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ABSTRACT

Human herpesvirus-8 (HHV-8) is the etiologic agent of Kaposi's sarcoma (KS), which occurs in epidemic form in human immunodeficiency virus (HIV)-infected individuals. Saliva is the only mucosal fluid in which infectious HHV-8 has been identified, although factors associated with HHV-8 salivary shedding remain unclear. Our study performed PCR analysis for HHV-8 DNA in saliva (and other body fluids) in 66 HIV- and HHV-8-co-infected women without KS so that we could examine predictors for HHV-8 DNA detection. CD4 count was the most significant predictor of HHV-8 salivary shedding, with increased prevalence of HHV-8 salivary DNA at higher CD4 counts. The odds of salivary HHV-8 shedding at CD4 counts ≥ 350 cells/ μ L was 63 times the odds of shedding at CD4 < 350 (95%CI, 1.3-3078), with an increase in effect size when the analysis was restricted to those with a CD4 nadir > 200 . Analysis of these data suggests an increased potential for HHV-8 transmission early in HIV infection, with implications for HHV-8 prevention.

KEY WORDS: HHV-8, Kaposi's sarcoma, HIV, salivary shedding, CD4 count.

Prevalence of Human Herpesvirus-8 Salivary Shedding in HIV Increases with CD4 Count

INTRODUCTION

In 1994, human herpesvirus-8 (HHV-8) was identified as the etiologic agent of Kaposi's sarcoma (KS) (Chang *et al.*, 1994). Despite 10 years of ensuing research, however, the mode(s) of HHV-8 transmission remains unclear, although the epidemiology of KS suggests both non-sexual and sexual routes. The endemic variant of KS occurs mainly in children and young adults in Africa, where HHV-8 seropositivity is detected early in life, suggesting a non-sexual route of transmission (Vitale *et al.*, 2000). When an epidemic form of KS emerged with the human immunodeficiency virus (HIV) pandemic, higher rates of HHV-8 infection were observed with a greater number of sexual partners in men who have sex with men (MSM), and a history of sexually transmitted diseases and HIV in both heterosexuals (Cannon *et al.*, 2001; Greenblatt *et al.*, 2001) and MSM (Martin *et al.*, 1998; Pauk *et al.*, 2000), suggesting sexual routes of transmission. Analysis of recent data shows that the prevalence of HHV-8 infection in MSM in San Francisco did not decrease from 1978 to 1996, despite reductions in HIV prevalence, AIDS incidence, and reported rates of unprotected anal intercourse during that period (Osmond *et al.*, 2002), suggesting persistence in HHV-8 transmission.

Although risk factors for the development of KS in HIV- and HHV-8-co-infected patients have been identified, including lower CD4 counts and higher HHV-8 cell-associated viral loads (Engels *et al.*, 2003), risk factors for HHV-8 transmission in the setting of HIV remain unclear. HHV-8 DNA is detected infrequently in genital secretions (Blackbourn and Levy, 1997; Calabrò *et al.*, 1999) and is seen in approximately 6-10% of peripheral blood mononuclear cells (PBMCs) (Campbell *et al.*, 1999; Cannon *et al.*, 2003) in HIV-infected individuals without clinical KS. HHV-8 DNA is seen most consistently and frequently in salivary secretions in HHV-8-seropositive patients. One study reports that HHV-8 DNA is detected in saliva in 75% of HIV-positive men with KS and in 15% of HIV-positive men without KS (Koelle *et al.*, 1997). Other studies report an 11-57% rate of salivary shedding of HHV-8 DNA in HIV- and HHV-8-co-infected patients without KS (Boldogh *et al.*, 1996; Blackbourn *et al.*, 1998; Pauk *et al.*, 2000; Corey *et al.*, 2002). Indeed, saliva is the only mucosal fluid in which infectious, transmissible HHV-8 virions have been identified (Vieira *et al.*, 1997). Epstein-Barr virus (EBV), the human herpesvirus most closely related to HHV-8, replicates in oropharyngeal epithelial cells and is transmissible through saliva (Ikuta *et al.*, 2000). EBV, however, is almost ubiquitously present in adults, whereas HHV-8 seroincidence varies by risk group, indicating differing transmission routes for the 2 herpesviruses (Macswen and Crawford, 2003).

Since saliva is the only body fluid in which infectious HHV-8 has been identified, factors associated with HHV-8 salivary shedding in HIV patients might inform prevention strategies for HHV-8 transmission. Although the

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presence of clinical KS is associated with increased rates of HHV-8 salivary DNA detection in HIV-positive individuals (Koelle *et al.*, 1997), predictors of HHV-8 salivary shedding in patients without KS are unknown. One small study suggests an association between higher peripheral CD4 counts and the detection of salivary HHV-8 DNA in HIV-positive men with active KS (Koelle *et al.*, 1997), but no study has examined the association of CD4 or other HIV progression markers with salivary HHV-8 detection in patients without KS. The present study reports rates of HHV-8 DNA shedding in various bodily fluid compartments in a cohort of HIV and HHV-8 dually infected women without KS and examines risk factors associated with HHV-8 salivary shedding.

METHODS

Study Population

The Women's Interagency HIV Study (WIHS) is a multicenter, prospective cohort study established in 1994 to investigate the progression and pathogenesis of HIV infection in US women (Barkan *et al.*, 1998); participants are seen at six-month intervals for core visits that include interviews, physical examination, and specimen collection. A WIHS substudy recruited 66 HIV-seropositive women with HHV-8 seroreactivity for an analysis of HHV-8 DNA detection in various body fluids. The criterion for HHV-8 seropositivity was the presence of antibodies to either latent or lytic antigens of HHV-8; methods of antibody detection have been described (Greenblatt *et al.*, 2001). Informed consent was obtained from all patients, and the Institutional Review Board at each of the five participating WIHS centers approved the study protocol. Human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of this study.

Study Procedures

Unstimulated and stimulated saliva was collected from participants on each of 3 days during a two-week period following a WIHS core visit between 1998 and 2000. Salivary stimulation involved the chewing of neutral gum at prescribed frequencies. Each patient participated in the three-day collection scheme over a total of 3 core WIHS visits (visits 10, 11, and 12), so that salivary shedding could be evaluated both for short-term consistency and for changes over time. Full oral examinations by study dentists were performed once for each cluster of three-day visits. Cervicovaginal (CVL) specimens were collected for each participant at each core visit. Anal swabs and blood samples for HHV-8 PCR were collected from participants at the San Francisco WIHS site only. Each collected sample was frozen at -70°C until PCR analysis for HHV-8 DNA could be performed. Lymphocyte subsets were assayed *via* flow cytometry.

PCR Methods

PCR for HHV-8 DNA detection was performed at the University of Washington in Seattle, and methods developed by this laboratory have been described in detail (Koelle *et al.*, 1997). Briefly, specimens are normalized for cellularity or volume, and DNA is isolated from the samples by phenol-chloroform extraction after overnight digestion at 50°C with 100 $\mu\text{g}/\text{mL}$ proteinase K, 0.5% SDS, 25 mM EDTA, 100 mM NaCl, and 100 mM Tris (pH 8.0). After precipitation with sodium acetate (0.25 M), glycogen (100 $\mu\text{g}/\text{mL}$), and 2 vol of ethanol, and re-suspension in 200 μL of Tris buffer, a 10- μL quantity (5%) of DNA was used for each PCR

reaction. Each 100- μL PCR mixture contained 50 mM KCl, 1.5 mM MgCl_2 , 2U AmpliTaq, 200 μM each dNTP and 0.83 μM each of HHV-8-specific primers designed by the laboratory (Koelle *et al.*, 1997). After the PCR reaction was run, HHV-8 gene products were detected by liquid hybridization with ^{32}P -labeled probes specific for the PCR product amplicons. PCR products (7 μL) and probe (10⁶ cpm) were heated in 25 μL of 1.2 M NaCl, 100 μM each dNTP, and 44% formamide at 97°C for 10 min. A 10- μL quantity of cooled hybridization reactions was analyzed on 6% acrylamide gels, dried, and autoradiographed. To ensure that negative results were not due to non-specific inhibition of PCR, we "spiked" each PCR reaction with an internal positive control DNA and associated primers. DNA from specimens exhibiting inhibition (failure to amplify the internal control DNA) was re-purified and re-amplified.

Statistical Methods

Data on demographics, immunologic and virologic markers, clinical status, and the use of highly active antiretroviral therapy (HAART) are collected at each core WIHS visit (Barkan *et al.*, 1998). The relationship between these factors and the outcome of HHV-8 salivary shedding at each visit was analyzed with the use of a multivariate logistic regression model that included a random subject effect. Statistical analyses were performed with use of the SAS software package (SAS Institute, Cary, NC, USA). All p values are two-tailed.

RESULTS

Saliva was collected for each of the 66 patients at five clinical WIHS sites on each of 3 days in the period proximate to 3 WIHS core visits; each three-day cluster was termed a subvisit, and 178 out of the anticipated 198 (66 x 3) subvisits were completed. A subvisit was classified as being positive for salivary HHV-8 shedding if HHV-8 DNA was detected in the saliva on at least 1 of the 3 collection days. CVL samples were collected once for each subvisit, and 167 of the anticipated 198 were collected. Anal swabs and blood samples were collected for 22 and 37 participants, respectively, from the San Francisco WIHS site. HHV-8 DNA was detected in 15.7% of the saliva specimens, 8.1% of PBMC samples, and in none of the CVL, anal swabs, or plasma or serum specimens. In terms of salivary HHV-8 shedding consistency over a subvisit, salivary HHV-8 DNA was detected in 3 out of 3 collection days in 31% of the subvisits, in 2 out of 3 days in 50% of subvisits, and in only 1 out of 3 days in 19% of subvisits.

The following factors were assessed for their association with the detection of HHV-8 DNA in saliva: age, race, CD4 cell count at time of collection, CD4 nadir count, current CD8 cell counts and CD4/CD8 ratios, current HIV viral loads and \log_{10} change since the recorded peak viral load, HAART use, any systemic symptoms or active infections, including systemic herpesvirus infections, smoking, active substance abuse (alcohol, heroin, cocaine, marijuana, amphetamines), presence of oral lesions, and salivary gland enlargement. Oral lesions recorded by study dentists included angular cheilitis, pseudomembranous candidiasis, erythematous candidiasis, oral hairy leukoplakia, oral warts, aphthous ulcers, abscesses, denture-related ulcers, herpetic lesions, and lichen planus. Each oral lesion was examined as an individual variable and then grouped into a composite variable for the analysis. None of the women in the study had clinical KS.

Each of the above factors was identified for a subvisit and examined in relation to the outcome of HHV-8 DNA salivary shedding, first in univariate analysis, and then in bivariate models. The only statistically significant predictor for HHV-8 DNA detection in the saliva in our model was the current CD4 cell count (Table). The odds of salivary HHV-8 shedding at current CD4 counts of ≥ 350 cells/ μ L was 63 times the odds of shedding at CD4 counts < 350 (95% CI, 1.3-3078; p value, 0.037). Most of the increased risk of salivary shedding occurred in participants with a current CD4 count ≥ 350 , who never had a CD4 count ≤ 200 cells/ μ L (OR, 74; 95%CI, 1.6-3419; p 0.029). Box plots compare the mean and median CD4 counts and the associated quartiles for HHV-8 salivary shedders (CD4_{mean} 419) with those for non-shedders (CD4_{mean} 278) (Fig.). A trend toward increased HHV-8 salivary shedding with HAART use at the time of salivary collection was observed. The effect of the CD4 > 350 variable remained similar when controlled for any one of the other predictors in two-predictor models. The small number of outcomes prevented any further multivariate analysis with 3 or more predictors.

DISCUSSION

HHV-8 DNA was detected more frequently in saliva than in blood, genital fluids, or anal swabs from HIV/HHV-8 co-infected women. The rates of HHV-8 DNA shedding in various bodily compartments in our study are similar to shedding rates for KS-negative patients in other studies (Koelle *et al.*, 1997; Calabrò *et al.*, 1999). We further demonstrate that HHV-8 salivary shedding is most frequent in individuals with the least perturbed CD4 cell populations (current CD4 counts of ≥ 350 cells/ μ L with a CD4 nadir that has never fallen below 200). Participants with current CD4 counts of ≥ 350 with a nadir of < 200 did not demonstrate a similar association, increasing the biological plausibility of more preserved CD4 cell populations positively influencing HHV-8 salivary shedding. Furthermore, the increase in effect size when more stringent CD4 count criteria are applied suggests that the association between a relative state of immunocompetence and HHV-8 DNA salivary shedding is real.

The increase in HHV-8 salivary detection rates at higher CD4 counts is consistent with data demonstrating an increased probability for HHV-8 DNA detection in plasma at higher CD4 ranges (odds ratio for detection of HHV-8 plasma DNA for CD4 ≥ 200 vs. CD4 < 200 is 7.24 [95%CI, 1.30-40.4; Tedeschi *et al.*, 2001]). Only one other published report has analyzed the correlation between CD4 counts and HHV-8 salivary DNA detection (Koelle *et al.*, 1997); this analysis was performed in a subset of HIV-positive men with clinical KS (sample size 23) and showed an odds ratio for salivary HHV-8 shedding at CD4 counts of ≥ 350 compared with CD4 counts < 350 of 2.3 (95%CI, 0.18-127; p 0.12). This study therefore allows for the possibility of a very strong association between CD4 counts and salivary shedding, consistent with our data. However, neither study was able to determine the precise magnitude of this effect, given the width of the confidence

Table. Odds Ratios of Salivary HHV-8 DNA Shedding Associated with Various Factors

Predictor	Odds Ratios of Salivary HHV-8 DNA Shedding ¹	p-value
CD4 _{current} ≥ 350 vs. < 350	63 (1.3-3079) ²	0.037
CD4 _{current} ≥ 350 and CD4 _{nadir} ≥ 200	74 (1.6-3419) ²	0.029
CD4 _{current} ≥ 350 and CD4 _{nadir} < 200	1.1 (0.003-409)	0.98
CD4 _{nadir} ≥ 200 vs. < 200	33 (0.8-1000)	0.061
HAART use	21 (0.5-881)	0.11
Smoking	0.67 (0.05-9.5)	0.77
Age (> 40 vs. ≤ 40)	0.48 (0.02-10.1)	0.63
Viral load, per log ₁₀ increase	1.07 (0.36-3.16)	0.91
Oral lesions, composite ³	0.47 (0.05-4.3)	0.50
Systemic symptoms ⁴	1.02 (0.06-16.6)	0.35
Systemic diseases ⁵	0.86 (0.11-6.67)	0.88

- 1 Numbers in parentheses indicate 95% confidence intervals.
- 2 Only statistically significant predictors of salivary shedding.
- 3 Oral lesions examined individually and in composite: angular cheilitis, pseudomembranous candidiasis, erythematous candidiasis, oral hairy leukoplakia, oral warts, aphthous ulcers, abscesses, denture-related ulcers, herpetic lesions, or lichen planus. Each lesion was examined individually and then in composite for the analysis.
- 4 Systemic symptoms examined individually and in composite: fever, weight loss, night sweats, diarrhea, lymphadenopathy, and/or wasting.
- 5 Systemic diseases examined individually and in composite: sinusitis, urinary tract infection, rheumatologic illnesses, liver disease, localized or disseminated zoster, visceral herpes simplex virus infection, cytomegalovirus infection, *Pneumocystis carinii* pneumonia, pneumonia, visceral candidal infection, mycobacterium avium complex infection, toxoplasmosis, meningitis, cryptococcal disease, endemic mycoses, and/or Salmonella infection.

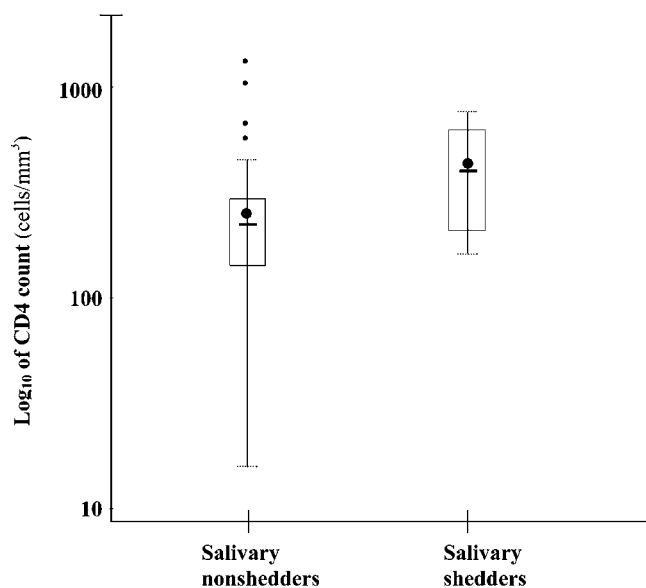


Figure. Box plots of CD4 counts for salivary shedders vs. non-shedders. Box plots depict the median CD4 count with the 25% and 75% quartiles and ranges for participants in the study, who are classified as salivary shedders (participants at first subvisit with salivary DNA detection, n = 17) and non-shedders (participants at first subvisit without salivary DNA detection, n = 49). Shedders: CD4_{mean} 419 cells/mm³; CD4_{median}, 399; interquartile range, 417; range, 521. Non-shedders: CD4_{mean} 278 cells/mm³; CD4_{median}, 227; interquartile range, 154; range, 1294. □ Box plots depicting 25% quartiles (lower edge of rectangle) and 75% quartile (upper edge of rectangle) of CD4 counts for each group. ● Mean CD4 counts. — Median CD4 counts. Range of CD4 counts for each group. • CD4 count outliers.

intervals. Our analysis represents the largest sample of HIV/HHV-8 co-infected individuals studied to date to analyze correlates of HHV-8 salivary shedding and is the only study to assess relationships between peripheral CD4 count and HHV-8 DNA salivary detection in individuals without KS.

HHV-8 DNA salivary shedding with more preserved CD4 counts suggests that CD4 cells may modulate HHV-8 replication in other cell types. One possible modulation site is within the germinal centers of lymphoid tissue, where CD4 cells form cognate interactions with B-cells (Kelsoe, 1996; Garside *et al.*, 1998); circulating B-cells are a primary reservoir for HHV-8 replication (Monini *et al.*, 1999). Such germinal centers are located in the palatine and lingual tonsillar tissue in the oral cavity. Local gingival crevicular fluid cytokine and immunoglobulin levels may vary with peripheral CD4 count (Grbic *et al.*, 1997) and might contribute to the amount of salivary HHV-8 replication in various states of immunosuppression. Moreover, enhanced HHV-8 replication may be secondary to a more effective Th1 helper response at higher CD4 counts. Interferon- γ (IFN- γ) has been shown to induce lytic replication of HHV-8 (Chang *et al.*, 2000; Mercader *et al.*, 2000). Since IFN- γ -producing T-cells decrease with CD4⁺ T-cell depletion (Kostense *et al.*, 2002), increases in salivary and plasma HHV-8 detection at higher CD4 counts may reflect more robust Th1 helper responses early in the course of HIV infection.

The presence of infectious HHV-8 in saliva and the proclivity of the virus for salivary shedding imply that oral secretions are involved in transmission. Continued HHV-8 transmission among MSM in San Francisco, despite reductions in unprotected anal intercourse and HIV prevalence from 1978-96 (Osmond *et al.*, 2002), may be explained by orogenital contact. Sexual activities perceived as lower-risk (*e.g.*, kissing, orogenital contact, oro-anal contact) may have continued in this population despite reductions in high-risk activities during that period. Orogenital contact is statistically associated with HHV-8 seroconversion in MSM (Dukers *et al.*, 2000). Analysis of our data suggests that HHV-8 transmission in HIV-infected individuals may be greater in early HIV infection, when CD4 cell populations are relatively intact. Since HHV-8 seroconversion in patients who are already HIV-infected increases the risk of developing KS compared with individuals who become HHV-8-infected prior to HIV (Jacobson *et al.*, 2000), further data on predictors for HHV-8 salivary shedding are greatly needed.

Our study indicates a continued risk of HHV-8 transmission in HIV-infected individuals, even in relative states of immunocompetence. Longitudinal studies to assess changes in frequency of HHV-8 salivary shedding in HIV patients over time and with immune reconstitution on HAART may further elucidate these findings. A more detailed analysis of immunomodulatory mechanisms and HHV-8 replication in the oral cavity may help explain the positive association between peripheral CD4 counts and the degree of HHV-8 salivary shedding in HIV patients. Further data on CD4 count correlates of HHV-8 salivary shedding may have implications for HHV-8 transmission and prevention in the HIV-infected population.

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