WOMEN'S INTERAGENCY HIV STUDY
SECTION 16: HHV-8 SUBSTUDY PROTOCOL

A. HYPOTHESES

Ha. HHV-8 DNA will be identified in saliva in 30% of women who are HHV-8 seropositive and in the cervico-vaginal fluid of 15% of women who are HHV-8 seropositive. The occurrence of Kaposi's sarcoma (KS) will be predicted by shedding in saliva and cervico-vaginal fluid, reactivity to latent antigen, and non-reactivity to lytic antigen alone.

Hb. Seropositivity for HHV-8 will predict both the detection of the organism in saliva and occurrence of oral lesions of KS.

Hc. Salivary and cervico-vaginal shedding of HHV-8 will be associated with CD4 cell depletion, detectable HIV RNA in plasma, the presence of oral soft tissue lesions associated with HIV infection, and cigarette smoking.

Hd. Persistent HHV-8 salivary shedding will be associated with HIV-disease progression and the presence of oral soft tissue lesions.

He. Location, oral/anal contact, receptive anal intercourse, bisexual male partners, lifetime number of sexual partners and use of crack/cocaine are independently associated with HHV-8 infection.

Hf. Newer serological test methods will eliminate false positive results (substudy to examine false negatives).

Hg. HHV-8 infection will be associated with adenopathy and increased rate of CD4 cell depletion, polyclonal gammopathy.

Hh. CD4 cell proliferation (and CD8 cells in the near future) will be detected in response to specific HHV-8 antigens compared with positive and negative controls.

Hi. Vigorous lymphocyte proliferation in response to HHV-8 antigens is associated with lower levels of HHV-8 shedding from mucosal sites, a lower level of plasma viremia and lower likelihood of Kaposi's Sarcoma.

Hj. The presence of HHV-8 DNA in oral lymphatic tissue among infected women will be associated with less lymphocyte proliferation in response to HHV-8 antigens, and greater levels of shedding in oral secretions.

B. SCIENTIFIC AIMS

1. To determine the clinical manifestations of HHV-8 infection among women with an emphasis on the oral cavity.

2. To determine the rate of HHV-8 DNA shedding from the mouth (saliva) and lower genital tract (CVL and anus) among women who are known to have serum antibody to lytic and/or latent antigens.

3. To identify factors associated with HHV-8 shedding in saliva and cervico-vaginal fluid.

C. BACKGROUND

Kaposi's sarcoma and lymphoma are the most frequent malignancies among persons with HIV infection. The risks of KS vary significantly with mode of transmission; prevalence rates are highest

among homosexual men (20-30%) and lowest among women (1-3%)[1-3]. Sexual transmission of an infectious co-factor in KS pathogenesis has been hypothesized[4,5]. Recently a novel herpesvirus (KS-associated herpesvirus or human herpesvirus-8, HHV-8) was identified in KS lesions and in B-cell lymphoma cells[6]. Serological reactivity is highly prevalent among patients with KS and parallels the epidemiological risk of KS[7]. The association between HHV-8 infection and risk of KS was further demonstrated in a WIHS I substudy of serological reactivity to latent antigens; we described a prevalence of 4% and 1% among HIV+ and HIV- CWWHS participants at baseline visits[7]. The two cohort participants with KS were seropositive. HHV-8 DNA has been consistently identified in saliva from male KS patients[8-11], a finding consistent with an oral site of viral replication and possible oral transmission of the pathogen. Oral transmission is an intriguing possibility given the relatively low prevalence of infection in broad population groups such as women. Modes of transmission, the prognostic value of serologic reactivity to latent and lytic antigens, rate of shedding at oral and genitourinary sites and the relationship between HHV-8 infection and non-KS malignancies remains largely unknown. WIHS II provides an ideal cohort for the investigation of these questions. Completion of serologic screening for reactivity to HHV-8 using both latent (nuclear) and lytic (cytoplasmic) antigens will be completed at the University of Washington during the final year of WIHS I. WIHS II can then initiate in-depth follow-up of HHV-8 infected women, including studies of oral and genital tract viral shedding. Study plans include longitudinal evaluation of HHV-8+/HIV+ women by the WIHS dental research team including an assessment of oral soft tissues including salivary glands and collection of unstimulated and stimulated saliva. PCR will be performed to identify HHV-8 DNA and RNA in stimulated saliva in the laboratory of Dr. Lawrence Corey at the University of Washington. Dr. Corey is an internationally recognized authority on herpesvirus infections; his laboratory was recently certified by the CDC to perform HHV-8 PCR (one of two US facilities to pass the certification procedure).

D. PRELIMINARY STUDIES

In previous studies, Kedes et al, 1997[7], in collaboration with the Northern California WIHS site, reported that the prevalence of serological reactivity to latent HHV-8 antigens was 3.4% among 387 participants. The prevalence of HHV-8 serum antibodies among the 302 HIV-infected women was 4%; the two women with active KS lesions were both seropositive. This prevalence was dramatically lower than that recently reported among HIV-infected homosexual and bisexual men (30%-35%). The Kedes study utilized a first generation serological technique that detected reactivity to latent (nuclear) antigens only. Other studies in Northern California[12] have shown that the prevalence of serologic reactivity among women can reach 20%, using assays that detect cytoplasmic antigen reactivity.

In 1997, Koelle et al.[13], of the University of Washington (co-investigator of the Substudy), reported the detection of HHV-8 DNA in saliva from 18 (75%) of 24 HIV-infected subjects with active or resolved KS, 1 of 1 HIV-negative patient with KS, and 3 (15%) of 20 HIV-infected men without KS, and none of 24 controls. HHV-8 DNA was detected in the PBMC of two separate HIV-infected homosexual or bisexual men without clinical KS, yielding a prevalence rate of 25% for HHV-8 infection among this population, as detected by PCR. Among HHV-8-infected and HIV-infected subjects, detection of HHV-8 DNA in saliva was associated with the presence of clinical KS. The intermittent nature of HHV-8 shedding in saliva and the small sample sizes in this study, however, prevented the authors from making firm conclusions concerning the relationship between antibody levels and salivary shedding of HHV-8 among patients with KS.

E. METHODS

1. OVERVIEW

   a. All participants who have serum antibody to HHV-8 lytic or latent antigens, or who have HHV-8 DNA detected in peripheral blood lymphocytes will be referred to the Oral HHV-8 Substudy and counseled about HHV-8.

   b. Stimulated saliva collection will be performed per Mahvash Navazesh's HHV-8 Saliva Collection Protocol (see Section F: Specimen Collection). For original recruits, a total of three collections over three visits in a two-week period within two months of a core WIHS visit will be done; for new recruits, a total of three collections in a two-week period will be done within two months of the baseline visit (visit 15 or 16). Two aliquots from three consecutive collections will be dedicated for HHV-8 DNA PCR.

   c. CVL will be collected per WIHS protocol at visits 10, 11 and 12 for original recruits, and at the baseline visit for new recruits; a single aliquot of the collection from three consecutive visits will be dedicated to HHV-8 DNA PCR.

   d. Detailed oral soft tissue examinations will be performed by the Oral Substudy group; gynecological examinations will continue as part of the core WIHS evaluation.

   e. In SAN FRANCISCO ONLY, phlebotomy will be performed at one of the three visits by the Oral Substudy staff. 20cc of ACD blood will provide two aliquots of 3 million PBMC, in addition to plasma. 9cc (1 large red top) will be collected for serum.

   f. A question regarding lifetime experience with oral/anal sex was added to the WIHS Visit 9 behavioral survey at the end of the male partner section. Anal sampling to detect anal shedding will be performed at WIHS core visits 10, 11 and 12 for original recruits and at baseline for new recruits per Dr. Joel Palefsky's Anal Sampling Protocol.

2. STUDY POPULATION

   WIHS participants who are determined to have serum antibody to HHV-8 latent (nuclear) or lytic (cytoplasmic) antigens in specimens collected at baseline will be offered enrollment in the WIHS Oral HHV-8 Substudy. In addition, any WIHS participant who has negative HHV-8 serologies but positive lymphocyte PCR for HHV-8 DNA will be offered enrollment.

3. SAMPLE SIZE (TO BE REWRITTEN BASED ON PARTICIPATION OF ONLY FOUR SITES)

   Over three observations, 15% and 30% of HHV-8 seropositive women will have HHV-8 DNA detected in cervico-vaginal fluid and saliva specimens respectively. Minimal sample size requirement has been calculated in the following manner: Our goal is to define the prevalence of salivary shedding of HHV-8 at the 95% confidence level. The total number of samples required is: $N = 4\ z_\alpha^2\ P\ (1\text{-}P) \div W^2$ where z_α = the standard normal deviate for a two-tailed α, where $(1\text{-}\alpha)$ is the confidence level, P is the expected proportion, and W is the total width of the confidence interval. With an expected proportion of 30%, a sample size of 323 will yield a confidence interval of 95%. Thus each of the oral substudy sites will need to offer 22 eligible women participation in this substudy and successfully collect specimens from 16 individuals to support this level of precision in estimates of prevalence of viral shedding.

   The five participating WIHS sites (calculations based on five sites, although **only four sites** will now be participating) saw 1336 subjects at visit 4. We anticipate that 10% of these women will have serum antibody to HHV-8 and another 2% will have positive lymphocyte PCR for HHV-8 DNA thus yielding a total eligible population of 160 women. If 70% of the eligible women agree

to participate, then a total of 112 women will be studied with three specimen collections (total of approximately 67 visits per site per six-month follow-up). Thus 336 samples will be available for analysis for each six monthly WIHS visit.

4. COMPENSATION

Eighty dollars ($90 in San Francisco) will be provided for a complete examination and set of three specimen collections in a ten-day period – $20 per visit for each of the first two soft tissue exam and saliva collection visits, $35 for the third visit, a single $5 on-time incentive, and, in San Francisco, $10 for phlebotomy.

5. CONSENT

Informed consent for the three Oral HHV-8 Substudy visits, including oral exam, saliva collection, anal swab collection and phlebotomy (SF only) will be obtained separately from WIHS II consent.

WIHS has developed a counseling protocol in conjunction with the National Community Advisory Board for informing participants of HHV-8 test results (see Appendix A). This counseling includes information regarding what HHV-8 is, what a positive serological or saliva test means in terms of risk of KS, and contagion and acquisition of HHV-8, as far as current understanding permits.

6. CLINICAL DATA COLLECTION

a. Medical history of prior Kaposi's sarcoma, lymphoma, myeloma and other malignancy (and Medical Record Abstraction confirmation of diagnoses) will be gleaned from the current WIHS medical history database. Additionally, all participants will be asked whether they have ever experienced anal/oral sexual activity.

b. WIHS physical examination data will be used to identify additional cases of KS and gynecological abnormalities, including genital KS, lymphadenopathy, and other dermatological findings.

c. Oral soft tissue examination data will be used to identify cases of oral KS and salivary gland enlargement. This examination will include completion of Oral Substudy form OP04/04a (Oral Mucosal Tissue Exam) which includes diagnostic categorization of lesions with graphic depiction and numeric coding of lesion location, characteristics such as pain, indication of smear collection, duration of lesion, and prior lesion history. In addition, Section C of OP04, Salivary Gland Examination, will be completed to identify glandular enlargement, tenderness, and lack of salivary expression. The Oral Substudy Protocol for completion of OP04/04a stipulates the method of data recording, order of examination, equipment needed, and method of salivary gland examination (see Section 13 of the Manual of Operations). In addition, gingival bleeding will be assessed and recorded on form OP13 at each visit.

F. SPECIMEN COLLECTION

1. SALIVA

The WIHS HHV-8 Saliva Collection Protocol will be used; this procedure is based on two reports by WIHS investigator Mahvash Navazesh[14, 15]. Form OP03 will be completed to provide tracking and specimen quality information.

1) Subjects are asked to fast (except water) for one hour prior to the test session.

2) Subjects are asked to remove lipstick with a 2x2-gauze square and any removable dental prosthesis. For stimulated whole saliva collection, if it would be more comfortable for the

participant to chew with her removable dental prosthesis, she may go ahead and replace them. However, this should be consistent at every visit; if the participant removes her dental prosthesis at her first Oral HHV-8 visit, she should remove them again at follow-up visits.

3) Subjects rest for five minutes (no talking or reading) before saliva collection begins.

4) Whole-stimulated saliva is collected over **five minutes** by the spitting method using a standard-size gum base as a stimulant. The frequency of stimulation is controlled by a metronome at about 70 chews per minute. The subject is asked to expectorate saliva into the graduated test tube once per minute. (Remind subjects not to spit out the gum base at end of each minute.)

5) Send the specimen to the local lab for processing. As long as the specimen is received by the local lab the same day, it is not necessary for it to be stored or transported on ice. It should get to the local lab the same day as collection and be aliquoted and frozen that day. The maximum waiting time between collection and aliquoting and freezing should be eight hours. If absolutely necessary, the sample can be put in the refrigerator overnight and aliquoted the next morning, but this should be discouraged.

2. PHLEBOTOMY (AT SAN FRANCISCO SITE ONLY)

a. Collect 20 cc ACD yellow top for PBMC and plasma.

b. Collect approximately 9cc, 1 large red top for serum.

3. ANAL SAMPLE COLLECTION

If the participant is HHV-8 positive and enrolled in the HHV-8 Substudy, the clinician will collect an anal sample during the Gynecological Exam at visit 10 for original recruits and at the baseline visit for new recruits. After examination of the external anus, the clinician will change gloves and then brush one dacron swab over the anorectal area to collect cells for HHV-8 DNA hybridization studies. It is acceptable to use a Dacroswab similar to those used for viral culture and break it off into a sterile, screw cap, plastic 1/5–2.0 ml tube. After collection, the swab should be returned to a sterile dry sleeve collector and transported at room temperature to the local lab.

G. LABORATORY METHODS

1. SPECIMEN HANDLING

a. Saliva Specimens

Pipet the saliva up and down to mix. Aliquot into appropriate vials. Use a P1000 pipetman and pipet tips that are sterile and DNA, Dnase and Rnase (nuclease) free – these are commercially available. If the saliva is too thick and hard to handle, a PipetAid device and disposable 1-ml plastic serologic pipettes can be used. It is OK if the volume is above 500 ul and is variable. THE LARGEST NUMBER OF ALIQUOTS TO BE SHIPPED SHOULD NOT EXCEED FOUR DUE TO STORAGE SHORTAGE; DISCARD EXTRA SAMPLES.

Store at -70° C with identifying information, including WIHS ID, collection date, type (stimulated saliva) and storage temperature (i.e., -70° C). Ship aliquots to BBI on dry ice with regular batch shipments of WIHS core specimens. In addition to identifying information, the label should include the HHV-8 visit number, i.e., 10A, 10B or 10C for original recruits and 15A, 15B, 15C, 16A, 16B or 16C for new recruits.

b. CVL specimens

CVL specimens will be accessed via the WIHS core specimen repository at BBI per availability.

c. Anal specimens

After being transported to the local lab, anal specimens should be stored at -70° C. It is acceptable to store samples at -20° C at the local collection site; however the sample should be archived at -70° C prior to shipment, if possible. Specimens should be shipped to BBI on dry ice with regular specimen shipments of WIHS core specimens.

d. PBMC and plasma specimens

PBMC and plasma specimens will be processed per the protocol provided by Dr. David Koelle. This protocol will be distributed only in San Francisco.

2. PCR ANALYSIS FOR SALIVA AND CVL

Specimen input will be normalized for cellularity or volume. 200- to 400-μL samples will be submitted for DNA extraction.

DNA will be isolated by phenol-chloroform extraction after overnight digestion at 50°C with 100 μg/mL proteinase K, 0.5% SDS, 25m*M* EDTA, 100m*M* NaCl, and 10 m*M* TRIS (pH 8.0); precipitated with sodium acetate (0.25 M), glycogen (100 μg.mL), and 2 vol of ethanol; and resuspended in 200 μL of 10 m*M* TRIS (pH 8.0). Ten μL (5%) of the DNA will be used for each PCR reaction, and semiquantitative results will be adjusted accordingly.

For HHV-8-specific PCR primer pairs will be KS-1 and –2, amplifying the $KS330Bam_{233}$ fragment of the ORF 26, and primer pairs of KS-A and –B (CGTCGGTCGTGTCGTGTGAT and GCATAATGTCTTCCTTGTGG), amplifying a 225-bp sequence within ORF 25.

Each 100-μL PCR mixture will contain 50 m*M* KCl, 1.5m*M* $MgCl_2$, 2 U Amplitaq (Perkin-Elmer, Foster City, CA), 200 μ*M* each dNTP, and 0.83 μ*M* each primer. KS-1 and –2 reactions will contain 15% glycerol; other primer pairs were used with 10% glycerol. PCR conditions for KS-1 and –2 include: 96°C for 3 min, 35 cycles at 98°C (30 s), 53°C (30 s), and 72°C (30 s), and 72°C for 5 min. Reaction conditions for KS-A and KS-B will be identical except for annealing temperatures (50°C for KS-A and KS-B).

PCR product detection will utilize liquid hybridization with ^{32}P-labeled probes. HHV-8-specific PCR products will be detected with probes KS-P and KS-P2 (CAACCCGTGGGCTTCGCAGC), specific for PCR amplimers generated by KS-1 and KS-2 and KS-A and KS-B, respectively. PCR products (7μL) and probe (10^6 cpm) were heated in 25μL of 1.2 m*M* NaCl, 100μ*M* each dNTP, and 44% formamide at 97°C for 5 min. Ten μL of cooled hybridization reactions were analyzed on 6% acrylamide gels, dried, and autoradiographed.

To quantitate KKV-8 PCR assays, a standard prepared by cloning KS-1 and –2 PCR product into TA vector (Invitrogen, San Diego, CA) will be used and quantitated by UV absorption. Specimen DNA and $\log_{10}$ dilutions of standard DNA (10^4-10^1 copies) were amplified using the same PCR master mix. The intensity of the specimen band at the predicted molecular weight is visually compared to the standard curve. Specimens will be rerun in $\log_{10}$ dilutions (in 10 m*M* TRIS, pH 8.0) when initial sample contained $>10^4$ DNA copies/mL. Results will be expressed as HHV-8 DNA copies per mL of sample. For tissue, 2×10^6 β-globin DNA copies were assigned the value of 1×10^6 cells. Previous experiments have shown that PBMC number measured by hemocytometry and quantitative β-globin PCR were concordant.

To insure that negative results are not due to nonspecific inhibition of PCR, internal positive controls, 50 copies of KS-fly2, will be included in each PCR amplification. DNA samples from specimens exhibiting inhibition will be repurified and reamplified. Determination of each negative KS-1 and KS-2 PCR result will require detection of KS-fly2DNA. To detect failure to isolate DNA, cellular specimens will analyzed by PCR for human β-globin. To detect false-positives, specimen sets will be processed in parallel (from DNA isolation through autoradiography) with aliquots of HSB2 cells. PCR reactions without DNA will also be included in each PCR run.

3. SALIVA FRACTIONATION AND ASSESSMENT OF VIRAL DNA SUSCEPTIBILITY

   Saliva (5mL) will be liquefied with 1m*M* of dithiothreitol for 10 min at room temperature, centrifuged (800 g) for 10 min, and filtered (0.8μm). The cell-free salivary fluid will then be ultracentrifuged at 90,000 *g* for 60 min, and pelleted material was resuspended in 100μL of one-third-strength PBS. DNA was extracted from the supernatant and pellet, and 5% was used for PCR with KS-1 and KS-2 primers. In order to determine the DNase sensitivity of the viral DNA, 10μL of the resuspended pellet fraction will be spiked with 3×10^4 copies of VZV DNA and treated for 1 h at 37°C with 10 U of RQ DNase (Promega, Madison, WI) in 100μL of buffer containing 10 m*M* NaCl, 2m*M* $MgCl_2$, 10 m*M* TRIS-HCl (pH8.0), and 1m*M* dithiothreitol. The DNase will be will be inactivated with 25m*M* EDTA and 2 vol of lysis buffer (6 *M* guanidinium thiocyanate, 37.5 m*M* sodium citrate, 0.75% n-lauroylsarcosine, 0.15 *M* β-mercaptoethanol). DNA was extracted as above.

4. CULTURE AND STRAIN IDENTIFICATION

   This specimen collection method should enable recovery of HHV-8 and strain identification for future research, should the WIHS choose to pursue this line of investigation.

H. DATA ANALYSIS

All completed HHV-8 data collection forms should be sent to the San Francisco WIHS site for data entry into an MS ACCESS-based data entry system per WIHS protocol. In addition, the Recruitment Outcome and Disenrollment Forms should be locally entered into Apollo so that persistent tables will reflect accurate substudy enrollment information. All data will be imported into SAS for analysis.

The initial univariate analyses will determine prevalence of shedding as defined as PCR positivity on any of the three sequentially collected specimens. Subsequently, longitudinal analysis of persistence of shedding over the two-year study follow-up will be performed. The second analytic activity will determine odds ratios for the association between salivary HHV-8 shedding and potential cofactors such as the presence of oral lesions, CD4 cell count, HIV viral load, history of KS, stage of illness, drug use and smoking history and HIV risk factor. In addition, bivariate analysis will examine factors associated with persistence of shedding. Finally, multivariate models of salivary HHV-8 shedding will be constructed to determine the contribution of multiple coexistent factors to viral shedding.

I. REFERENCES

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APPENDIX A: HHV-8 COUNSELING PROTOCOL

Your Human Herpesvirus 8 Results from the WIHS

What they mean to you

Words to know:
Herpesvirus: A group of viruses that are common in people; herpesviruses cause many routine illnesses, such as genital herpes, chicken pox, CMV and cold sores. The viruses tend to remain latent (present but inactive) in people who are infected. These inactive viruses can sometimes become active again and this can cause new illness such as recurrent cold sores, genital herpes outbreaks or shingles. Often the viruses become active without causing any symptoms (asymptomatic shedding or reactivation).

Kaposi's sarcoma: KS is a type of cancer that is associated with HIV infection. Kaposi's sarcoma is much more common among men, especially gay men, than women. It can produce purple-colored skin lesions, and can involve internal organs. It is treated with chemotherapy but appears to have decreased in frequency in people who have been on HAART (highly active antiretroviral treatment), such as protease inhibitors.

Human herpesvirus 8 (HHV-8) is a virus that was discovered only a few years ago. The virus was found in tissue from Kaposi's sarcoma (KS) lesions. More recently, antibodies to HHV-8 have been commonly found in KS patients and in HIV-negative or HIV-infected gay men. HHV-8 has also been found in patients with a very rare type of lymphoma, another type of cancer. We know that most people with KS have antibodies to HHV-8. However, having antibodies to HHV-8 does not mean that KS, or other kinds of cancer, will definitely occur.

Little information is currently available about HHV-8 infection in women. The test results you are receiving today have come from screening tests done through the WIHS. These tests looked for the presence of two kinds of antibody to HHV-8 in blood samples: "lytic" and "latent." Both lytic (meaning active virus) and latent (meaning inactive or sleeping virus) antibodies indicate that HHV-8 infection has occurred; other differences between people who have each type of antibody are not yet known. In all of WIHS, 6% of women had latent antibody and 13% had lytic antibody. KS does occur in women, but very infrequently. Among the 2600 women in WIHS, only about 25 women have had KS. We think that women who have antibody to HHV-8 are at risk for KS, but that risk is probably very low. Many other aspects of HHV-8 are unknown, such as how the virus is spread, and what parts of the body are actually infected with the virus.

In order to find out more about HHV-8 in women, WIHS is conducting a new study of HHV-8 in saliva. This study may help us understand how HHV-8 is spread to others. You may be asked to participate in this study.

Summary:
- HHV-8 is a new herpesvirus that is found in people with Kaposi's sarcoma but also in people who do not have KS.
- KS is uncommon in women.
- HHV-8 infection occurs in women, but there are many questions remaining about this infection.
- Presence of antibody to HHV-8 means that you have been infected with this virus, but does not necessarily mean that you will get KS or lymphoma.
- Your medical provider or WIHS clinician can answer your questions or concerns about your test result.
- We will give you more information about HHV-8 as we learn about it – check your newsletters from your site.