Human Herpesvirus 8 Seropositivity and Risk of Kaposi’s Sarcoma and Other Acquired Immunodeficiency Syndrome-Related Diseases

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For the Italian Seroconversion Study

Background: The incidence of Kaposi’s sarcoma (KS) is increased severalfold in individuals infected with human immunodeficiency virus-1 (HIV). Human herpesvirus 8 (HHV8) has also been implicated in KS. We investigated several factors that may determine the onset of KS, particularly HHV8 infection in individuals after becoming seropositive for HIV. Methods: We studied 366 individuals belonging to different HIV-exposure categories (i.e., homosexual activity, intravenous drug use, and heterosexual contact) for whom a negative HIV serologic test and then a positive HIV serologic test were available within a 2-year period. HHV8 antibody testing was performed by use of an immunofluorescence assay on the first serum sample available after the first positive HIV test. Actuarial rates of progression of KS and of other acquired immunodeficiency syndrome (AIDS)-defining diseases were estimated by use of time-to-event statistical methods. All statistical tests were two-sided. Results: Twenty-one of the 366 study participants developed AIDS-related KS, and 83 developed AIDS without KS. One hundred forty (38.3%) participants had detectable anti-HHV8 antibodies. The actuarial progression rate to KS among persons co-infected with HIV/HHV8 was nearly 30% by 10 years after HIV seroconversion. Increasing HHV8 antibody titers increased the risk of developing KS (for seronegative versus highest titer [1:125 serum dilution], adjusted relative hazard [RH] = 51.82; 95% confidence interval [CI] = 6.08–441.33) but not of other AIDS-defining diseases (adjusted RH = 1.14; 95% CI = 0.72–1.80). HHV8-seropositive homosexual men compared with HHV8-seropositive participants from other HIV-exposure categories showed an increased risk of KS that approached statistical significance (adjusted RH = 6.93; 95% CI = 0.88–54.84). Conclusions: Approximately one third of individuals co-infected with HIV/HHV8 developed KS within 10 years after HIV seroconversion. Progression to KS increased with time after HIV seroconversion. Higher antibody titers to HHV8 appear to be related to faster progression to KS but not to other AIDS-defining diseases. [J Natl Cancer Inst 1999;91:1468–74]
ried and to identify cofactors for disease development, we examined 366 individuals from different HIV-exposure categories with known dates of HIV seroconversion for the presence of antibodies against HHV8 antigens expressed during the lytic phase of viral productive replication (anti-HHV8 antibodies). We evaluated both KS and other outcomes, including progression to HIV-related immunosuppression.

**Materials and Methods**

### Study Population

We analyzed data derived from a prospective cohort study of HIV-positive individuals belonging to different exposure categories and with established dates of HIV seroconversion. Details on the methods have been reported elsewhere (18,19). In brief, participants were recruited from 10 clinical centers located throughout Italy. The main criterion for inclusion in the cohort was the availability of a documented negative HIV test followed by a positive HIV test within a 2-year period; the HIV seroconversion date was estimated as the midpoint in time between the dates of the last negative and the first positive test. For HHV8 testing, only those individuals from whom at least one stored serum sample was available after HIV seroconversion were included in the study.

### Data Collection and Laboratory Procedures

Clinical information and laboratory data were collected at follow-up visits conducted approximately every 6 months. In particular, diagnosis of KS was confirmed by histologic examination in all cases diagnosed up to 1987; beginning in 1987, presumptive diagnostic criteria (gross appearance of an erythematous or violaceous plaque-like lesion on the skin or mucosa) were also used, as indicated in the 1987 revision of the AIDS case definition (20). T-cell subsets were evaluated by flow cytometry, as described previously (19).

### Serologic Tests for Anti-HHV8 Antibodies and Anti-Epstein-Barr Virus Antibodies

Anti-HHV8 antibodies were detected by means of an immunofluorescence assay by use of the BCBL-1 cell line (obtained from Drs. M. McGrath and D. Ganem through the AIDS Research and Reference Reagent Program, Division of Acquired Immunodeficiency Syndrome, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), derived from a body-cavity-based B-cell lymphoma. These cells are Epstein-Barr virus (EBV) negative and are latently infected with HHV8. Cells were grown in RPMI-1640 medium with 10% heat-inactivated fetal calf serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 5 × 10⁻⁵ M 2-mercaptoethanol. All reagents were obtained from GIBCO–BRL, Paisley, U.K. Cells were subcultured at a 1 : 5 dilution twice a week. For the immunofluorescence assay, BCBL-1 cells were treated for 48 hours with 20 ng/mL phorbol-12-myristate-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO) to reactivate HHV8 and induce expression of antigens expressed during the lytic phase of viral replication. The immunofluorescence assay was performed as described elsewhere (21,22). Briefly, 10 μL of a suspension of 4 × 10⁶ cells/mL was smeared on coverslips, rapidly air-dried, and fixed in an acetone–methanol (50%–50%) solution for 10 minutes. Fixed smears (duplicate sample) were incubated with the serum samples diluted 1 : 5 for 30 minutes at 37 °C. The cell smears were washed with phosphate-buffered saline and then incubated with fluorescein-labeled affinity-purified goat antibodies to human immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Titrations were done after five-fold serial dilutions of patients’ sera. All of the microscopic examinations were conducted by two different investigators and were done on coded samples without knowledge of the disease outcome of the individuals. Serum samples that yielded a bright cytoplasmic staining after a dilution of 1 : 5 or more were considered positive. Serum samples from infants 8–12 months old and from HHV-seronegative HHV8-seropositive KS patients were used as negative and positive controls, respectively.

To exclude cross-reactions with antibodies against EBV, we also tested all sera for the presence of antibodies directed against Epstein-Barr nuclear antigen (EBNA) and virus capsid antigen (VCA) (enzyme-linked immunosorbent assay; Gull Laboratory, Inc., Salt Lake City, UT). The association between HHV8 and EBV seropositivity was explored through the use of 2 × 2 tables and standardized statistical tests.

### Statistical Analysis

The study population was stratified on the basis of the results of a single HHV8-serologic measurement performed at a median time of 1 year after HIV seroconversion. HIV-exposure categories (i.e., homosexual activity, intravenous drug use, and heterosexual contact) were assigned by use of a mutually exclusive hierarchy. Intravenous drug users with risk from heterosexual contact were classified as intravenous drug users. The clinical end points were diagnosis of KS, diagnosis of non-Hodgkin’s lymphoma or primary lymphoma of the brain, and diagnosis of AIDS-defining diseases other than KS. For each of these end points, event-free time was estimated as the interval between the estimated date of HIV seroconversion and the earliest of the following dates: the first diagnosis of one of the three end points, the date of death, or the cutoff date (i.e., December 31, 1995). The cumulative probability distribution of progression to clinical AIDS [i.e., as described by the 1993 European definition (23)] and of progression to specific AIDS-defining tumors (i.e., KS, non-Hodgkin’s lymphoma, and primary lymphoma of the brain) was estimated by use of the Kaplan–Meier method. Comparison of actuarial progression curves of individuals infected with HIV alone with those of individuals co-infected with HIV/HHV8 was performed by the logrank test. The Cox proportional hazards regression model was used to calculate crude and adjusted (i.e., for age at HIV seroconversion) relative hazards (RHs) (24). To investigate age at seroconversion and CD4⁺ T-cell counts as possible determinants of KS, we also performed multivariate analysis by the Cox proportional hazards model for

### Results

#### General Characteristics of the Participants

The analysis included 366 persons who seroconverted to HIV during the period from 1983 through 1995. Two hundred seventy-nine (76.2%) participants were males, and 87 (23.8%) were females. With regard to the HIV-exposure category, 171 (46.7%) were homosexual men, 133 (36.3%) were intravenous drug users, 56 (15.3%) were heterosexual contacts, and six (1.6%) had an unknown mode of HIV transmission. The median age at the estimated date of HIV seroconversion was 28 years (range, 16–66 years). When the study population was stratified by exposure category, the median age at HIV seroconversion was 34 years for homosexual men, 25 years for intravenous drug users, and 26 years for heterosexual contacts. One serum sample collected within 1 year (average) from HIV seroconversion (median time, 0.96 year; interquartile range, 0.48–2.20 years) was available for each of these individuals. Antibodies to HHV8 were detected in 140 (38.2%) participants; the remaining 226 (61.7%) individuals were HHV8 seronegative. The demographic characteristics and the number of specific end points among individuals infected with HIV alone and those co-infected with HIV/HHV8 are shown in Table 1. Homosexual men were more likely to be HHV8 positive (61.4%) than individuals who were infected with HIV through heterosexual contact (21.4%) and intravenous drug users (15.0%). In all exposure categories, the proportion of HHV8-positive individuals tended to increase with increasing age at seroconversion (results not shown).

No difference was observed in the distribution of CD4⁺ or CD8⁺ T-cell counts between individuals infected with HIV alone and those who were co-infected either around the date of HIV seroconversion or at the time of serum collection (data not shown).
Association Between Anti-HHV8 and Anti-EBV Antibodies

There was no evident association between HHV8 and EBV seropositivity. Of 17 individuals who were positive for EBNA, only four (23.5%) were seropositive for HHV8. Of the 196 individuals with high EBV VCA titers (>1:2000), only 86 (43.9%) were also seropositive for HHV8.

HIV/HHV8 Co-infection and Progression to KS and AIDS

The median follow-up time of the participants was 6.0 years (range, 0.4–10.7 years). Twenty-one individuals developed KS during the study period: Twenty (95.2%) of these individuals (19 homosexual men and one heterosexual contact) were HHV8 positive. The one individual who was HHV8 negative at the time of the initial testing was a homosexual man; a targeted follow-up of samples from this man showed that he seroconverted at least 1 year before he developed KS. The median time to develop KS was 5.6 years (range, 2.2–9.9 years) from the time of HIV seroconversion and 4.2 years (range, 1.4–7.6 years) from the positive HHV8 test. Fig. 1, A, illustrates the Kaplan–Meier curves of the progression to KS for HIV-infected individuals with or without co-infection with HHV8. The cumulative incidence of KS among co-infected persons within 8 years after HIV seroconversion was 19.5% (95% CI = 10.3%–28.7%) among HHV8-positive individuals and 1.4% (95% CI = 0.0%–4.1%) among HHV8-negative individuals (P < .001, logrank test).

Fig. 1, B, shows the cumulative incidence curves of KS according to the HHV8 anti-lytic antibody titer. The incidence of KS among antibody titers of 1:125 had a higher cumulative incidence of KS than those with an antibody titer ranging between 1:5 and 1:25. Specifically, cumulative incidence within 8 years was 30.9% (95% CI = 7.4%–54.4%) and 17.3% (95% CI = 7.2%–27.4%), respectively (P < .001, logrank test).

Fig. 1, C, shows the Kaplan–Meier curves of the risk of developing AIDS-defining diseases other than KS in both HHV8-positive and HHV8-negative individuals. The difference between curves was not statistically significant (P = .276, logrank test).

Cox models were applied to perform univariate and bivariate analyses, including the presence of HHV8 infection and the age at seroconversion as covariates. At the univariate level and considering the overall study population, the RH of KS in individuals co-infected with HIV/HHV8 compared with those in individuals infected with only HIV was 39.17 (95% CI = 5.24–293.03) (Table 2, group A). The RH of KS estimated for increments of 10 years of age at seroconversion was equal to 2.24 (95% CI = 1.53–3.28). In the bivariate analysis, the RH of KS adjusted for age at seroconversion decreased to 29.56 (95% CI = 3.90–224.32) (Table 2, group A), and the RH for age at seroconversion was basically equal to that obtained with the univariate analysis (data not shown).

Similar models were applied by including HHV8 antibody titer levels as variables (Table 2, group A). With the use of HHV8-seropositive individuals as a reference group, the unadjusted RH of KS for those with antibody titers between 1:5 and 1:25 was 30.01 (95% CI = 3.91–230.25), and that of those with antibody titers equal to 1:125 was 83.85 (95% CI = 10.31–682.06). When adjusting for age at seroconversion, the RH was 24.81 (95% CI = 3.19–192.62) for the group with lower antibody HHV8 antibody levels (1:5 to 1:25) and 51.82 (95% CI = 6.08–441.33) for the group with higher antibody levels (1:125).

Eighty-three persons developed AIDS without presenting with a diagnosis of KS during the study period. The RH of AIDS without KS was 1.28 (95% CI = 0.82–1.98) (Table 2, group B). When adjustment was made for age at seroconversion, the RH decreased toward unity (Table 2, group B). The RH for age at seroconversion was 1.29 (95% CI = 1.02–1.64) in the univariate analysis and 1.27 (95% CI = 0.99–1.62) in the bivariate analysis. When the previous models were repeated (selecting only homosexual men), the results did not change (data not shown).

Two individuals developed non-Hodgkin’s lymphoma (one HHV8-positive homosexual man and one HHV8-negative heterosexual contact), and two developed primary lymphomas of the brain (one HHV8-positive homosexual man and one HHV8-negative intravenous drug user). The cumulative incidence of HIV-related lymphomas was 2.1% (95% CI = 0.0%–6.1%) among individuals co-infected with HIV/HHV8 and 0.6% (95% CI = 0.0%–1.8%) among HHV8-negative individuals within 5 years of HIV seroconversion (P = .523, logrank test).
CD4+ T-Cell Decline Following HIV/HHV8 Co-infection

To evaluate the rate of CD4+ T-cell decline, we examined 335 individuals (91.5% of the study population) for whom at least three CD4+ T-cell counts were available after the date of HIV seroconversion. The distribution of the CD+ T cells over time (slopes) was similar between the HHV8-seropositive or HHV8-seronegative groups. The median rate of decline of CD4+ T cells was −4.8 (interquartile range: −8.8; −2.0) and −5.6 (interquartile range: −10.0; −2.6) for HHV8-positive and HHV8-negative individuals, respectively (P = .304, logrank test). Similar results were obtained by stratifying by age (≤28 years old and >28 years old) at the estimated date of HIV seroconversion (data not shown).

Determinants of KS Among Individuals Co-infected With HIV/HHV8

Table 3 shows the univariate and multivariate analyses for possible determinants of KS, including exposure group, age at seroconversion, use of anti-herpetic treatment known to be active against HHV8 (foscarnet or ganciclovir) (25,26), and CD4+ T-cell counts. In the univariate analysis, the RHs due to all variables were significantly higher than 1 (Table 3). In particular, the RH of KS for homosexual men was 6.93 (95% CI = 0.88–54.84) compared with other risk groups. In the multivariate analyses, the RH of KS for homosexual men decreased and was not statistically significant; the magnitude of the RH did not change substantially after adjustment was made for other variables. The tendency of a higher risk for homosexual men persisted also after including in the model HHV8 anti-lytic titers (high versus low). The titers against latency-associated nuclear antigens could not be determined in all the individuals and hence were not included as variables in any models (results not shown).

**DISCUSSION**

This study provides accurate estimates of the long-term risk of developing KS in HHV8-seropositive individuals with known dates of HIV seroconversion, belonging to different HIV-exposure categories. After 10 years from HIV seroconversion, approximately 30% of HHV8-positive individuals developed KS. The incidence was low within the
first 3 years of HIV seroconversion and increased dramatically after 7 years. The risk of KS increased with increasing anti-HHV8 antibody titers, reaching more than 40% by 10 years after HIV seroconversion for individuals with an antibody titer of 1:125 or higher. This finding suggests that the incubation time of KS is variable and possibly long, and it is consistent with the results of previous retrospective studies of KS patients (27) from whom sequential specimens were available. Only one individual who was HHV8 seronegative at the time of the first testing developed KS during the study period; however, antibodies were detected in a specimen collected 1 year before the diagnosis of KS. A previous study (28) reported that HHV8 seroconversion may occur a few months before the onset of KS.

Our data are not directly comparable with those of other studies, which used other techniques to identify HHV8-infected persons among individuals with an undefined duration of HIV infection. In a previous study of 143 HIV-positive individuals followed for a median time of 30 months (9), more than 50% of those who initially had HHV8 sequences detectable by PCR in the PBMCs progressed to KS within 3–5 years. The high KS incidence in HHV8-infected individuals found in that study is likely due to the specific methodology and study design. PCR testing of PBMCs is less sensitive than is serology (29) and may identify only a subset of the individuals who have been exposed to HHV8, presumably those at the highest risk of developing KS in a brief period of time. Moreover, the expected incidence of KS is higher among individuals recruited in advanced stages of AIDS (as in prevalent cohort studies) than in recent HIV seroconverters. This is also suggested by our finding that, in contrast to previous hypotheses (13), the risk of developing KS during the course of HIV infection is not constant but tends to increase over time after HIV seroconversion.

Direct comparisons are possible with studies of HIV seroconverters with a known HHV8 serostatus. One study (14) showed faster progression rates (49.6% after 10 years); however, KS also developed in about 25% of HHV8-negative individuals. To explain the latter finding, the authors argued that seroconversion might have occurred later in most of the initially HHV8-negative persons but also that false-negative results occurred because of the insufficient sensitivity of the assay for antibodies against latency-associated nuclear antigens. The lower prognostic value of these antibodies is also confirmed by our results, showing that only nine of the 20 participants who developed KS had anti-latent antibodies (data not shown). Another study (15) gave similar results but also suggested that individuals who seroconvert to HHV8 during HIV infection are at a higher risk of KS than those who are infected prior to HIV seroconversion.

In agreement with another study (15), we did not find an excess risk of other opportunistic diseases, after excluding KS, among HHV8-positive individuals compared with HHV8-negative individuals. The CD4+ T-cell decline was also similar in the two groups, excluding other possible effects on the course of HIV infection and on the development of immunosuppression.

HHV8-positive individuals had a tendency to be at higher risk of non-Hodgkin's lymphoma and primary lymphoma of the brain compared with HHV8-negative individuals; however, the difference was not statistically significant. A possible role of HHV8 in lymphoproliferative disorders, such as multicentric Castleman’s disease and BCBLs, has already been shown (8,30,31), although an association between non-Hodgkin’s lymphomas other than BCBLs and HHV8 has not been consistently found (31). However, we cannot exclude that the small number of events in our study may have limited the power to detect small differences.

We were able to identify cofactors that may influence the course of HIV disease and the development of KS among individuals co-infected with HHV8. Homosexual men tended to have an excess risk of KS compared with the risk in other participants. This difference, which was statistically significant only at the univariate level, was not completely explained by HHV8 co-infection; however, the data could not be adjusted for the duration of HHV8 infection before HIV seroconversion. Older age was associated with a faster progression to KS in the univariate analysis; however, when adjusted for the CD4+ T-cell counts, the age effect tended to decrease, since it was mostly explained.
by the immunodeficiency. These data confirm the age effect observed in studies that did not take into account HHV8 infection (32), allowing for more power quantification of the RH.

The risk of KS increased at lower CD4+ T-cell counts. This result is consistent with results of studies showing an increased risk of KS among HIV-infected individuals with fewer than 100 CD4+ T cells/µL (15). In fact, even if KS tends to occur at higher CD4+ T-cell counts in comparison to other AIDS-defining diseases at first AIDS diagnosis (33), it still occurs at a median CD4+ T-cell count of fewer than 50 cells/µL (34).

No reduction in the risk of KS was found in individuals treated with anti-herpetic drugs that are known to inhibit HHV8 lytic infection, after adjustment was made for other confounding variables. Our results seem to confirm those of other studies that found neither a preventive effect (35) nor an influence of different antiviral drugs on the course of KS (36). These findings are also supported by in vitro studies that continued to detect HHV8 in the PBMCs of patients receiving antiviral drugs, such as foscarin and/or ganciclovir (37). However, they are in contrast with the results of other studies suggesting some effects of these drugs on KS treatment (38) or prevention (39,40).

In our opinion, only controlled clinical trials can provide reliable information on this issue.

Our study is limited, in that we did not have HHV8 results from sequential sera, which were not available. In fact, it is possible that some participants not developing KS seroconverted during the follow-up period. However, this problem did not greatly affect the main objective of our study, which was to estimate the long-term risk of KS among co-infected individuals. The small number of lymphomas occurring during the study period did not allow us to exclude a possible role of HHV8 in the etiopathogenesis of these HIV-related tumors.

We used TPA-induced cells to detect anti-lytic HHV8 antibodies. We have found that this test is more sensitive than the anti-latent assay. Although a cross-reactivity with EBV was excluded (see the “Materials and Methods” section), we cannot rule out the possible occurrence of false-positive results from other sources, particularly for results that were based on low antibody titers. This could explain, in part, the differences observed when comparing progression curves of individuals with different antibody titers. However, at worst, these reactions would dilute the associations observed, resulting in more conservative results. Finally, from a statistical point of view, we need to specify that the methods we applied assume that the risk of death before the end point can be removed. This assumption may have biased some of our results.

In conclusion, approximately 30% of the individuals co-infected with HIV/HHV8 develop KS within 10 years after HIV seroconversion. Homosexual men appear to have a higher prevalence of HHV8 infection. The tendency to an excess risk of KS in homosexual men compared with other co-infected individuals could suggest the presence of other cofactors but could also be due to longer duration of HHV8 infection. HHV8 does not seem to modify other parameters of the natural history of HIV disease or to influence the decline of CD4+ T cells.

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Notes

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