Longitudinal Sero-Reactivity to Human Herpesvirus 8 (KSHV) in the Swiss HIV Cohort 4.7 Years Before KS

E. Byrd Quinlivan,1,2* Rui Xue Wang,2 Paul W. Stewart,3 Chulaluk Kolmoltri,3 Nicolas Regamey,4 Peter Erb,4 Pietro L. Vernazza5 and the Swiss HIV Cohort Study

1Department of Medicine, University of North Carolina, Chapel Hill, North Carolina
2Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina
3Department of Biostatistics, University of North Carolina, Chapel Hill, North Carolina
4Institute for Medical Microbiology, University of Basel, Basel, Switzerland
5Departments of Medicine and Microbiology, Kantonsspital, St. Gallen, Switzerland

The relationship between viral infection with Kaposi sarcoma-associated herpesvirus (KSHV) and the onset of Kaposi sarcoma (KS) in AIDS patients is incompletely understood. This study investigates the use of three serological assays to predict the development of KS in HIV-positive patients. Serially collected serum samples from 36 patients with KS and matched controls in the Swiss HIV Cohort Study (SHCS) were analyzed in a case control study. Three serologic assays to detect antibodies against KSHV (nuclear and membrane antigen immunofluorescence assay, N-IFA, M-IFA and ORF 65.2 ELISA) were used to determine the predictive value of KSHV-seropositivity. Serial samples from the cases were also analyzed to determine longitudinal patterns of seroreactivity and identify cases of seroconversion. Assay sensitivity for detection of KSHV antibodies was highest for M-IFA (83%), followed by N-IFA (74%) and 65.2 ELISA (52%). At the time of initial serum sampling (median 4.7 years before KS), only the N-IFA distinguished case and control sera (61% vs. 32%) and no assay was clearly predictive of subsequent onset of clinical KS. Moreover, an unexpectedly high rate of reversions to seronegativity were observed by N-IFA (27/33) as well as by 65.2 ELISA (11/26) in the longitudinal analysis. Analysis of the ORF65.2 ELISA index indicated that these reversions before the clinical onset of KS were associated with antibody levels that frequently hovered around the level of detectability. A marked increase in ORF 65.2 antibody titer occurred in a third of the patients at the time of KS diagnosis. Only two seroconversions were documented. KSHV infection within the SHCS is likely to have preceded HIV infection. KSHV infection alone is not highly predictive of KS development in this cohort of HIV-infected homosexual men as compared with matched controls. Three KSHV serologic assays, though sensitive at the time of clinical KS are inconsistently positive before the development of AIDS-related KS. J. Med. Virol. 64:157–166, 2001. © 2001 Wiley-Liss, Inc.

KEY WORDS: KSHV; Kaposi sarcoma; human herpesvirus 8; human immuno-deficiency virus type 1; seroprevalence; antibody testing

INTRODUCTION

In the 1980s AIDS suddenly thrust a little-known malignancy, Kaposi sarcoma (KS), into the forefront of medicine [Beral et al., 1991]. Historically, the incidence of KS varied significantly with the geographic region and ethnic background of the individual. Highest prevalence rates of KS were restricted to central Africa, southern Europe, and eastern European Jews. In the United States, before the AIDS epidemic, KS was

The members of the Swiss HIV Cohort Study are M. Bättig, (Chairman of the Scientific Board), E. Bernasconi, P.H. Bürgiesser, M. Egger, P. Erb, W. Fierz, M. Flepp (Chairman of the Group Clinics), P. Francioli (President of the SHCS, Centre Hospitalier Universitaire Vaudois, Lausanne), H.J. Furrer, P. Grob, B. Hirschel (Chairman of the Scientific Board), B. Ledergerber, R. Malinverni, L. Matter (Chairman of the Group Laboratories), A. Meynard, M. Opravil, F. Paccoud, G. Pantaleo, L. Perrin, W. Pichler, J-C. Piffaretti, M. Rickenbach (Head of Data Center), P. Sudre, J. Schupbach, A. Telenti, P. Vernazza, and R. Weber.

Grant sponsor: Swiss Federal Office of Public Health; Grant sponsor: Swiss National Science Foundation; Grant numbers: 3900.010.2, 3233-48902.96, 3200-49193.96, 3100-51397.97; Grant sponsor: National Cancer Institute; Grant numbers: CA-74779-01, CA75976-02; Grant sponsor: University of North Carolina Center for AIDS Research; Grant number: NIH P30-HD37260.

*Correspondence to: E. Byrd Quinlivan, MD, CB 7030, 547 Burnett-Womack, University of North Carolina, Chapel Hill, NC 27599-7030. E-mail: eby@med.unc.edu

Accepted 25 August 2000

© 2001 WILEY-LISS, INC.
limited to patients with African, Mediterranean or eastern European ancestry. In the European patients KS was a very slowly progressive skin cancer that occurred on the lower extremities of men. In 1981, young American men developed an aggressive, fatal form of the illness. Epidemiology studies of these men revealed high numbers of male sexual partners and a high incidence of sexually transmitted diseases. The Acquired Immunodeficiency Syndrome was recognized and a sexually transmitted "KS agent " was hypothesized. This agent, identified in 1994 in Yuan Chang and Patrick Moore's laboratory, has been designated as human herpesvirus 8, or Kaposi sarcoma-associated herpesvirus, (Family Herpesviridae, genus Rhadinovirus, species human herpesvirus 8, abbreviated: KSHV, HHV8) [Chang et al., 1994]. KSHV DNA was subsequently found to be present in over 95% of all KS tissue samples, regardless of the type of KS or the geographic origin of the patient [Ambroziak et al., 1995; Boshoff et al., 1995; Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995; Su et al., 1995; Chang et al., 1996].

European countries with an incidence of classic KS (CKS) greater than five cases per million population are Italy, Greece, and surprisingly the Faroe Islands [reviewed in Iscovich et al., 1998]. The two large Italian islands in the Mediterranean Sea, Sicily and Sardinia, are the specific regions of Italy where KS is most commonly seen. Increased rates of CKS, however, are also seen in the Po river valley in northern Italy [Franceschi et al., 1997]. The central and northern European countries report lower rates of CKS.

Time-related as well as geographic variation has been noted in several countries. Israeli rates increased by 2-fold from 1960 to 1974. The authors did not think immigration explained this change, as the increase was similar for Israeli-born as well as the immigrant populations. Although Sweden has a much lower rate of CKS, rates were noted to double from the 1960s to 1970s. Explanations for the changes seen in these countries have not become apparent [Bendsoe et al., 1990; Iscovich et al., 1998].

Switzerland is a landlocked central European country that borders Italy as well as central European countries with low rates of CKS. In the canton of Vaud, Switzerland, between 1974 and 1983, there were no cases of KS registered. In 1983, the first cases of both AIDS and KS in Vaud occurred [Levi et al., 1993]. Based on this study, Switzerland is thought to have a very low incidence of CKS. Of note, this report was limited to a French-speaking region of Switzerland. An analysis of geographic variations of KS incidence using cancer registries from other regions (bordering Germany and Italy) of Switzerland have not been reported. It is quite possible that a different incidence exists in those regions. Currently, the only report on CKS and Switzerland suggests that CKS is quite rare in this country.

Longitudinal cohort studies of individuals at risk for HIV are ongoing in a number of countries, including Switzerland [Melbye, 1984; de Wolfe et al., 1988; Ledergerber et al., 1994]. Early in the AIDS epidemic in Europe, AIDS frequently presented with aggressive disseminated KS. Now that HIV is recognized in risk groups other than homosexual males, KS is noted less frequently. The incidence of AIDS-related KS also varies in each region of Europe. The percentage of men with AIDS-related KS is slightly higher in the northern and central regions of Europe as opposed to southern Europe (Italy, Spain, Portugal, Greece) [Ebrahim et al., 1997]. When these regions are examined more closely, the highest incidence rates are in Central Europe, even when adjusted for risk group, year of diagnosis, and CD4 count [Hermans et al., 1996].

In Switzerland, KS was the presenting condition in 16 of the first 30 AIDS cases [Somadin, 1984; Vogt et al., 1984]. The incidence rate of AIDS-related KS in Switzerland is over 10 per million population. The only European country with a higher incidence rate is France. KS and other AIDS complications have been shown to sharply decline after the initiation of active antiretroviral therapy [Ledergerber et al., 1999a,b]. In France and Switzerland the incidence rate showed at most a slight decrease by 1994, in contrast to the sharp decrease seen in the United States [Franceschi et al., 1997]. Hence, the epidemic of AIDS-KS in Europe may have different characteristics than the epidemic in the United States.

The prevalence of antibodies to KSHV parallels the prevalence of KS. Geographic regions with high rates of KS have higher rates of KSHV nuclear antigen seroreactivity [Gao et al., 1996a]. HIV-infected persons with increased risk of KS have high rates of KSHV infection as well [Kedes et al., 1996; Martin et al., 1998]. In Switzerland approximately 30% of HIV-infected men who have sex with men (MSM) are infected with KSHV and HIV negative MSM have 20% seroprevalence [Regamey et al., 1998a].

The relationship between KSHV infection and onset of KS is incompletely understood. A full understanding of the KSHV contribution to KS will require characterization of incubation time, latency, viral gene expression patterns and the presence of other co-factors. The serologic response over time of HIV- infected individuals to the antigens used in these newly developed assays has not been thoroughly investigated. In this study we examined three different serological assays for the detection of antibodies against KSHV. We selected matched pairs of patients with and without KS, enrolled in the Swiss HIV Cohort Study (SCHS) and evaluated serial KSHV sero-positivity before the onset of disease.

MATERIALS AND METHODS
Patient and Sample Selection
We selected 36 patients in the Swiss HIV Cohort Study (SHCS) [Quinlivan et al., 1997; Rutschmann
et al., 1998; Ledergerber et al., 1994) who were followed for more than 3 years before a diagnosis of KS earlier than March 1996. All human subjects provided voluntary consent and the study was reviewed by the institutional review boards of both the American and Swiss institutions at which the study was conducted. An initial serum sample at the earliest observation time (about 7 to 3 years before KS: referred to in the manuscript as -7 year, -3 year, etc.) and all subsequent serum samples up to the time of KS were obtained from the repository. These are the samples from KS patients at enrollment (n = 36). One patient did not have a sample in the repository at or near the time of KS diagnosis (KS patients at diagnosis, n = 35).

A serum sample from patients without KS, matched for observation time, CD4 count, age, gender, and HIV risk factor was also selected from the SHCS repository. Appropriate matches could not be identified for two patients and five matched control sera were missing from the repository or insufficient for the serologic examination (control patients at enrollment, n = 29).

Complete sets of sera were available from 28 case/control pairs.

Immunofluorescence (IFA)

Cells infected with human herpesvirus 8, (KSHV, Family Herpesviridae, genus Rhadinovirus, species human herpesvirus 8) were used for antibody detection. BCBL-1 cells [Renne et al., 1996] were stimulated with phorbol-ester and harvested after 4 days, washed twice with phosphate buffered saline (PBS) and plated onto glass slides, fixed with acetone and stored at 4°C [Lennette et al., 1996]. Slides with DG75 cells (an KSHV, EBV negative B cell line) were prepared in a similar manner. Sera were diluted 1:10 with PBS, and placed on the fixed cells. Monoclonal enhancement of the signal was performed with mouse anti-human IgG (Zymed Inc., San Francisco, CA) diluted to 1:100. Antibody detection was performed with FITC-labeled goat anti-mouse IgG (Zymed, Inc., San Francisco, CA). All three antibodies were incubated at 37°C and the excess removed by washing three times with PBS. Only those sera that showed a distinctive membrane (M-IFA) or speckled nuclear (N-IFA) pattern of reactivity with the BCBL-1 cells, and not with the DG75 cells, were scored as positive. Inclusion of the DG75 analysis was added to improve the specificity of the M-IFA. Sera were scored blindly by two independent observers; discrepancies were resolved by joint review of the slide and consensus determination. Sera from United States AIDS-related KS patients were examined blindly using this method: 21/23 (91%) were reactive to the membrane antigens and 14/23 (61%) were reactive to the nuclear antigen. Specificity of these assays was high; none of 18 sera from HIV-negative hospitalized United States patients reacted with the nuclear antigen and only two sera reacted with the membrane antigen (100% and 89% respectively). These sensitivity and specificity results were comparable to those reported [Lennette et al., 1996] except the sensitivity of the M-IFA was somewhat reduced in our modified protocol.

ELISA

Seroreactivity to a bacterially expressed ORF 65.2 peptide to in an ELISA (65.2 ELISA) was examined earlier using over 500 sera from different patient groups in Switzerland and we performed the ELISA as previously described [Regamey et al., 1998a,b]. Briefly, recombinant ORF 65.2 proteins expressed in M14 bacteria were purified using a nickel column (Qiagen, Basel, Switzerland). Sera were diluted in PBS containing 0.15% Tween 20 at 1:100. A positive result was a value above the upper “cut-off” calculated from blood donor sera as the mean plus five standard deviations. A negative value was a value below the lower “cut-off” calculated from blood donor sera as the mean plus three standard deviations. Indeterminate values were between these two. To adjust for inter-assay variability, the same five negative Swiss blood donor sera were analyzed in each plate and these results were used to determine the cut-off for each plate. An index was determined by dividing the sample value by the five standard deviations cut-off value. Two reactive sera from Swiss patients with KS were included per plate as positive controls. Only the data from those plates in which these KS sera produced higher values than the cut-off value were accepted. All sera were tested twice blindly without knowledge of the IFA results. Using this procedure with sera from 26 KS patients (unrelated to current study), 24 (92%) were found to be positive [Regamey et al., 1998a].

Statistical Analysis

Matched pairs were compared with test the primary a priori hypothesis that seropositive KSHV status is associated with subsequent KS development. The null hypothesis, “none of the three assays is predictive” was tested by performing three separate assay-specific McNemar’s tests. A P-value of 0.0166 (α = 0.05/3) was considered statistically significant. Additional graphical and model-based methods were used to 1) verify the robustness of the primary result to perturbations of the methods and assumptions, and 2) to fully explore the data with respect to longitudinal patterns of seroreactivity. Conditional logistic regression was used to characterize the probability of developing KS as a function of initial serum assay and elapsed time (years post assay). This model, fitted separately for each of the three assays using maximum likelihood methods, provided the necessary framework for estimating odds ratios (OR) and testing secondary hypotheses. The logistic models were also fitted with and without assay-by-time interaction terms. Grizzle-Starmer-Koch (GSK) methods, generalized estimating equation (GEE) methods, methods such as Fisher’s exact test that ignore the matching, and simplistic descriptive methods were also used. McNemar’s and GSK used only complete pairs, whereas GEE was able to include all data, including
incomplete pairs and longitudinal case data. For GSK and GEE, models were fitted with and without inclusion of time (years elapsed after assay), with and without group-by-time interaction terms, and either separately for each assay or jointly for all three assays. Statistical computations were performed using the SAS System (SAS Institute, Inc., Cary, NC).

**RESULTS**

**Sample Selection and Matching**

Thirty-six patients with KS and at least 36 months of observation time between enrollment and KS diagnosis were identified. Seven patients did not have matched control sera for analysis and one patient did not have sera from the time of KS diagnosis. Thus, 28 matched case-control pairs were identified. The median collection time for case sera was 4.7 years before the date of KS diagnosis and 4.6 years for the control sera. The median time elapsed from control to case blood-draw dates was 8.5 days. Five to 12 serum samples (depending on the length of follow-up) were obtained per case and the final sample was obtained within 79 days, on average, of the diagnosis of KS (SD = 81 days; median 19 days before KS). The CD4 counts were quite similar in the 2 groups (median = 20 in both; cases: range = 0–316 and controls: range = 0–300 cells/mm³). HIV RNA data was not available. The cases and controls were not matched by year of infection but the median date of matched samples was comparable (cases: median = 8/1989, range = 11/1988–11/1992; and controls: median = 12/1989, range = 10/1988–12/1992).

**Assay Sensitivity**

To confirm the sensitivity of the assays used in this study, samples (n = 35) obtained within 6 months of KS diagnosis were used. Reactivity was present in the KS samples in 74% (26/35) by N-IFA, 83% (29/35) of the KS samples by M-IFA, and 49% (17/35) by 65.2 ELISA (Fig. 1). The IFA sensitivities in this cohort were similar to the sensitivities found using the standardization sera (see Materials and Methods). The sensitivity of the 65.2 ELISA was surprisingly lower than seen previously [Regamey et al., 1998a].

**Prediction of KS After Assay Seroreactivity**

To determine the predictive value of KSHV seropositivity, the results from the initial serum in cases and the corresponding sample from the controls were analyzed (Table I). N-IFA seemed to be the most discriminating as 61% of the KS patients had detectable KSHV antibodies compared with 32% for the matched control samples. The corresponding results were 71% vs. 61% for M-IFA and 18% in cases and controls for 65.2 ELISA.

The hypothesis that sero-positivity in one of three assays would be predictive of subsequent KS development was tested. The differences described above were not statistically significant at the $\alpha = 0.05/3$ level by McNemar’s test (Table I). Incomplete pairs (n = 8) and samples with indeterminate results (n = 7) with the ELISA were excluded from statistical analysis (Table I). Conditional logistic regression (and all other auxiliary analyses performed) gave concordant results. The odds ratio was 3.0 for a positive N-IFA assay and 1.7 and 1.2 for M-IFA and ELISA, respectively. In an analysis of the individual assays, the N-IFA was statistically significant at the $\alpha = 0.05$ level but the other two assays did not reach even this level of significance. For both sero-positive patients and sero-negative patients, the estimated probability of KS increased slightly with each year of additional follow-up. This apparent time trend was not statistically significant ($P > 0.145$).

**Changes in Seroreactivity Over Time**

To determine patterns of change in seroreactivity to KSHV antigen, the percent positive samples in each year was determined for both cases and controls (Fig. 2). The nuclear antigen reactivity in cases steadily increased throughout the observation time, whereas,
KSHV in the Swiss HIV Cohort

TABLE I. Predictive Value of KSHV Serology

<table>
<thead>
<tr>
<th>Test of predictive value</th>
<th>McNemar's test</th>
<th>Logistic regression for matched pairs^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroreactivity in the initial matched pairs (cases, controls)</td>
<td>(P,N)^b</td>
<td>(N,N)</td>
</tr>
<tr>
<td>N-IFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-IFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65.2 ELISA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aNumber of case-control pairs. ELISA results with OD values between 3 and 5 standard deviations from the mean were excluded from this analysis.

^bP, positive; N, negative for the assay.

^cEach test at level α/3, and with α = 0.05 (ns = not significant).

^dThree separate assay-specific models.

the percentage of positive controls remained stable. Low rates of seroreactivity were seen in the 65.2 ELISA for cases as well as controls (21%). Reactivity to the membrane antigen was 83% in both cases and 89% controls. No appreciable difference is seen between cases and controls for either M-IFA or 65.2 ELISA. Only the N-IFA distinguished between cases and controls.

Seroconversion

To determine the rate of seroconversion to KSHV antigens during the observation time, a longitudinal analysis was performed. The timepoint of the first positive serum sample for each patient was identified (Table II). Patients were considered positive at all control patients. The table in the bottom of each figure presents the actual number of positive samples/total samples for each timepoint. Only those timepoints with five or more samples were graphed. Error bars = SE. A: M-IFA reactivity. B: N-IFA reactivity. C: 65.2 ELISA.
points in time after the initial positive sample, and the proportion of patients per year who were KSHV-positive before KS was determined for each assay independently. The patient without a sample at KS was excluded from this analysis. At 5 years before KS, 18/23 (78%), 19/22 (86%), and 8/23 (35%) patients were positive by N-IFA, M-IFA and 65.2 ELISA, respectively. The proportion of positive patients increased steadily to reach 94%, 97%, and 74%, respectively, by the time of KS diagnosis. The ELISA was the only assay that showed a large increase in positive patients during the observation period.

At entry, only seven of the 36 patients were negative by all three assays. Only two of these seven patients demonstrated a classic seroconversion pattern with reactivity in all three assays by the time of KS diagnosis. KSHV DNA in peripheral blood mononuclear cells was previously examined in one of these two patients and found to be persistently positive [Quinlivan et al., 1997]. The other five patients were negative or only inconsistently positive with the three assays, suggestive of a poor immune response. In the Swiss cohort, patients with acute infection were not readily identified during the approximately 5 years preceding KS diagnosis.

**Intermittent Seroreactivity**

A surprising result of the longitudinal analysis was the frequent occurrence of transient reversions to seronegativity. A seronegative episode was defined when a negative assay result followed a positive one from an earlier timepoint. Excluding the patients who never developed an antibody response, reversion occurred in 27 of 33 patients with the N-IFA (Fig. 3A). Reversions were also seen with M-IFA (14/33) and 65.2 ELISA (11/26) (data not shown).

To determine how the observed high frequency of seroreversion was related to antibody titer, we plotted the sample 65.2 ELISA index over time (Fig. 3B). At enrollment, four of the cases and three controls had a strongly positive serum sample (index > 2.0). During this time the majority of the sample OD values clustered around the cut-off value, despite being classified as both positive and negative. At diagnosis, 14 of the cases demonstrated a markedly elevated index value, whereas the values for the other cases continued to cluster around the threshold. The increased frequency of reversion to seronegativity was associated with low 65.2 ELISA index values.

Antibody titers to the latent nuclear antigen were determined in Patients 3, 5, 11, and 12 and peak values were 1:≥ 1,600, 1:≥ 3,200, 1:400, and 1:200, respectively (data not shown). The patient with the highest titers (1:≥ 3,200) had no episodes of seronegativity. In the two patients with low peak titers (1:200 and 1:400) frequent transient episodes of seronegativity were seen. In the fourth patient, one episode with sequential negative samples occurred. At the same time the M-IFA was also negative and all previous samples were negative by the 65.2 ELISA. In this episode actual “sero-reversion” seems to have occurred. In the majority of the seronegative episodes examined in these patients, these episodes were preceded by low antibody titers as detected by these assays. Yet in one of four patients examined, the seronegative episodes were not explained by low antibody levels.

**DISCUSSION**

**Seroconversions in Cohorts of Homosexual Men**

The appearance of KS in young homosexual men where it was previously unknown suggests a recent entry of KSHV into this group. This idea is supported by the evidence for recent seroconversions to KSHV in several of the HIV cohorts [Gao et al., 1996a; Melbye et al., 1998; Renwick et al., 1998]. In the United States Multicenter AIDS Cohort (MAC) study the median seroconversion time was 36 months before KS. Seroconversions in the Amsterdam cohort occurred in 215 of 735 KSHV seropositive individuals identified during the years 1984 to 1996. In Denmark, in a similar period of time (1981–1996), 43 seroconversion events were observed and KSHV was seroprevalent in 52 of the 246 homosexual men. These rates are much higher than that seen in our study and may be due to the fact that the study period in these cohorts spanned the early to mid-1980s whereas our observation time began in 1988.
It is not due to inclusion of HIV negative men in these cohorts because in both, seroconversion was associated with HIV infection. If a similar rate of seroconversion was occurring in the SHCS population after HIV, we should have seen evidence of seroconversion occurring in 10–15 patients with KS rather than in two. The relative absence of seroconversions suggest that the SHCS population represents a cohort in which KSHV entered before HIV.

**Seroreactivity in Relation to KS Development**

A picture is emerging from the Swiss cohort regarding KSHV infection in Swiss men who have sex with men (MSM) when the data from our previous study is compared with that from the current study [Regamey et al., 1998a]. Progressive seroreactivity is seen with the 65.2 ELISA in conjunction with clinical progression toward active KS. This progression in seroreactivity rates is as follows: 1) HIV negative MSM = 20%; 2) HIV-positive MSM = 30% in our previous study and 23% in the current study (28/124 cases and controls, year = 8 through = 4, see Fig. 2C); 3) HIV-positive MSM with early preclinical KS = 26% (year = 3 and = 2); 4) HIV-positive MSM at KS diagnosis = 49% (year = 1 and 0); and 5) HIV MSM with well established KS = 92% in our previous study. Unfortunately, the median duration of KS for the patients in the previous study was not available, so at present this trend remains a hypothesis. The seroreactivity rate is fairly flat through the early preclinical period (before = 3 year), until it increases from 23–30% to approximately 50% in the 36 months before KS. Apparently, this sharp increase continues after clinical KS is present, until over 90% of AIDS-related KS patients develop antibodies to ORF65.2. Seroprevalence rates were higher in HIV-positive MSM in Italy (83%) [Rezza et al., 1998] and Netherlands (50%) but were similar for Denmark (30%). Despite the use of different immunoassays, it is likely that some of the geographic variations are real. The
time-related antibody changes after KS diagnosis have not been characterized to date.

**Intensity of Antibody Response**

We found persistently elevated LANA antibody titers in one of two of the seroconverting patients. A much more persistent antibody response was detected in seroconverting patients in the MAC study where the nuclear antigen was detected using BC-1 cells (a different KSHV-infected cell line) and the serum was diluted to 1:100 rather than 1:10. In this study, three of the six patients had titers greater than 1:1,000 for months (persistence of peak titers for 30 to 80 months) [Gao et al., 1996a]. This suggests that higher antibody titers occur if HIV precedes infection by KSHV. This again supports the hypothesis that KSHV infection in the Swiss cohort preceded HIV.

Antibody titers to ORF 65.2 were markedly elevated at KS diagnosis in about a third of the KS patients. This is similar to the changes in antibody titers that occur in Epstein-Barr-related nasopharyngeal carcinoma [Tam and Murray, 1990; Littler et al., 1991; Sigel et al., 1994]. In this case, the occurrence of a rising IgA titer to the EBV replicative protein, EAD, can actually be used to predict the development of a relapse of nasopharyngeal carcinoma. It could be therefore hypothesiz-

**Risk of KS Development**

The specific aim of the case control study was to evaluate the predictive value of KSHV sero-positivity for the development of KS in a cohort of European HIV-positive homosexual males after matching. The difference between cases and controls was small for the M-IFA and 65.2 ELISA. N-IFA seemed to be more discriminating but the association between the N-IFA result and future development of KS was weak ($P = 0.046$). Our study suggests that the true magnitudes of the assay-specific odds ratios are less than 3.5.

The study had adequate power to detect OR of 3.5 or greater. A retrospective analysis of the statistical power of the McNemar's procedure with 28 matched pairs indicated that the study had at least an 80% chance of rejecting the null hypothesis (which states that “OR = 1”) if the true value of the three assay-specific odds ratios was 3.5 or larger. For an OR of 9.2 or greater, this study had roughly a 99% chance of rejecting the null hypothesis. These results provide evidence that the long-term (> 4 years) KSHV seroreactivity was not highly predictive of AIDS-related KS in the Swiss HIV-positive population.
KSHV in the Swiss HIV Cohort

The low OR is in contrast to the OR reported with transplant associated KS [Parravicini et al., 1997; Frances et al., 1999; Regamey, 1998b]. In an Italian case-control study of KSHV sero-positivity and the subsequent development of KS after organ-transplantation, 10 of 11 patients who developed KS after transplantation were KSHV-seropositive before transplantation. This is in contrast to the two sero-positive individuals of 17 matched control patients who did not develop KS after transplantation. The OR calculated for this study was 75 (95% CI 4.5–3,500).

An odds ratio of 8.9 was reported for the Amsterdam cohort. The risk of KS after KSHV seropositivity in the Amsterdam cohort was greater in those individuals seroconverting after HIV infection (HR = 5.64) compared with individuals infected with KSHV at the time of HIV infection (HR = 3.17). One half of these individuals developed KS within 3 years after seroconversion and all of the KS developed by 6 years post seroconversion despite a 10-year follow-up. In the KSHV seroprevalent group a steady rate of KS development was seen after enrollment and half of the cases of KS did not occur until after 6 years of follow-up. When multivariate analysis was done, CD4 count but not HIV RNA remained an independent risk factor. The control population in our study was matched for CD4 count, removing the contribution of immunosuppression to the risk of KS. The CD4 matching and the low seroconversion rate both contribute to the low relative risk we found. It is clear that the risk associated with KSHV sero-positivity varies according to the population being studied and that the risk after organ transplantation is much greater than that found in HIV-infected men.

Reversion to Seronegativity

Consistent with the reduced sensitivity and the low ELISA index values, we found a relatively high frequency of reversion to seronegativity. Seroreversion to KSHV antigens in a few patients has been previously described in both the United States MACS and the SHCS [Gao et al., 1996a; Regamey et al., 1998a]. Of note transient drops in antibody concentrations were reported in 18% of the seroconverting patients in the Dutch cohort. This was not determined for the seroprevalent group. Possible explanations for the frequency of this observation include differences in the assays as performed, differences in the patient populations being studied and factors relating to serum storage.

First, we investigated the possibility of problems in serum storage. The median storage time for the positive samples was only 2 months less than the median storage time for the negative samples (data not shown). Serum from a US AIDS KS patient was subjected to 10 freeze/thaw events without altering the reactivity in the N-IFA assay (data not shown). In addition, no clustering of negative samples was revealed by analysis of the calendar year of collection or the length of storage time (data not shown).

Next we considered potential differences in the immunologic function in our patients. The patients in this series were recruited to the Swiss cohort before the widespread use of protease inhibitors. Therefore, reconstitution of immune function is unlikely to explain the fluctuating reactivity. EBV seroreactivity has been reported as positive in 100% of the patients in this cohort [Telenti et al., 1998]. This demonstrates that the population at large is able to respond to latent herpesvirus infections.

We considered the effects of changes in assay procedures. We used the monoclonal enhanced IFA protocol [Lennette et al., 1996]. This assay enables M-IFA and N-IFA to be interpreted simultaneously but reduces the sensitivity of the N-IFA (from greater than 80%–50%). A reduction in sensitivity of the M-IFA but increased specificity occurred when the use of an internal negative control cell line was included. No alterations in the ELISA protocol occurred. Therefore, changes in procedure may explain some of the difference we found with the IFA but do not explain the ELISA results.

The final consideration was the populations being studied. Immunoreactivity to KSHV antigens may be quite different in early KS (i.e., around diagnosis) compared with late KS. This is a potential explanation for the differences between our earlier study and this one. We observed an association between KSHV seroreactivity and clinical progression to KS. We hypothesized that different amounts of and duration of KS in different groups explain some of the differences in antigen reactivity between studies, including ours.

In conclusion, the strongly seropositive individual can be readily identified but identification of the seronegative individual from the weakly seropositive one, is less certain. We observed seroconversion infrequently despite over four years of observation time. The ELISA index values and N-IFA titers suggested that our patients have a reduced seroreactivity compared with patients who become infected with KSHV after HIV infection. These data suggest that KSHV infection within the SHCS commonly preceded HIV infection and in this setting KSHV infection alone is not highly predictive of KS.

ACKNOWLEDGMENTS

We appreciate the technical help from Zhuwei Zhu and help with manuscript preparation from Trellis Stewart and Diianne Feldman.

REFERENCES


