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Kaposi's Sarcoma-Associated Herpesvirus and Multiple Myeloma: Lack of Criteria for Causality

By Karin Tarte, Yuan Chang, and Bernard Klein

RETTIG ET AL[1] RECENTLY reported the detection of Kaposi's sarcoma-associated herpesvirus (KSHV) DNA as well as KSHV viral interleukin-6 (vIL-6) transcripts in cultured bone marrow (BM) stromal dendritic cells (DC) of patients with multiple myeloma (MM). In a follow-up study, this same group used KSHV ORF72 (v-CYC) in situ hybridization and KS330$_{233}$ polymerase chain reaction (PCR) to detect the virus in fresh BM biopsies of MM patients.[2] Given the general consensus that IL-6, mainly produced by BM stromal cells, is a major growth factor for malignant plasma cells in the active stage of the MM[3] and because the viral homolog of IL-6 is able to substain survival and proliferation of myeloma cells,[4] these data provided a plausible and convenient model for MM pathogenesis. The putative role of KSHV in the emergence of MM was also emphasized by the detection of KSHV DNA in cultured stromal DC of 2 of 8 patients with monoclonal gammopathy of undetermined significance (MGUS), a finding that could be used to explain transformation to MM in 25% of MGUS.[1] Berenson's group also reported detection of KSHV DNA in peripheral blood DC[5] and in apheresis cells from patients treated with cyclophosphamide and growth factors for hematopoietic precursor mobilization.[6] Furthermore, Berenson's group has claimed that KSHV not only is involved in the pathogenesis of MM, but also is involved in another late B-cell disorder, Waldenstrom macroglobulinemia.[7]

The determination of whether an infectious agent is causally related to a disease was first formally codified by Robert Koch in the famed Koch's postulates. These criteria served early microbiologists well. However, in the present era, when newly discovered pathogens are not easily cultivated and the detection of agents may depend on exquisitely sensitive molecular techniques, scientists have looked to other guidelines. A.B. Hill's epidemiologic criteria for causation, now widely used to distinguish a causal from a noncausal association, is one such set of guidelines.[8] According to Hill's criteria, evidence for causality depends on (1) strength of association, (2) specificity, (3) temporality, (4) consistency/reproducibility, (5) biologic

gradient, (6) biologic plausibility, and (7) experimental evidence. Clearly, all of these criteria cannot be fulfilled for every proposed disease association; however, they can provide a systematic approach in examining this issue. In a little over 1 year since the initial report of Rettig et al[1] that hypothesized that KSHV causes MM through infection of BM DC and elaboration of vIL-6 paracrine effects, various laboratories have conducted investigations predominently dealing with the establishment of strength of association and consistency/reproducibility. The majority of the studies that have accumulated fail to confirm this attractive hypothesis. Because KSHV has been controversially involved in several diseases,[9] we will summarize the published data in an attempt to clarify the association between KSHV and MM.

KSHV AND BM STROMAL CELL CULTURES

In their first report, Rettig et al[1] described that stromal cell cultures of MM patients contained KSHV ORF26 DNA by using unnested PCR to amplify a 233-bp product ($KS330_{233}$) and expressed vIL-6 by using reverse transcription-PCR (RT-PCR) for ORF K2. This suggested that at least two noncontiguous KSHV ORFs were present in BM stromal cell cultures of patients with MM. In a subsequent report, the same group detected and sequenced KSHV ORF65 and ORF72 in MM samples. In addition to detecting KSHV DNA by PCR, in situ hybridization of KSHV ORF26 resulted in positive cytoplasmic localization in a majority (nearly 100%) of stromal cultured cells.[1] This high rate of positivity of a lytic phase gene in the cytoplasm is incompatible with sustainable maintenance of these stromal cell cultures, because viral lytic program equates with imminent host cell lysis. Aside from virologic considerations, the presence of at least one copy of KSHV in each of the cultured stromal cells would allow for Southern detection of viral DNA, such as can be seen in KS lesions. To date, no evidence of positivity has been produced by Southern analysis.

Four independent studies have failed to confirm the high rate of infection reported initially. In three studies, KSHV DNA was detected in only 1 of a total of 34 stromal cell cultures.[10-12] The single positive case was only positive using unnested $KS330_{233}$ PCR, which allows detection of approximately 10 KSHV genome copies per 0.2 µg of DNA,[13] but was negative by PCR using primers amplifying other ORFs and on immunostaining for vIL-6. In the fourth study, Tisdale et al[14] obtained BM stromal cells from 25 normal subjects and 30 MM patients and assayed them for KSHV infection. BM stromal cells were obtained using various culture conditions.[14] In a nested PCR assay determined to detect approximately 3 KSHV genome copies per 200,000 cells, 60% of MM samples were positive for KSHV ORF26 DNA (KS330). However, the same sequence was also detected in 44% of samples from normal human controls and in 85% of samples from rhesus macaques. Only 2 of 15 MM and 2 of 21 control samples gave a reproducible weak signal in repeat experiments. Nested PCR is exquisitely sensitive but also prone to contamination, and these results suggest that, if the detection of KS330 was not a false-positive, the target abundance was at the limit of PCR detection. Furthermore, despite similar nested PCR sensitivities, Tisdale et al[14] failed to amplify two other KSHV ORF sequences (ORF75 and ORF72). To explain these results, Tisdale et al[14] suggest the presence of another herpesvirus that shares the KS330 sequence with KSHV and that is found in very low levels in healthy donors' and MM patients' BM stromal cells. Finally, an argument that detection of KSHV depends on culture conditions is not supported by the preceeding reports, because the percentage of KSHV-positive samples was not higher when stromal cells were cultured according to Rettig's protocol. Taken together, these data from four independent studies do not confirm a majority of cultured stromal dendritic cells from MM patients to be infected with KSHV and neither can at least two KSHV ORFs be reproducibly detected.

KSHV AND FRESH BM SAMPLES

In seven studies, including the initial work of Rettig et al, KSHV DNA was undetectable in fresh BM aspirates (1 positive sample of 133) by unnested PCR.[1,10-12,15-17] The only patient positive for KSHV by PCR in these studies was also seropositive for the virus.[17] In response, Berenson has suggested that heparin, which was used to harvest BM, could inhibit Taq polymerase and must be removed by heparinase to allow KSHV sequence amplification. However, Perna et al[12] performed their PCR on 22 DNA samples extracted from EDTA-treated BM aspirates, and two recent studies have clearly demonstrated the absence of a KSHV PCR inhibitor in negative BM samples.[18,24] Thus, no study has reported the presence of KSHV DNA in BM aspirates from MM patients, despite the use of very sensitive PCR assays.

To explain these negative results, it has been further suggested that the KSHV-infected cells adhere to bone and cannot be harvested by aspiration. In agreement with this explanation, Said et al[2] showed by ORF72 in situ hybridization that 2% to 10% of cells in BM biopsies were infected with KSHV in 17 of 20 MM patients. By using either Southern blotting of unnested PCR amplification products or nested PCR, two studies failed to amplify the KS330 sequence (ORF26) in BM biopsies from MM patients (1 positive sample of 18).[11,19] In two other studies, ORF26 amplification could be detected in a majority of patients (23/30); however, detection required either two rounds of amplification (2×30 cycles)[20] or 45 cycles of PCR.[21] Because the sensitivity of the PCR was not assessed and amplification of other ORFs was not verified in these reports, it is not possible to draw a definitive conclusion concerning KSHV infection in these cells. Nevertheless, no report has confirmed that a high percentage of cells (2% to 10% according to Said et al[2]) are infected with KSHV in BM biopsies of MM patients.

The lack of KSHV detection in patients with MM could be due to strong humoral and cell-mediated immune control of this infection. Such an immune control is well-documented in acquired immunodeficiency syndrome (AIDS)-related and post-transplant KS patients.[22,23] The lack of serological prevalence in MM does not support a strong humoral response (see below). Furthermore, the lack of KSHV DNA detection in MM patients with severe T-cell deficiency does not support a strong T-cell–mediated control. Indeed, we studied 10 patients treated with double high-dose chemotherapy associated with autologous transplantation of purified $CD34^+$ cells.[24] These patients had less than 200 $CD4^+/µL$ for up to 1 year in association with biological and clinical reactivation of herpesvirus infections (2 varicella, 1 herpes simplex, 1 herpes zoster, and 6 patients with

antigen positivity for cytomegalovirus). However, despite the use of a sensitive PCR (detection of <5 KSHV genome copies in 150,000 cells) and the lack of KSHV PCR inhibitors, KSHV DNA could not be detected in any of the 30 BM aspirates collected 90, 180, and 360 days after the second autograft. Berenson reported that KSHV was present in BM biopsies from untreated patients and from patients in relapse but not in BM biopsies from patients in remission.[2] However, the lack of KSHV detection in these patients with T-cell immunodeficiency could not be explained by an induction of complete remission, because 4 of the 10 patients relapsed during the first year after treatment.[24]

EPIDEMIOLOGICAL AND SEROLOGICAL STUDIES ARGUE AGAINST AN ASSOCIATION BETWEEN KSHV AND MM

KSHV and non-AIDS KS are found at a higher incidence in Italy,[25] but this is not the case for MM.[26] Recently, a comparison of the incidence rates of KS and MM in different populations worldwide, including the United States, indicates that they do not correlate.[27] Immunofluorescence and immunoblot seroassays allow the detection of antibodies against KSHV in 80% to 90% of KS patients at an early stage of infection.[25,28-30] Among the 15 serological studies published to date, only one reported an increased seroprevalence for KSHV in MM patients (81%) in association with low antibody titers.[31] In this study, antibodies against KSHV were also found more frequently in sera from control cancer patients (22%) than in sera from blood donors (6%). The investigators concluded that KSHV could be associated with some other cancer types. By compiling the data from the other studies, antibodies against KSHV were found in 20 of 447 MM (4.5%) and 28 of 404 normal donors (6.6%).[10-12,14-17,19,21,22,32-34] When tested, the MM patients had a normal humoral response against Epstein-Barr virus (EBV) and cytomegalovirus (CMV),[10,15,17,32-34] excluding a generalized immune defect as responsible for the seronegativity. In addition, patients with MGUS, who are not immunocompromised, have a KSHV seroprevalence of only 4.5% (4/89 seropositive patients). No serological data are currently available for the patients evaluated by Berenson's group. In agreement with the comments given above, one simple hypothesis to explain the lack of seroprevalence to KSHV in MM contrary to other KSHV-related disease is that KSHV is not involved in MM. Another hypothesis proposed by Berenson is that KSHV infected DC and may thus, as the measle virus,[35] compromise the immune response to KSHV proteins. For this reason, we have investigated whether functional DC were infected with KSHV.

FUNCTIONAL DC ARE NOT INFECTED WITH KSHV IN MM

The initial finding of Rettig et al suggested that DC could be a reservoir for KSHV in MM patients, as it was shown for monocytes in patients with KS.[36] DC are essentially defined by their unique ability to capture soluble and particulate antigens and to present, with great efficiency, antigenic peptides to T lymphocytes, including naive T cells.[37] In Rettig et al's study, the dendritic origin of the KSHV-infected cells was assumed only on the basis of certain phenotypic characteristics (CD68$^+$, CD83$^+$, Fascin$^+$), but no functional assay was performed to demonstrate it.[1] CD68 is expressed by a wide variety of cells of the dendritic/monocyte lineage. Fascin is also expressed on B cells infected with EBV, another herpesvirus.[38] Thus, there is no evidence showing that a putative KSHV-infected cell in MM patients is of DC origin. To generate DC for cancer immunotherapy, two main precursor cells can be used: CD34$^+$ cells and monocytes. We and others were unable to detect KSHV DNA in total apheresis cells (collected on ACD) or purified CD34$^+$ cells (0/33 and 0/12 positive samples, respectively)[16,18] collected after hematopoietic growth factor and/or cyclophosphamide treatment, contrary to Berenson's data (15/32 and 3/30 positive samples, respectively).[6] We have also shown that true functional DC (CD68$^+$, CD83$^+$), generated in clinical-grade conditions from adherent apheresis cells, were not infected with KSHV in 10 of 11 patients with MM.[18] These results have been further confirmed in four additional studies that report the absence of KSHV DNA in DC generated either from peripheral blood adherent cells (0/17 positive samples) or from CD34$^+$ purified cells (0/10 positive samples).[16,39-41] Thus, in MM as in other cancers, DC could be safely generated in vitro; pulsed with tumoral cell, peptide, or RNA; and reinjected as an antitumoral cell vaccine.

CONCLUSION

In their initial reports, Berenson et al showed, using PCR and in situ hybridization, that a majority of BM stromal cells, either generated by in vitro culture or present in BM biopsies, were infected with KSHV.[1,2] They concluded that KSHV was present on the basis not only of ORF26 detection, but also of the presence of ORF72, ORF65, and ORF K2. These data lead to the attractive concept of KSHV involvement in MM, particularly because KSHV encodes for a viral homolog of IL-6 that is a major survival and growth factor in this disease. Eighteen months after the initial publication, all studies published so far fail to confirm a widespread infection of BM stromal cells in MM patients. In addition, 14 of 15 studies showed a lack of KSHV seroprevalence in this disease, contrary to other KSHV-related diseases. However, confusion still exists, because in 3 studies some amplification of the KS330 sequence related to ORF26 was confirmed from either BM cultures or BM biopsies.[11,17,21] Detection of the KS330 sequence required very sensitive PCR, indicating that only a few KSHV genome copies were present. These data cannot be reconciled with the high proportion of virus-infected cells reported by Berenson's group (2% to 10% of cells from BM biopsies and 100% of stromal cultured cells).[2] In addition, when it was investigated, the investigators failed to amplify KSHV-related ORF other than ORF26, despite the use of PCR with similar sensitivity. Taken as a whole, these results emphasize that KSHV is not involved in the physiopathology of MM.

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Rebuttal to Tarte, Chang, and Klein

TARTE ET AL REFER to three studies, two letters to the editor and one recent publication from their group, that have failed to show HHV-8 in multiple myeloma (MM) bone marrow stroma cells (BMSCs).[1-3] Importantly, the types of BMSCs produced by long-term marrow culture are highly heterogeneous, and the cell populations that were evaluated for the presence of HHV-8 were not fully characterized in these studies. In addition, no positive controls (Kaposi's sarcoma [KS] patients) were included in the analysis; thus, the sensitivity of the polymerase chain reaction (PCR) assay could not be assessed.[1-3] Anderson's group[4] has recently confirmed the presence of HHV-8 in MM BMSCs with a similar immunophenotype to our own.[5] Although the Tisdale group was able to amplify ORF26 but not ORF72 products in most MM BM samples,[6] we and Anderson's group have easily amplified product in these patients using primers from both of these ORFs in addition to other HHV-8 ORFs. The inability to find HHV-8 in BM aspirates from myeloma patients is consistent with our own experience.[5] In contrast, we and others have detected HHV-8 in the BM biopsies from most MM patients.[7,8] We have used primer pairs from many HHV-8 ORFs and have found consistent detection in fresh BM biopsies from MM patients but not in normal subjects or in patients with other malignancies. Importantly, the conditions necessary to amplify product are optimized for each of our primer pairs before use. In addition, the integrity of the sample DNA is assured by serially diluting it until only one copy of it is present and then performing PCR amplification with actin primers. The lack of viral interleukin-6 (vIL-6) expression in the BM biopsies[3] is consistent with our recent report showing the infrequent expression of this viral homologue in fresh BM biopsies from these patients.[9]

Tarte et al also showed lack of HHV-8 in 30 BM aspirates from MM patients collected after a second high-dose therapy procedure.[10] This is consistent with our previous results on BM aspirates posttransplant.[5] Unfortunately, studies using fresh BM biopsies have not been reported by this group, except in a single case in which the clinical status of the patient is not recorded.[3] In addition, the lack of HHV-8 in BM aspirates obtained from the 4 patients who relapsed after high-dose therapy is consistent with our ability to detect HHV-8 in aspirates from less than 10% of similarly treated patients.

It is intriguing that MM patients have weak[11] or no detectable antibodies[12,13] against HHV-8. This finding may reflect the relative general immunodeficiency that is the hallmark of MM or a specific immune defect that does not allow generation of antibodies against HHV-8. A more interesting possibility is that the type of HHV-8 present in MM patients is different than that present in KS and body cavity lymphoma (BCL) patients. In support of this, we have identified in MM patients consistent changes in the HHV-8 sequence present in regions of the virus that are largely responsible for the immune response.[9] These differences may help explain the inability to identify antibodies that were developed using KS tissues. Recently, specific viral strains of another gammaherpesvirus, Epstein-Barr, have been associated with the development of lymphoid malignancies.[14] Similarly, HHV-8 derived from KS tissues show distinct genetic differences from BCL-derived viruses, and these different viral isolates show different biological effects.[15] Moreover, recent data from KS patients show that certain subtypes of HHV-8 may even predict more aggressive clinical characteristics of the tumor.[16]

The investigators suggest that the HHV-8–infected BMSCs in myeloma patients are not actually dendritic cells (DCs), because they have not been demonstrated to provide antigen-presenting cell function. Quite to the contrary, Anderson's group has shown that these virally infected DCs are, in fact, functional.[17] There is also marked heterogeneity in the type of DCs generated in these different culture systems.[18] To assume that the DCs analyzed for viral presence by other groups were identical to those shown to contain virus in our and Anderson's long-term marrow cultures is a leap of faith. The studies from Tarte et al[19] and Yi et al[20] characterized their DCs as CD1a and CD4-expressing cells, in contrast to the HHV-8–containing DCs in our and Anderson's studies that lacked expression of both of these markers.[4,5] In addition, the DCs in Yi et al's study also lacked CD83, which was found on the HHV-8–containing DCs. Consistent with Tarte et al[19] and others[21] and the absence of CD34 on the BMSCs infected with HHV-8 from our and Anderson's groups,[4,5] we also have rarely found HHV-8 in CD34-enriched autograft material.[22] We certainly believe that it may be possible to generate functional DCs from CD34-selected and other autograft material for clinical use based on our results and these other studies as well as the recent report from Raje et al.[17]

Thus, our studies support the presence of HHV-8 in the vast majority of bone marrow and peripheral blood samples from MM patients. It also suggests that the viral strain may be unique in these patients and may help explain the weak or lack of a

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Kaposi's Sarcoma-Associated Herpesvirus and Multiple Myeloma: Lack of Criteria for Causality

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