A 210-bp DNA segment specific to the human herpesvirus 8 (HHV-8) genome was amplified by nested polymerase chain reaction from 10 of 14 archived oral biopsy samples of HIV-positive patients in London who had no evidence of oral Kaposi's sarcoma (KS). Various oral sites were represented. Oral tissues from 20 general dental patients not known to be HIV-infected were negative. When DNA sequences of these products were compared with sequences derived from 5 oral KS tissues of AIDS patients in London and 10 skin biopsies of Italian patients with Mediterranean KS (total number of positive tissues = 25), 11 were found to be unique. DNA and predicted peptide motifs of these sequences were also different from those in 28 of 36 HHV-8-positive lesions previously reported from American and African patients. HHV-8 is tropic for the oral mucosa of HIV-infected persons, and HHV-8 variants, though diverse, may be geographically restricted.

Unique DNA sequences homologous to a capsid protein gene of γ-herpesviruses have been identified in cutaneous Kaposi's sarcoma (KS) lesions [1–3], blood of human immunodeficiency virus (HIV)-infected patients with KS [4], effusions of patients with AIDS-related body cavity–based lymphoma [5], semen [6], prostatic and urogenital tissue [6], and lymph nodes [7]. The putative virus from which these sequences derive has been assigned to the Rhabdinovirus genus [8]. The virus has been described as KS-associated herpesvirus or, more formally, human herpesvirus 8 (HHV-8). Here, we investigate the presence of HHV-8 DNA sequences in oral tissues of HIV-infected people in London and compare these with sequences identified in cutaneous KS lesions of Italian patients and those reported from HHV-8–infected tissues of patients in the United States and Africa.

Materials and Methods

Specimens. We examined formalin-fixed, paraffin-embedded tissues of 25 incisional oral biopsy samples taken from 23 patients with HIV infection who attended the oral medicine clinic of a genitourinary medicine department in central London. Of these, 11 had histologic features consistent with KS, 10 nonspecific ulceration, and 4 other diseases. Various sites of the oral mucosa were represented. Also studied were paraffin blocks of 16 skin biopsy samples from Italian patients with classic (Mediterranean) KS and 20 diagnostic biopsy samples from patients with various oral diseases attending general dental clinics (including 9 with oral squamous cell carcinoma). The HIV antibody status of the patients in the latter 2 groups was unknown.
Specimen preparation. Multiple sections, totaling 30–50 μm in thickness, were cut from each tissue block using disposable microtome blades that were changed between samples. The sections were deparaffinized, and DNA was extracted using GeneClean (Bio 101, Vista, CA).

HHV-8 DNA amplification. About 100 ng of DNA from each extract was processed for HHV-8 DNA amplification. Conditions and primers used to amplify a 233-bp fragment (the KS330 region), which is located in open-reading frame (ORF) 26 of the HHV-8 genome [8], were as described previously [1]. A nested polymerase chain reaction (PCR) was applied to an aliquot of the first-round amplificate to generate 210-bp products. The DNA sequence of the sense inner primer was 5'-TTCCACCATTGT-GCTCGAAT (from positions 366–385 of ORF 26); that of the antisense inner primer was 5'-TACGTCCAGGCATATGTTGC (positions 457–456). Cycling conditions were identical to those of first-round PCR. The nested PCR procedure is capable of amplifying, in a reaction tube, as little as 5 zg of the KS330 fragment ligated to a plasmid vector (data not shown); this is equivalent to 1 copy of the target sequence. The presence of DNA in each tissue extract was verified by amplifying a 110-bp fragment of the β-globin gene. As positive control, we used either extracts of KS tissue from an Italian patient or DNA from the BCBL-1 cell line (provided by E. Cesarman, Cornell University, Ithaca, NY). PCR detection of HHV-8 DNA was repeated at least once from each sample extract.

Characterization of HHV-8 DNA polymorphism. Amplified HHV-8 DNA was processed for single-strand conformation polymorphism (SSCP) analysis as previously described [9]. DNA sequencing of HHV-8 PCR products, without prior cloning, was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A DNA Sequencer (Perkin Elmer, Forest City, CA). Routinely, two opposing strands from each product were sequenced.

Amplification of DNA from other human herpesviruses. Extracts from oral tissues subsequently found to be HHV-8-positive were subjected to nested PCR procedures to amplify DNA specific to Epstein-Barr virus (EBV), cytomegalovirus (CMV), and HHV-7. For EBV, the primer sequences were 5'-TATGGGAGCCAGCC-GCAAAAGG-3' and 5'-GAAACCAGGAGCAATCTACT-3' (outer primers) and 5'-CGGAAAAGAGGAGGTGTGTTT-3' and 5'-CATCGTCAAAGCTGCACACAG-3' (inner primers); these flank a 470-bp segment in the BamHI K region of the genome (coordinates 109311–109780). For CMV, the primer sequences were 5'-GGAAACGTGTCCGTCTTCGA-3' and 5'-GAAACCAGGGAGGCAAATCTACT-3' (outer primers) and 5'-GTGTTCCTGGGATGGTTGG-3' and 5'-TAGATTTTTTGAAAAAGATTTAT-3' (inner primers), which flank a 245-bp segment in the glycoprotein B-coding region. For HHV-7, the primer sequences were 5'-TTCTCGAATAGGTATTTCATATAGTTAC-3' and 5'-GGCTGATTAGGATTAGATAGCATATGTTACAT-3' (outer primers) and 5'-GGAAACGTGTCCGTCTTCGA-3' and 5'-GGCGCGGCAATCGG-3' (inner primers) which flank a 124-bp region within the genome.

Heminested PCR was used to amplify a 176-bp segment of the HHV-8 genome, which is within the large tegument protein-coding region; the sequences of the primers were 5'-GATCCGACGCCTACAAACAC-3' (sense first- and second-round primer), 5'-TACGTCCAGGCATATGTTGC (antisense first-round primer), and 5'-GGCTGATTAGGATTAGATAGCATATGTTAC-3' (antisense second-round primer). Specificity controls used were DNA extracted from Namalwa cells (European Collection of Animal Cell Cultures [ECACC] catalog no. 87060801) for EBV and SUP-T1 cells (gift of R. Jarret, University of Cambridge) for HHV-7, and plasmids sp64/gB for CMV (gift of J. Sinclair, University of Cambridge) and HD9 for HHV-6 (ECACC catalog no. P91013124).

Results

HHV-8 DNA could be amplified from 5 of 11 KS and 11 of 14 non-KS oral tissues from HIV-positive patients, 10 of 16 classic KS tissues, and none of 20 oral samples taken from general dental patients. SSCP analyses and DNA sequencing demonstrated that HHV-8 sequences of 11 of the 25 positive tissue samples were unique. A common HHV-8 DNA sequence was identified in 3 KS oral samples, 5 non-KS oral samples, and 1 cutaneous KS sample. Another sequence was common to 5 KS tissues (2 oral and 3 cutaneous). Figure 1 illustrates
### Table 1

<table>
<thead>
<tr>
<th>Variant group</th>
<th>Molecular basis for grouping</th>
<th>Distribution of variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UK/Italy</td>
</tr>
<tr>
<td>A</td>
<td>NGPD P VPMHPVQPQLGHAIlQQQVyhyskisagap D DVMMAEDLYLYTVNIVSFMPQQ</td>
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</tr>
<tr>
<td>B</td>
<td>... L ... ctc ... ... D ... gat</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>... I ... atc ... ... G ... ggt</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>... T ... acc ... ... G ... ggt</td>
<td>0</td>
</tr>
<tr>
<td>others</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>(Total)</td>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>

**Figure 2.** Molecular basis of HHV-8 grouping and geographic distribution of HHV-8 variants. Predicted group A peptide sequence, codon positions 130–186, is shown in full, using single-letter code. Amino acid residues whose codons are sites of 95% of mutations are in bold. Residues that allow classification of variants into groups B, C, and D are shown in alignment with group A sequence; dots signify residues that are identical to group A. Codons that specify group-specific amino acid changes are depicted as lower-case letters below corresponding amino acid residues. Not shown are codons in which mutations are silent and amino acid changes in group of variants that cannot be classified as A, B, C, or D.

sequence polymorphism of HHV-8 DNA amplified from all of the oral non-KS tissue samples and from representative KS samples. EBV, CMV, and HHV-6 and -7 DNA were not amplified from any of the oral tissue samples.

From each of the 26 HHV-8-positive tissues, the sequence of the ORF 26 segment encompassed by nested primers (170 bp in length, representing 57 codons) was aligned and compared with the others and with 36 sequences reported from previous studies involving patients from the United States and Africa [1–3, 5]. Genetic variation within the segment was not, in most instances, random. Using the prototype virus sequence [1] as the basis for comparison, there were altogether 109 base changes among the 61 sequences examined. Of these changes, 102 (95%) were confined to six positions in 5 codons: 134, 141, 152, 167, and 169 (numbered according to [1]). In codons 134 and 167, nucleotide substitutions occurred at positions that lead to amino acid changes (figure 2). Substitutions in codons 141 (G→T at the third base), 152 (C→T at the first base), and 169 (A→C at the third base) are silent. Based on amino acid changes predicted from substitutions in codons 134 and 167, all sequences, including those that were unique and possessed mutations in other codons, could be segregated into 4 main groups, labeled A–D, and a miscellaneous group (figure 2).

Figure 2 also shows the geographic distribution of variants. Group A and D variants were exclusive to tissues of patients from the United States and Africa. Group B and C variants were also identified in American and African tissues (group B: 6/36 [17%]; group C: 2/36 [6%]) but were less prevalent than in tissues of London and Italian patients (group B: 16/25 [64%]; group C: 6/25 [24%]). Of the 10 oral non-KS tissues from the London patients, 70% belonged to group B.

Of the 109 base substitutions, the 7 that did not involve the 5 codons aforementioned were located at codons 130 (A→G at the second base), 138 (A→T at the first base), 142 (A→T at the second base), 146 (G→T at the third base), 163 (C→T at the third base), 164 (G→A at the third base), and 168 (T→C at the third base). Only the substitutions at codons 138 and 142 are predicted to lead to amino acid changes. Tissue extracts whose PCR products yielded these base changes were reamplified and resequenced. The changes remained the same, discounting the possibility that Taq polymerase misincorporation had led to the detection of unique sequences.

**Discussion**

This study identifies the oral mucosa as another site of HHV-8 infection in people who are HIV-infected. In designing this study, we were aware of factors that could lead to false-negative results after PCR amplification for HHV-8 DNA from fixed archived tissues: fragmentation of target DNA [10] and formaldehyde-mediated cross-linking of nucleic acid polymers to each other and to proteins [11]. We therefore chose to use a nested approach in searching for HHV-8 DNA (to amplify the population of target DNA that had escaped reaction with formalin) and to adopt KS330 primer sets as outer primers (to keep the segment of amplifiable DNA short, thereby increasing the chance of detecting nonfragmented target DNA). Nevertheless, 6 of 11 oral KS and 4 of 15 cutaneous KS tissues were negative for HHV-8 DNA in our nested PCR assay. This is likely to reflect the combined effects of nucleic acid polymer degradation [10] and formalin [11] on the specimens. We note that
most other PCR assays for HHV-8 DNA in KS tissues [1–3] have used snap-frozen or fresh biopsy specimens.

The overall rate of positive results in our study is, notwithstanding, high. It is unlikely to be due to carryover PCR contamination, because an array of unique sequences was found. We are thus confident that the results obtained from the KS samples and, in particular, the oral non-KS tissues arose from HHV-8 DNA that was already present in tissue samples before the study.

That HHV-8 may be present in oral non-KS, albeit pathologic, tissues of people who are HIV-infected points to the oral mucosa of such persons as a site of HHV-8 infection. The pleiotropism of HHV-8 in the mouth of HIV-positive persons is similar to that of CMV [12] and contrasts with EBV, which preferentially replicates in the tongue [13]. However, unlike CMV and EBV, it is unclear if the oral mucosa can support active HHV-8 replication.

HIV-8 DNA was detected exclusively in oral tissues of people with HIV infection but not in those who were presumed to be uninfected. As this study has not defined which cell types in the oral compartment were infected, the possibility that the detection of the viral genome in the oral tissues is due to the presence of HHV-8–infected mononuclear cells [4] cannot be excluded. However, <10% of HIV-positive patients without KS are positive for HHV-8 DNA in their peripheral blood mononuclear cells [4]; this contrasts with the 70% positivity rate in the oral non-KS tissues examined here. It is, hence, unlikely that the high positivity rate found in the oral tissues was entirely due to infiltration by HHV-8–infected blood cells. In situ hybridization studies are in progress to define precisely which cells in the oral mucosa harbor HHV-8 DNA.

The presence of HHV-8 in oral tissues may be related to the immunosuppressive state that follows HIV infection. That immunosuppression per se may heighten the activity of HIV-8 is suggested by the relatively quick appearance of KS lesions in patients who undergo immunosuppressive therapy and their resolution after discontinuation of therapy [14]. However, as HIV-1 proviral DNA can be located in oral epithelial cells [15], HIV may play a more direct role, for example, by heterologous (HIV–HHV-8) transactivation, in promoting HHV-8 replication.

Comprehensive PCR studies of saliva, and of viral RNA and protein expression in tissues, will be necessary to establish if saliva is a vehicle of HHV-8 transmission.

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The absence of EBV DNA in the oral tissues examined was surprising. The small sample size and the consequent susceptibility to sampling errors are probable contributory factors. Furthermore, none of the samples showed histologic changes suggestive of oral hairy leukoplakia. The absence of DNA from the other herpesviruses known to be shed into saliva is less remarkable, since they are not considered to persist to any significant extent in the oral mucosa of people who are HIV-infected. In contrast, the finding that HHV-8 DNA is found in the majority of this small set of tissues does underscore the prominence of HHV-8, relative to the other herpesviruses, in oral tissues of HIV-positive patients.

We found diversity in the HHV-8 DNA sequences derived from our study samples: 11 of 25 sequences were different from each other. Polymorphism in this region of the viral genome has also been observed in a study of American and African KS patients [3], and another study [5] has demonstrated the hyper-variability of the KS330–133 fragment compared to other regions in the 965-bp ORF. As the genome of DNA viruses is relatively immutable, the high degree of polymorphism in this middle segment of ORF 26 is likely to confer some selective advantage to the virus. The putative peptide encoded from this region might be an immunologic target.

Despite the polymorphism of the HHV-8 DNA ORF 26 fragment, the presence of nucleotide and peptide motifs in it has permitted HHV-8 variants to be grouped. Although the 170-bp stretch of DNA examined is too short and the number of base differences too few to allow a formal typing system, it is intriguing to observe that certain motifs correlate with the locale in which the hosts seek health care, suggesting that HHV-8 variants circulate in geographically restricted areas. Sequence analysis of this short segment of ORF 26 may allow the molecular epidemiology of HHV-8 to be studied in archived tissues.

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References

Early Detection of Perinatal Human Immunodeficiency Virus (HIV) Type 1 Infection Using HIV RNA Amplification and Detection


Early diagnosis of perinatally transmitted human immunodeficiency virus type 1 (HIV) infection can guide early interventions. HIV coculture and DNA polymerase chain reaction (DNA-PCR) detect few HIV-infected infants at birth and 90%-100% by age 3 months. Because extracellular HIV RNA may appear soon after infection, a plasma HIV RNA assay was compared with DNA-PCR for early detection of perinatally infected infants. Blood-draw specimens (108) obtained at the same time from 49 HIV-infected infants and 10 specimens from 8 uninfected infants were tested. HIV RNA and DNA-PCR positivity rates were 56% and 33%, respectively, in 36 specimens from 36 infants <28 days of age (binomial test, \( P = .001 \)). Among 81 specimens obtained after age 14 days, 79 (98%) were positive by HIV RNA testing. No HIV-infected infant specimens were DNA-PCR–positive and HIV RNA–negative. All specimens from 8 uninfected infants were HIV RNA–negative. These results suggest that plasma HIV RNA was detectable earlier and more reliably than HIV DNA in perinatal infection.

In the United States, nearly all new human immunodeficiency virus (HIV) infections in children are acquired through perinatal transmission. The accurate and timely detection of HIV in early infancy has become increasingly important because strategies for the medical management of children born to HIV-infected mothers [1] (prevention of opportunistic infections [2], treatment strategies, and inclusion in clinical trials) rely on knowledge of HIV infection status. However, because maternal antibodies are passively transferred to the infant, HIV antibody testing cannot be used to diagnose infection until the second year of life [3]. Efforts to overcome this problem have included evaluation of new bands on serial Western blots [4], monitoring of antibody titer to detect increases indicative of infant infection [5], and evaluation of infant-specific (IgM and IgA) antibodies to HIV [4, 6]. Other efforts have focused on testing for virus (culture) [7], viral antigen (p24) [8], or proviral DNA using polymerase chain reaction (DNA-PCR) [9]. Culture and DNA-PCR testing are highly sensitive [7, 9] and specific [7, 10, 11] in infants by age 3–6 months and possibly earlier (1–2 months) [10].

Because extracellular HIV RNA increases to high levels soon after infection [12], the sensitivity of tests detecting HIV RNA may be adequate for early diagnosis of perinatal infection. We used diagnostic DNA-PCR to detect infant infection and