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HYPOTHESIS AND DEBATE

Interleukin-6 stimulates HHV-8 replication in bone marrow cultures and infected cell lines

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Introduction: The significance of HHV-8 DNA detection in bone marrow stromal cells from patients with multiple myeloma is still controversial. Since IL-6 plays a key role in the pathogenesis of myeloma, we studied the effect of this lymphokine on HHV-8 DNA detection.
Materials and methods: Amplification of HHV-8 DNA from long-term bone marrow cultures established from normal individuals in the presence or absence of 1 ng/ml IL-6 and from an HHV-8 infected ISI cell line.
Results and conclusions: IL-6 increased HHV-8 replication in seven of ten bone marrow cultures as well as in the ISI cell line. Quantitative PCR showed a 3–100-fold increase in HHV-8 DNA copy number/μg DNA. These data suggest that when IL-6 is present in the micro-environment, HHV-8 replicates and may be amplified in the absence of systemic infection in patients without cellular immune deficiency.
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Introduction

Two years after the initial report from Rettig *et al*.,[1] there is still controversy about the significance of HHV-8 DNA detection in bone marrow from patients with multiple myeloma. The following points pertain to this ongoing debate: (1) the amplification of HHV-8 DNA in bone marrow from myeloma patients could not be confirmed in a number of different laboratories;[1–5] (2) in all but one study, no antibodies to HHV-8 were detected in patients' sera;[6] and (3) the malignancies most certainly related to HHV-8 infection are not observed in patients with myeloma.

Several hypotheses have been put forward to account for these discrepancies. First, myeloma patients might be infected by an HHV-8 related virus as suggested by sequence data;[7] however, multiple ORFs from the original virus could be amplified.[1] Second, the methods used to perform bone marrow cultures may be crucial as the reproducibility of these cultures is quite different from one laboratory to another. Indeed no other study has reported the unusually high yield of dendritic cells in myeloma bone marrow cultures found in the initial study of Rettig *et al*.[1] However, this explanation would not apply to the detection of HHV-8 DNA in bone marrow biopsies as reported by some groups but not others.[3,5,8,9] Third, the amplification of HHV-8 DNA may require exquisitely sensitive polymerase chain reaction (PCR) assays which, aside from contamination pitfalls, need optimized conditions for each primer pair. Therefore, the controversies about the presence and the role of HHV-8 in myeloma have not been satisfactorily resolved to date.

We offer a new hypothesis which relies on the role of one key factor in the biology of myeloma, that of interleukin-6. We show that HHV-8 infected B cells actively replicate HHV-8, when cultured with this lymphokine. Furthermore, when long-term bone marrow cultures from normal individuals were performed in a medium enriched with Interleukin-6 (IL-6), HHV-8 DNA was easily amplified in more than half of the cases.

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Materials and methods

Long-term bone marrow culture (LTC)

Normal bone marrow was obtained after informed consent from ten healthy HHV-8 seronegative bone marrow transplant donors. The samples were diluted (1/3) with PBS and nucleated cells separated by centrifugation over Ficoll (d 1.119 Sigma), before being washed and counted. LTCs were performed in Myelocult H5100 (Terry Fox Laboratory) with hydrocortisone (H4001 Sigma) at 2×10^6 viable cells/ml in 25 cm^2 flasks (5 ml/flask). For each experiment, two LTCs were performed, in one IL-6 was added at 1 ng/ml; the other was a control. After the first week, 50% of the supernatant was replaced twice a week with fresh media with or without IL-6. After three weeks of cultures, the adherent stromal cells were washed three times and then trypsinized. Coded samples (without indication of the presence or not of IL-6 during culture) were further processed for DNA amplification.

In three experiments, stromal cell suspensions were stained with fluorescein-conjugated monoclonal antibody to CD1a. Flow cytometry was performed on a FACScalibur. A total of 20 000 events were analyzed.

DNA extraction

Genomic DNA was extracted from cells harvested from both treated and untreated IL-6 cultures obtained from each individual included in this study (QIAmp blood kit; Quiagen, Courtaboeuf, France). Eluate volume was adjusted to 1 μg DNA/5 μl after optical density measure by spectro-photometry at 260 nm absorbance.

HHV-8 genome amplification

Two gene fragments, ORF 26 (105 bp) and ORF 39 (189 bp), were amplified by single PCR using the primers shown in Table 1. The primer set ORF 26 was within the $KS330_{233}$ sequence previously described by Chang *et al*.[10] The primer set ORF 39 was designed using the Perkin Elmer computer program and located on HHV-8 sequence previously described by Russo *et al*.[11] PCR conditions for amplification of 1 μg of genomic DNA were as follows: denaturing step at 94°C for 3 min followed by 45 cycles of amplification including denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 30 s, then a final extension step at 72°C for 5 min. Reaction mixtures contained 50 pmoles of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 50 mM KCl and 2.5 U of ampli Taq DNA polymerase (Perkin Elmer) in a final volume of 50 μl. For primer set ORF 26, the reaction mixture also contained 0.5 U Uracil N-glycosylase (UNG) and 400 μM dUTP instead of dTTP. PCR assay was performed in the 9600 thermocycler (Perkin Elmer, Cetus). The specificity of products from primer set ORF 26 was confirmed with a 5-biotinylated internal probe (set position: 47 369-399) according to DNA enzyme immunoassay kit (DiaSorin, Salugia, Italy). PCR products from ORF 39 were analyzed by electrophoresis on 3% Nusieve 3 : 1 agarose gel stained with ethidium bromide. The integrity of each DNA sample was appreciated by amplification of the human β-globin gene. Negative controls, including reaction mixtures with or without HHV-8 negative human DNA, were analyzed in each reaction.

Quantitative PCR by Taqman assay

Quantitative PCR detection of HHV-8 was performed with the primer set ORF 26. The internal probe (nt positions: 47 369-399) was synthetized and dual-labeled with fluorescent reporter dye (FAM) at the 5′ end and quencher dye (TAMRA) at the 3′ end (Perkin Elmer, Courtaboeuf, France). Using components supplied in the Taqman core reagent kit (Perkin Elmer), the reaction volume of 50 μl contained: 5 μl of 10 × buffer, 10 pmoles of each primer, 5 mM $MgCl_2$ (optimal concentration), 200 μM of dATP, dCTP and dGTP, 400 μM of dUTP, 1.25 U AmpliTaq Gold, 0.5 U AmpErase Uracil N-glycosylase (UNG), 15 pmoles of dual-labeled probe and 1 μg DNA (5 μl) of each sample. Thermal cycling on ABIPRISM sequence detection system 7700 (Perkin Elmer) was initiated with 2 min incubation at 50°C followed by

Table 1 Primers used for PCR amplification of HHV-8 genes

Gene	*Primer*	*Genomic positions*	*Product size*	*Comments*
ORF 26	5′-CCAACGggATTTgACCTCgTg-3′	47 318–337	105 bp	Sense primer
	5′-CggCCgATATTTTggAgTAgAT	47 422–401		Antisense primer
				The sequence was located within the $KS330_{233}$[10]
ORF 39	5′-CgCggATCCgCgAATgAATATCATTgCgTTTCgTCCATTTCACTgTCACTT-3′	58 976–59 015	189 bp	Sense primer
	5′-CgCggATCCgCgTATgCCTCTggCgACgTCggCgCCgACggTCgATA-3′	59 164–131		Antisense primer
				The primers are associated with *Bam*HI-restricted sequence at 5′ end (underlined sequence)

10 min denaturation at 95°C and then 45 cycles each at 95°C for 15 s and at 60°C for 1 min.

Quantitative analysis of the standard viral load curve was based on a PCR standard curve established with DNA copy numbers ranging from 3 to 7.5×10^6 copies calculated from a relevant cloned plasmid containing the same KSHV sequence $KS330_{233}$.

Culture of HHV-8 infected cell (ISI) and cytokines

The ISI cell line infected by HHV-8 in the absence EBV[12] was used to evaluate the effect of cytokines on viral replication.

Cells were cultured in RPMI 1640 medium containing 20% heat-inactivated calf serum, 100 μg/ml of ampicillin, 50 μg/ml of streptomycin sulfate, 2 mM glutamin and supplemented without or with cytokines (0.5×10^6 cells with IL-6 (1 ng), IL-2 (100 U), IL-4 (100 U) and GM-CSF (100 U)). After 3 days of culture, the cells were centrifuged, then the pellet lysed for DNA extraction. Eluate volume was adjusted to 1 μg DNA/5 μl for quantitative and qualitative PCRs.

Results and discussion

The role of IL-6 in the triggering and/or maintenance of plasma cell growth in myeloma has been firmly established. Stimulation of cell growth may be autocrine[13] or paracrine[14] since bone marrow stromal cells were shown to secrete high amounts of IL-6. Furthermore there is a cross talk between malignant plasma cells and the bone marrow micro-environment since addition of cells from myeloma cell lines on long-term bone marrow cultures from normal donors triggers IL-6 secretion.[15]

According to data from Rettig *et al*.,[1] HHV-8 DNA may be amplified from bone marrow long-term cultures from over 80% of patients with myeloma whereas similar studies using cultures from normal donors were negative. We reasoned that one major difference between myeloma and normal bone marrow *in vitro* cultures might be the high concentration of IL-6 in the former cultures. We established cultures from ten normal donors with or without addition of 1 ng IL-6 twice a week during 3 weeks of cultures. Cells were therefore exposed to a constant concentration of IL-6 during the culture period, independently of constitutive IL-6 secretion which peaked between 40–600 pg/ml in control cultures. Cells were thereafter collected and their DNA amplified for HHV-8 ORF 26 to obtain quantitative estimates of the number of ORF 26 copies/μg DNA. As shown in Table 2, there was no HHV-8 DNA amplification in three cases, regardless of the culture conditions. In four cases, HHV-8 DNA was amplified only in samples cultured with IL-6. Finally, in three cases, there was a 3–30-fold increase in the number of HHV-8 copies amplified from DNA that was extracted from cultures performed in the presence of IL-6. These results were confirmed by the qualitative PCR using primer set ORF 26 and by a second set of experiments using another couple of primers for the HHV-8 ORF 39 (data not shown). We did not study the cell type which replicates HHV-8 in our cultures; however, the percentage of dendritic cells did account for 20–40% of the cells at the end of the culture period, and it did not differ according to the presence or absence of IL-6. Therefore, although IL-6 can modulate dendritic cell maturation, we did not observe such a high percentage of dendritic cells as was noted in cultures from myeloma bone marrow in Berenson's group.[1]

These data strongly suggest that IL-6 present in myeloma bone marrow environment increases HHV-8 replication to a PCR-detectable threshold. In view of these results, we expected that IL-6 might be able to trigger virus replication in HHV-8 infected cell lines. We cultivated the ISI cell line for 3 days with IL-6 (1 ng/ml), IL-4 (100 U/ml), IL-2 (100 U/ml), or GM-CSF (100 U/ml). Only the cultures performed in the presence of IL-6 featured an increase in viral DNA content (Figure 1). Indeed, the increase in HHV-8 ORF 26 DNA copy number was more than 100-fold

Table 2 Quantitative estimates of HHV-8 ORF 26 copies in long-term bone marrow cultures from normal individuals

	Long-term culture sample									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Medium	0*	0	70	107	0	1506	0	0	0	0
Medium + IL6	4949	141	732	308	1591	40135	1066	0	0	0

*Number of ORF 26 copies/μg DNA (see Materials and methods)

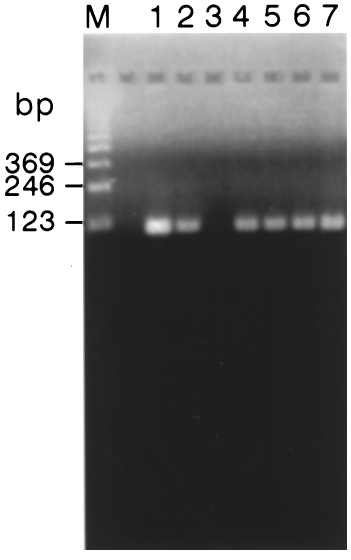

Figure 1 Amplification of DNA from ISI cell line (see Materials and methods). M: marker. ISI cells cultured in medium with IL-6 (lane 1), medium alone (lane 2), IL-2 (lane 4), IL-4 (lane 5), GM-CSF (lane 6). Control uninfected blood cells (lane 3), HHV-8 infected blood cells (lane 7)

(2.6×10^6 *versus* 1.3×10^4 copies/μg DNA) when IL-6 was added to the culture medium.

Obviously, these data may be germane to the present controversy on HHV-8 infection in multiple myeloma. Long-term cultures of bone marrow cells may harbor a detectable load of HHV-8 DNA because of the presence of endogenous IL-6 in myeloma or because of the addition of IL-6 to cultures from normal individuals. In myeloma, this would fit with the frequent detection of HHV-8 during progressive disease and its lower incidence in smoldering disease, as well as in patients with remission after high-dose chemotherapy.[16] Of note, we detected a low number of HHV-8 copies in three of ten normal bone marrow cultured cells in accordance with one report using a sensitive nested PCR for HHV-8 detection.[17] This figure indicates that latent HHV-8 infection in normal individuals may be higher than currently thought from its seroprevalence.

Altogether, our data suggest that minor technical variations such as PCR-sensitivity and *in vitro* culture conditions (which may or may not favor IL-6 secretion by bone marrow cells) may explain some of the discrepancies between previous studies. This also holds true for the detection of HHV-8 DNA by PCR on fresh bone marrow biopsies, if one considers that the viral load in myeloma bone marrow remains close to the detection threshold despite the production of IL-6 by the bone marrow micro-environment.

In this context, the absence of HHV-8 antibodies in patients with myeloma, as reported in all but one study, is still puzzling. We speculate that immune defenses against this virus are mainly mediated by T-cell dependent effectors which could maintain a latent infection in both normal individuals and myeloma patients, without the development of antibodies, at least with the currently available assays. This hypothesis is compatible with the absence of HHV-8-linked diseases in myeloma patients who have a normal cellular immunity and with the relatively low incidence of HHV-8 antibodies in the normal caucasian population.

In contrast, either the cellular deficiency in patients with human immune deficiency retroviral infection or the huge systemic IL-6 overproduction in patients with Castleman's disease[18] may favor systemic HHV-8 infection leading in some patients to the development of Kaposi's sarcoma or cavity-based lymphomas. With respect to this latter observation, we observed systemic HHV-8 infection or the development of Kaposi's sarcoma in two patients who received high-dose chemotherapy followed by blood stem cell grafts (purified CD34 progenitors in one myeloma patient).

Whether or not IL-6 overproduction in myeloma may resolve all controversies about HHV-8 infection will need further experiments. Currently, we believe that HHV-8 infection does not play a crucial role in the pathogenesis of myeloma, although its contribution cannot be definitively ruled out. However, myeloma patients with chemotherapy-induced cellular immune deficiency should be closely followed for evidence of HHV-8 infection.

Finally, the present data raise new questions on the biology of HHV-8 infection and extend the significance of recent reports on the importance of cytokines in the control of HHV-8 replication.[19]

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