all the other published strains, and to 20% variation between the DMKS strains (Fig. 2).

The sequences were further characterized by multiple alignment analyses in order to identify possible diagnostic sites of variation in this new grouping of HHV-8 strains and also sites of coding differences which may correlate with biological features. In Fig. 4, the multiple alignment of the nucleotide sequences from the K1 N terminal region is presented. There are 39 variable sites with 28 coding changes. Of the 39 variable sites, more than from the other three genes put together, 25 sites appear to correlate with the FL/KS-CE grouping. These sites are indicated on the alignment (Fig. 4b).

In order to investigate this K1 variation further, the entire encoded extracellular domain of K1 (K1EX) was PCR-amplified and sequenced from one KS-CE sample and compared to prototype strains BCBL and BC1 from USA PELs and KS-FN.
Fig. 4. Multiple alignment of the nucleotide sequence (a) or amino acid sequence (b) of the N-terminal-encoded K1 (K1N) from FI/KS-CE strains compared to DMKS strains and prototype BC1, BCBL and KS-FN strains. Dashes indicate identity. Asterisks indicate substitutions which cluster within the FI and KS-CE strains. Substitutions are additionally marked where they are exclusive for the FI and KS-CE strains (FI13, 14 and 351; KS-CE A to J). Changes in the FI and KS-CE strains which result in the loss of one glycosylation site and the gain of a new site are underlined.

Interestingly, the K1 region also has a higher ratio of non-synonymous (coding) to synonymous substitutions, which may be indicative of some form of selection taking place at this locus (Niewiesk & Bangham, 1996). Of the four regions examined, only K1 and gH have this property, although K1 has higher variation, particularly at the N-terminal region (Table 2). In this region, a cluster of diagnostic amino acid changes from a German AIDS KS. In Fig. 5(a) the alignment of the nucleotide sequences shows 55 sites of variation with an in-frame deletion of 15 nucleotides in KS-FN absent from the other strains. Of all these substitutions, four are exclusive to the KS-CE sample and these are within the N-terminal domain. In Fig. 5(b) the alignment of the encoded amino acid sequences shows that of the 55 sites, most are coding changes (42) and the identified diagnostic substitutions for KS-CE are coding changes in the N-terminal domain. This shows that these changes are clustered in the N-terminal domain, which may correlate with specific biological properties of this new grouping of FI and KS-CE strains.
between residues 54–56 shows coding changes which introduce a new potential site for N-linked glycosylation into the molecule (NQS) and which could have marked effects on the processing, cellular localization and function of this molecule (see Fig. 4b).

Further analyses of K1 in HIV-seropositive Fl and childhood KS showed similar results as that shown for the seronegative group (Fig. 6). This indicates that the strains infecting the HIV-negative children are similar to those infecting children with the now more prevalent AIDS KS.

Discussion

This report identifies HHV-8 strains present in HIV-negative childhood endemic versions of KS and shows that they co-segregate with strains identified in infections of Fl in the absence of KS. These strains were distinct from previously described HHV-8. This grouping was shown for 14 HHV-8 strains identified in samples from HIV-seronegative Fl and KS-CE distinct from previously published data for strains from adult endemic KS, classic KS, AIDS KS and PELs as well as additional European AIDS KS (DM) analysed here (Huang et al., 1995; Lagunoff & Ganem, 1997; Neipel et al., 1997a; Russo et al., 1996; Zong et al., 1997). This has been determined from sequence analyses of five PCR products of between 220 and 616 bp amplified from four distinct regions of the genome. Comparisons were made to all other published data as well as the DM AIDS KS presented here, which showed up to 2% nucleotide sequence variation in ORF22 (gH), ORF26 (minor capsid protein, KS233) and ORF75 (tegument protein, KS631). Most variation was in the HHV-8-specific K1 gene, where there was up to 10% nucleotide variation encoding up to a 25% difference in amino acid sequences. In contrast, comparisons between the strains from the Fl and KS-CE group showed, on the whole, less than 2% variation in all regions, including K1. However, each strain was distinct in that not one strain was identical to another across all regions examined and similar relationships were shown by phylogenetic analyses of all the regions linked together. The new Fl and KS-CE grouping was distinct from previous classification schemes.

There were minor exceptions in the ORF26 and gH gene analyses, although these may be due to the low variability at these loci. Sample A in ORF26 analysis grouped with classified strains (groups A and C), whereas in ORF75 analysis it grouped with KS-CE, and in gH analysis it grouped with classified strains. Conversely, Fl 13 grouped with KS-CE in ORF26 and ORF75 analyses, but with classified strains (groups A and C) in gH analysis. In the more variable K1, both these samples clearly segregate with KS-CE. In these two cases it could be that these strains are recombinants or it could be that the level of variation within the two regions used for previous strain classifications (ORF26 and ORF75) border on the random fluctuation that is present within the HHV-8 genome and would be insufficient for unambiguous grouping. In the related gammaherpesvirus, EBV, as well as HVS, there are also examples where there is greater local genomic variation which relates to biology and grouping whereas in other regions the variation is low (Albrecht et al., 1992; Jenkins & Farrell, 1996; Sample et al., 1990).

Overall the data show that the strains identified in the non-KS Fl with HHV-8 infections are closely related to those identified in the KS children, both in the absence of HIV infection. These samples are from Zambia, a region of sub-Saharan Africa where KS is endemic and can present as KS-CE. The Zambian strains were distinct from previously described strains or strain grouping and had low variation between them, even in K1 and even between the non-KS Fl (blood) and KS-CE strains. The clustering of the KS-CE could be due to sampling from one hospital; however, the UTH is a national reference centre for Zambia and receives KS referrals from most (approximately 60%) of the population. Although no regional clustering was identified in the hospital records, UTH rarely sees cases from the north and deals with mainly central, southern, eastern and western regions. The co-segregation of the non-KS infant and KS-CE strains suggests a relationship. This close relationship of HHV-8 Fl and KS-CE strains appeared significant in that children co-infected with HHV-6 had different strain variants (Kasolo et al., 1997). Furthermore, HHV-8 strains identified in Zambian HIV-seropositive Fl and KS-CE show a similar close relationship (Fig. 6; F. Kasolo, M. Monze & U. A. Gompels, unpublished results), whereas the proviral HIV sequences were divergent (Nanteza et al., 1998). This indicates that in Zambia the virus strains identified in the HIV-negative children with or without KS-CE are related to the strains transmitted in the now more prevalent AIDS KS. This geographical variation may result in virulence differences, possibly with different transmission patterns. This is supported by the observation that since the advent of epidemic HIV/AIDS in Zambia there has been an increase in the occurrence of childhood KS (Chintu et al., 1995). However, this is still a relatively rare disorder with the incidence published at 6 per million children per year (Athale et al., 1995). The identification of HHV-8 in 8% of Fl in this population suggests infection with this virus may be more widespread (Kasolo et al., 1997).

African KS-CE presents in different forms to the predominant skin-associated adult AIDS KS in Western patients (Matondo & Zumla, 1996). KS-CE is a more disseminated condition with various degrees of lymphadenopathy and a rapidly progressing fatal form (Athale et al., 1995). Interestingly, some data suggest that KS-CE in Zambia presents differently from neighbouring KS endemic African countries in that cases affect mostly a younger age group (< 5 years old), with a lower male/female ratio and lower prevalence (Athale et al., 1995). Here we have shown that similar strains can be present in peripheral blood of Fl as well as within the sarcoma tissue of older children with endemic KS in Zambia. In this study the mean age of the children with KS
African childhood endemic KS and HHV-8 variants

Fig. 5. Multiple alignment of the nucleotide sequence (a) or amino acid sequence (b) for the encoded K1 external domain of KS-CE strain C compared to prototypes BC1, BCBL and KS-FN. Only the substitutions exclusive to KS-CE are marked.

Table 2. Non-coding and coding substitutions used to calculate the ratio of non-synonymous changes

<table>
<thead>
<tr>
<th></th>
<th>Nucleic acid changes/Total (%)</th>
<th>Amino acid changes/Total (%)</th>
<th>Non-synonymous changes/Total (Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1(N)</td>
<td>37/221 (17)</td>
<td>28/74 (38)</td>
<td>28/37 (76)</td>
</tr>
<tr>
<td>K1(EX)</td>
<td>55/616 (9)</td>
<td>42/205 (21)</td>
<td>42/55 (76)</td>
</tr>
<tr>
<td>ORF22</td>
<td>6/330 (2)</td>
<td>6/110 (6)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>ORF26</td>
<td>8/233 (3)</td>
<td>3/77 (4)</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td>ORF75</td>
<td>10/423 (2)</td>
<td>5/141 (4)</td>
<td>5/10 (50)</td>
</tr>
</tbody>
</table>

Fig. 6. Comparison of K1 phylogeny for both HIV-negative and -positive FI and CE-KS samples. Based on multiple alignments of the K1N region amino acid sequences. The '+' symbol indicates HIV-positive samples from FI and CE-KS samples.

was 9-4 years (Kasolo et al., 1997), although the mean age in an earlier prevalence study of Zambian KS was 5-4 years (Athale et al., 1995). If infection was taking place during infancy, these figures may reflect the incubation period to development of KS-CE. The time-scale resembles development of nasopharyngeal carcinoma, a neoplasm which involves multiple factors including a geographical component and infection with a gammaherpesvirus, EBV (Sun, 1995). Our data suggest that infection as an infant could be related to development of KS-CE in a particular geographical area and thus needs to be evaluated as a risk factor in these regions.

The finding of a separate grouping for the Zambian FI and
KS-CE strains, here designated as ‘Z’, raises the issues of differences in virulence and biological determinants which may correlate with disease associations. Only K1 had sufficient coding changes which correlated exclusively with this grouping, although there are likely to be other genes. K1 also had a high proportion of non-synonymous changes indicative of selection (Niewiesk & Bangham, 1996). These changes may affect biological differences. In the HHV-8-related HVS, the left end of the genome is the ‘transforming region’, which includes the STP and Tip oncogenes. These genes are in the same loci in HVS as K1 in HHV-8 and their presence correlates with transforming ability in vitro as well as oncogenicity in animal models (Kretschmer et al., 1996; Lee et al., 1997). K1 has been shown to functionally complement this transforming region of HVS (Lee et al., 1998). The gene encodes a product with characteristics of a transmembrane glycoprotein with similarity to the immunoglobulin superfamily and may have possible roles in signal transduction, as has been shown for the transforming region in HVS (Lund et al., 1997). The amino acid substitutions in the Z strain group identified here may affect the function of K1 or its immune recognition, which may contribute to differences in virulence and associations with the development of KS.

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References


African childhood endemic KS and HHV-8 variants


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