Kaposi’s Sarcoma–Associated Herpesvirus Infection and Multiple Myeloma

M. B. Rettig et al. (1) describe evidence of Kaposi’s sarcoma–associated herpesvirus (KSHV) infection in long-term cultured bone marrow dendritic stromal cells in 15 out of 15 samples obtained from patients with multiple myeloma (MM) and in two out of eight samples from patients with monoclonal gammapathy of undetermined significance (MGUS). Rettig et al. used polymerase chain reaction (PCR) and in situ hybridization techniques. In the same study, three out of three long-term cultures of bone marrow stromal cells obtained from patients with MM were also found to contain KSHV transcripts coding for vIL-6, a viral protein homologous to human interleukin-6. Because vIL-6 may stimulate in vitro the proliferation of plasmocytoid cell lines, Rettig et al. postulate that infected bone marrow dendritic stromal cells may sustain in vivo the growth of uninfected plasma cell clones, promoting transformation from MGUS to MM. The validity of this attractive hypothesis is challenged, however, by the fact that Rettig et al., using PCR, did not find KSHV in bone marrow aspirates from any of the 15 patients with MM. Rettig et al. ascribed this discrepancy between in vivo and ex vivo results to four concomitant factors: low number of stromal cells, heavy contamination by peripheral blood, presence of up to 90% uninfected neoplastic plasma cells in bone marrow aspirates, and insufficient sensitivity of the PCR assay.

To evaluate the proposed link between KSHV (also known as human herpesvirus-8, or HHV-8) and MM without the limitations of a purely PCR-based experimental design, we studied a series of 40 Italian patients with MM by combining a nested PCR method that achieves single copy sensitivity (2) with serologic assays for antibodies against KSHV. For each case, 1 μg of DNA extracted from freshly purified bone marrow mononuclear cells (10 times the amount used by Rettig et al.) was tested in triplicate by nested PCR. Serum samples obtained at diagnosis (that is before therapy began) were available for half (20) of the patients and were tested by protein immunoblot (3) and immunoperoxidase (4) assays for the presence of KSHV-specific antibodies against orf65 lytic and latency-associated nuclear antigens, respectively. In addition, all sera were screened for antibodies against cytomegalovirus (HCMV) and hepatitis B (HBV) (5). We performed these assays in order to exclude false negative results since, as Rettig et al. correctly observe (1), patients with MM often exhibit a profound inhibition of the humoral immune response (Table 1).

Our results show that none of the 40 patients with MM had KSHV DNA sequences that were detectable in bone marrow aspirates and that only one out of 20 (5%) had antibodies against KSHV (Table 1). By contrast, antibodies to HCMV and HBV were detectable in 18 out of 20 (90%) and 6 out of 15 (38%) of the sera samples, and seroprevalence rates were similar to those found in earlier studies of the Italian general population (6). The latter findings make it unlikely that the low prevalence of KSHV antibodies in MM patients was a result of concomitant inhibition of the humoral response, unless one assumes that this inhibition selectively involved the production of antibodies against KSHV antigens. In fact, KSHV seroprevalence rates are similar in MM patients (5%) and in Italian blood donors of similar geographic origin (4%) (7). Therefore, our findings indicate that most MM patients are not infected by KSHV and argue against there being a link between KSHV infection and the development of MM.

Table 1. Demographic and serologic features of 20 Italian patients with MM. Ab, antibody; Neg., negative; Pos., positive; ND, no data.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Monoclonal component</th>
<th>Decreased serum polyclonal Ig classes*</th>
<th>KSHV+ LANA</th>
<th>KSHV orf65</th>
<th>HBV Ab</th>
<th>HCMV Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1015</td>
<td>F</td>
<td>71</td>
<td>A,k</td>
<td>ND</td>
<td>Neg.</td>
<td>Neg.</td>
<td>ND</td>
<td>Pos.</td>
</tr>
<tr>
<td>1017</td>
<td>F</td>
<td>80</td>
<td>G,λ</td>
<td>ND</td>
<td>Neg.</td>
<td>Neg.</td>
<td>ND</td>
<td>Pos.</td>
</tr>
<tr>
<td>1021</td>
<td>F</td>
<td>54</td>
<td>A,k</td>
<td>0</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Pos.</td>
</tr>
<tr>
<td>167</td>
<td>F</td>
<td>67</td>
<td>k</td>
<td>2</td>
<td>Neg.</td>
<td>Neg.</td>
<td>ND</td>
<td>Pos.</td>
</tr>
<tr>
<td>1739</td>
<td>F</td>
<td>63</td>
<td>A,k</td>
<td>0</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Pos.</td>
</tr>
<tr>
<td>1740</td>
<td>F</td>
<td>67</td>
<td>A,k</td>
<td>0</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Pos.</td>
</tr>
</tbody>
</table>

Positive/total sample (Percent positive) 1/20 (5%) 1/20 (5%) 6/16 (38%) 19/20 (90%)

*Serum polyclonal Ig classes were evaluated by nephelometry (BNA, Behring, Dusseldorf, Germany); 0, no Ig reduction; 1, reduction of one serum polyclonal Ig class; 2, reduction of two serum polyclonal Ig classes.

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2. Bone marrow mononuclear cells were obtained from bone marrow aspirates by Ficoll density centrifugation and their DNA extracted with the use of standard protocols (J. Sambrook et al., in Molecular Cloning: A Lab-

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KSHV is associated with Kaposi’s sarcoma (KS), Castleman’s disease, and a rare form of body cavity lymphoma (1–4). Rettig et al. (5) found KSHV (also known as HHV-8) in bone marrow stromal cells, but not in malignant plasma cells, in all 15 patients with MM that they examined. Their results suggest that KSHV may be required for the transformation of plasma cells. However, their study was limited to examination of bone marrow stromal and mononuclear cells by PCR for KSHV and showed viral DNA only in the bone marrow stromal cells. Bone marrow cells before stromal cell isolation were found not to contain KSHV. Serology for KSHV was not given for these patients with MM (5).

We blindly analyzed serum samples from patients with MM or with epidemic (classic) KS, and from healthy adult donors for antibodies against γ [KSHV-8 and Epstein-Barr virus (EBV)] and β (HHV-6 and HHV-7) herpesviruses. We used (i) an enzyme-linked immunosorbent assay (ELISA) to detect whole virus lysate (2 × 10^9 viral particles per liter) that contained the majority of the viral structural proteins (1.5 mg of viral protein per milliliter) and (ii) an indirect immunofluorescence assay (IFA) to detect KSHV lytic antigens with the KS-1 cell line (which comes from human immunodeficiency virus (HIV)–and EBV-negative body cavity lymphomas).

**Table 1.** Antibody assays of immune response.

<table>
<thead>
<tr>
<th>Virus</th>
<th>MM</th>
<th>KS</th>
<th>Blood donor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number positive/number tested (percent positive)</td>
<td>Number positive/number tested (percent positive)</td>
<td>Number positive/number tested (percent positive)</td>
</tr>
<tr>
<td>KSHV</td>
<td>2/28 (7)</td>
<td>22/25 (88)</td>
<td>2/24 (8)</td>
</tr>
<tr>
<td>EBV</td>
<td>26/28 (93)</td>
<td>20/25 (80)</td>
<td>20/24 (83)</td>
</tr>
<tr>
<td>HHV-6</td>
<td>22/28 (79)</td>
<td>21/25 (84)</td>
<td>21/24 (88)</td>
</tr>
<tr>
<td>HHV-7</td>
<td>24/28 (86)</td>
<td>22/25 (88)</td>
<td>23/24 (96)</td>
</tr>
</tbody>
</table>

*More than 60% of the samples were evaluated by IFA as well as by ELISA; IFA samples were tested at a 1:20 dilution; ELISA samples were tested at a 1:100 dilution. Appropriate negative and positive control sera for each herpes virus were tested simultaneously.

**Table 2.** Detection of KSHV DNA by PCR or by IFA. PB, peripheral blood; MNC, mononuclear cell; BM, bone marrow; ND, not done.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Method</th>
<th>MM</th>
<th>Hematologic malignancies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number positive/number tested</td>
<td>Number positive/number tested</td>
</tr>
<tr>
<td>PB-MNC</td>
<td>PCR</td>
<td>0/6</td>
<td>ND</td>
</tr>
<tr>
<td>BM-MNC</td>
<td>PCR</td>
<td>0/5</td>
<td>0/11</td>
</tr>
<tr>
<td>BM stroma*</td>
<td>PCR</td>
<td>0/5</td>
<td>0/11</td>
</tr>
<tr>
<td>Cell lines</td>
<td>PCR</td>
<td>0/2</td>
<td>0/5</td>
</tr>
<tr>
<td>BM stroma</td>
<td>IFA</td>
<td>0/5</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Short-term cultures of bone marrow stromal cells were established and successive subpassages (P_1–P_3) were tested for presence of KSHV antigen by IFA with the use of polyclonal KSHV-positive and KSHV-negative human serum. Hematologic malignancy cell lines included HUT 78, Raj cells, U937, Daudi cells (all from the American Type Culture Collection), and 23-2 (a lymphoma cell line). Bone marrow mononuclear cells from hematologic malignancies consisted of chronic myelogenous leukemia and Hodgkin’s disease.
phoma] as a substrate (6). The IFA to detect HHV-6, HHV-7, and EBV (also called HHV-4) was performed as described previously (7). We also examined peripheral blood mononuclear cells (six cases) and bone marrow mononuclear cells (five cases), bone marrow stromal cells (five cases) from patients with MM, and two myeloma cell lines for KSHV DNA with the use of PCR. Bone marrow stromal cells were isolated and studied as described previously (5) and were also analyzed for the presence of KSHV by IFA, with the use of the polyclonal antibody against KSHV (6). We did not observe any decline in KSHV antibody when we tested the polyclonal reference serum (which came from a patient with classic KS) for absorption of EBV antibodies with the EBV-producing cell line P3HR1 (Advanced Biotechnologies, Inc., Columbia, Maryland). Antibodies against EBV, HHV-6, and HHV-7 IgG were detected in the majority of the sera from MM patients (Table 1). However, antibodies against KSHV were detected in only 7% of MM sera samples, as compared with 8% of samples from healthy adult donors, and over 85% of samples from patients with acquired immunodeficiency virus (AIDS) and KS. Thus, these patients with MM were able to mount an immune response to highly prevalent lymphotropic human herpesviruses (EBV, HHV-6, HHV-7). The low seropositivity to KSHV in MM is similar to that of the general population in North America and is not secondary to the lack of a humoral response. Furthermore, mononuclear cells (from peripheral blood and from bone marrow) from the 11 patients with MM that we tested were found not to contain KSHV DNA; all of these patients were also seronegative for KSHV serum antibodies (Tables 1 and 2). Last, the short term cultures of myeloma bone marrow stromal cells from five of these cases were found not to contain KSHV with the use of DNA PCR and IFA (Table 2, last row, and Fig. 1).

These data contradict the study by Retting et al. (1) in which KSHV DNA was detected with the use of PCR in all 15 samples of bone marrow stromal cells taken from MM patients (5).

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Retting et al. propose (1) that KSHV (2) is involved in the development of MM from its more indolent precursor MGUS. They argue that KSHV may influence the switch from benign MGUS to malignant myeloma through the secretion and induction of autocrine growth factors, because the virus itself is not present in the clonal plasma cells. This is an attractive hypothesis because KSHV encodes a homolog of one of the cytokines, IL-6 (3), that is involved in MM pathogenesis. A further link might be that patients with multicentric Castleman's disease, another lymphoproliferation associated with KSHV (4), often have immunoglobulin dyscrasias, and occasionally develop MM.

The curious epidemiologic distribution of KS implies that an agent causing this disease would certainly not be the same agent inducing myeloma, which is a common hematological cancer with a much more even distribution throughout different populations. Furthermore, current molecular and serologic assays indicate that KSHV is, in northern Europe and the United States at least, predominantly sexually acquired and is not like most other herpesviruses, which are common pathogens (5) (6). The incidence rates of MM are homogenous in the various regions of northern and southern Italy (7), in contrast to the rate of classic KS (8), which correlates with the seroprevalence rates of KSHV in the different regions (9).

An immunofluorescence assay (IFA) that detects antibodies against the latent nuclear antigen (LNA-1) of KSHV is the most sensitive and specific serologic assay that we know of for revealing past or present infection with this virus (10). The decrease in polyclonal immunoglobulins associated with MM may be global or specific, and antibodies against KSHV may therefore not be detectable in infected patients, even if they have latent or lytic-specific antibodies against other herpesviruses. Antibodies against viruses in patients with MGUS are not suppressed, as a near normal immunoglobulin profile distinguishes MGUS from MM. If KSHV plays any major and common pathogenic role in MM and a subset of MGUS cases, we would expect to find increased serologic markers of infection, particularly in those with MGUS.

We therefore investigated the prevalence of antibodies against KSHV LNA-1 by IFA in patients with MM or MGUS from the Po valley of northern Italy, an area with one of the highest incidence rates of classic (HIV-negative) KS in the world. We compared the detection rate of antibodies against KSHV in these patients with that in patients with lymphoma (Hodgkin's disease and non-Hodgkin's lymphoma) and blood donors, all from the same Po valley area (Table 1). Sera were tested blindly at a dilution of 1:100 as previously described (11).

The prevalence of antibodies against KSHV in the sera of patients with MM, with MGUS, with lymphoma, and in sera from blood donors was not significantly different (P > 0.01; Table 1). The detection rate in Po valley blood donors is significantly higher (12.9% as opposed to <3.0%) than that found with the use of the same assay in blood donors in the United States or the United Kingdom (6) (11), which reflects the much higher incidence of classic KS there.

Four of the MGUS samples with no detectable antibodies were from patients who subsequently developed MM, whereas the only two MGUS patients with detectable KSHV antibodies had no evidence of MM after 36 and 48 months, respectively.

Thus, we did not find evidence for an increased detection rate of antibodies

Table 1. Results from sera of patients and blood donors living in the Po valley of Italy, tested with an IFA specific for antibodies against KSHV.

<table>
<thead>
<tr>
<th>Sera type</th>
<th>Number tested</th>
<th>Number positive (percent positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloma</td>
<td>37</td>
<td>4 (10.8)</td>
</tr>
<tr>
<td>MGUS</td>
<td>36</td>
<td>2 (5.5)</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>42</td>
<td>7 (16.7)</td>
</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>60</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>Blood donors</td>
<td>139</td>
<td>18 (12.9)</td>
</tr>
</tbody>
</table>
against KSHV in patients with MM, and even less so for those with MGUS. Our data do not support a role for KSHV in the evolution of MGUS to MM.

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2. Y. Chang et al., ibid. 266, 1885 (1994).
6. D. H. Kedes et al., ibid., p. 918.
9. D. Whitby et al., ibid., in press.
12. Sera provided by G. Longo, Second Medical Clinic, Modena, and D. Vallisa and L. Cavanna, First Medical Division, Section of Hematology, Piacenza, Italy. Supported by the Medical Research Council and the Cancer Research Campaign.

As a clinical dermatologist involved in the study of classic KS, I (F.C.) have followed about 200 cases of this disease over 20 years in the University Hospital of Sassari, which is the referral center for this disease in northern Sardinia. My epidemiological study has shown a standardized incidence of 1.58 per 100,000 inhabitants per year (2.43 for males and 0.77 for females) (1). This incidence is significantly higher than that observed in nine other Italian regions (1.25 for males and 0.27 for females) (2). I have found cases of KS associated with Hodgkin’s disease, chronic lymphocytic leukemia, and Castleman’s disease, but not with MM. Furthermore, the incidence of MM in the Sassari district is 4.51 per 100,000 inhabitants per year (4.57 for males and 4.45 for females), which is no different from that observed in seven other Italian regions where classic KS is not frequently detected (incidence range from 3.3 to 7.6 for males and from 3.7 to 7.3 for females) (3).

With regard to KSHV epidemiology in northern Sardinia, we found that 12 out of 13 KS patients had KSHV in their peripheral blood as detected by PCR (4). We have recently found that 95% of KS patients had sera with antibodies against KSHV and that the healthy northern Sardinian population has a higher prevalence of antibodies against KSHV in the serum (18%) than do people in the rest of Italy (4%) (5). If KSHV had a role in MM, as suggested by Rettig et al. (6), then we would expect to find an increased rate of MM in the Sassari district and, therefore, a more frequent association of classic KS with MM. Our observations (although made on a small number of cases because of the rarity of classic KS) do not seem to support such an association.

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REFERENCES AND NOTES

7. I would like to thank an anonymous reviewer for helpful remarks.

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REFERENCES AND NOTES

7. I would like to thank an anonymous reviewer for helpful remarks.

Contrary to the report by Rettig et al. (1), other groups have not found antibodies against KSHV in serum from patients with MM (2).

We took acetone-fixed and paraffin-embedded bone marrow biopsies from patients with MM and performed PCR analysis in an attempt to detect KSHV. We investigated 20 cases of MM (at different stages) with a protocol previously described (3) with the use of the primer set within open reading frame (ORF) 26 (KS33023). We carried out two rounds of amplification (2 × 30 cycles). This protocol yielded a positive amplification of 233 base pairs (bp) in 18 out of 20 patients. We also analyzed 15 bone marrow biopsy samples from patients with follicular lymphomas and 5 samples from patients with reactive processes; these samples did not show the presence of KSHV. In all cases, the integrity of the DNA was confirmed after amplification of a 258-bp-long fragment corresponding to the c-raf-1 gene as previously described (4). The specificity of the KS33023 PCR was checked by sequencing the amplification products of five randomly selected cases. We consider our results to be specific because there were different point-mutations in each case as compared with the original sequence (3). These results are similar to those obtained by Rettig et al. (1). Our method would appear to eliminate technical problems such as contamination as a source of the results. Overall, we confirm that, at least in our French series of cases, KSHV is strongly associated with MM.

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REFERENCES

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Response: Several groups raise questions regarding our report that KSHV (also called HHV-8) infects the bone marrow dendritic cells of myeloma patients and may be involved in myeloma pathogenesis (1). Whitby et al. performed a serologic assay, and both Parravicini et al. and Massoud et al. used both serologic and PCR assays to conclude that a lack of evidence exists for the association of KSHV and myeloma. Cottoni et al. demonstrate a lack of clinical correlation between the incidence of classic KS and MM in a cohort of patients from Sardinia.

A possible mechanism for the lack of serological evidence for KSHV in MM relates to a change of environment for B cell development in these patients. Myeloma patients demonstrate panhypogammaglobulinemia (that is, decreased concentrations of antibodies other than the monoclonal antibody produced by the malignant plasma cells), which may reduce the titer of KSHV antibodies. In addition, the infection of dendritic cells within the bone marrow, the site of early B cell development, may lead to B cell tolerance to KSHV antigens. Clonal deletion, functional inactivation, and antigen receptor editing have been shown to be mechanisms for preventing the maturation of B cells that have been presented antigen in the bone marrow (2). Thus, the combination of global and viral antigen-specific immunological defects may contribute to the decreased seroprevalence to KSHV in myeloma patients.

With the use of PCR amplification, Parravicini et al. and Massoud et al. were unable to detect viral sequences in mononuclear cells obtained from aspirated bone marrow, peripheral blood mononuclear cells, or bone marrow stromal cells. The results from bone marrow mononuclear cells are consistent with our
Kaposi’s Sarcoma-Associated Herpesvirus Infection and Multiple Myeloma

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