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Frequent demonstration of human herpesvirus 8 (HHV-8) in bone marrow biopsy samples from Turkish patients with multiple myeloma (MM)

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In order to investigate the frequency of HHV-8 in MM patients from another geographic location, we obtained fresh bone marrow (BM) biopsies from Turkish patients with MM ($n = 21$), monoclonal gammopathy of undetermined significance (MGUS) ($n = 2$), plasmacytoma ($n = 1$) with BM plasma cell infiltration, various hematological disorders ($n = 6$), and five healthy Turkish controls. The frequency of HHV-8 was analyzed by polymerase chain reaction (PCR) in two independent laboratories in the USA and in Turkey. Using fresh BM biopsies, 17/21 MM patients were positive for HHV-8 whereas all five healthy controls, and six patients with other hematological disorders were negative. Two patients with MGUS, and one patient with a solitary plasmacytoma were also negative. The data from the two laboratories were completely concordant. Also using primer pairs for v IRF and v IL-8R confirmed the results observed with the KS330₂₃₃ primers. Furthermore, sequence analysis demonstrated a C3 strain pattern in the ORF26 region which was also found in MM patients from the US. Thus, HHV-8 is present in the majority of Turkish MM patients, and the absence of the virus in healthy controls further supports its role in the pathogenesis of MM. *Leukemia* (2001) 15, 1268–1273. **Keywords:** HHV-8; multiple myeloma; frequency and sequence; Turkish patients

Introduction

HHV-8 is a new member of the gamma herpesvirus family originally discovered in tumor tissue derived from patients with Kaposi's sarcoma.¹ The virus is known to produce homologues to human cytokines such as IL-6 and IL-8R that could potentially promote tumor cell growth.² Recently, the virus was identified in nonmalignant stromal cells cultured from the bone marrow (BM) of patients with multiple myeloma (MM).³ Subsequent analysis of fresh BM biopsies and peripheral blood mononuclear cells enriched for the dendritic markers CD68 and CD83 also revealed HHV-8 DNA in most of these patients.^{4–6} Similar cultures and samples derived from healthy controls and patients with other hematological malignancies only rarely showed detectable virus. These initial findings have been confirmed by other investigators within the USA and France^{7,8} but refuted by other groups from the UK, France and Sweden.^{9–11} These conflicting results may be the consequence of technical problems or epidemiological differences in the patient populations studied. Thus, in order to clarify the role of epidemiological differences, we studied a patient population with a different ethnic origin. For this purpose, samples from MM cases and normal subjects living in Turkey were selected.

Patients and methods

Patients

Twenty-four patients with monoclonal gammopathies referred from different geographical regions and admitted to the Department of Hematology at Ibni Sina Hospital, Ankara University School of Medicine were included in the study. Sampling was done at the time of diagnosis in 18 patients, and following treatment in six patients. All patients with MM ($n = 21$) had an increased frequency of clonal plasma cells in the BM except for a patient who was in remission following autologous peripheral blood stem cell transplantation (PBSCT). In addition, uninvolved marrow from a patient with a solitary plasmacytoma, and two patients with monoclonal gammopathy of undetermined significance (MGUS) were also included in the study. Previously treated patients ($n = 6$) had received either melphalan and prednisolone or VAD but were either refractory or had relapsed at the time of analysis.

As controls, five individuals (related donors for BM transplants ($n = 3$) and healthy subjects following treatment of iron deficiency anemia ($n = 2$)) were analyzed. In addition, six patients with other hematological disorders were included in the study: one patient each with polyclonal gammopathy, nephrotic syndrome and monoclonal gammopathy, ITP, CML in second chronic phase, and two patients with non-Hodgkin's lymphoma.

Methods

BM biopsies were obtained from the posterior iliac crest in all subjects, transferred in RPMI 1640 medium containing 10% DMSO, and stored in liquid nitrogen. Samples were shipped in dry ice to the West LA VA Medical Center where molecular studies were performed in a blinded fashion. Furthermore, 14 of the same samples were also analyzed in the Department of Molecular Biology and Genetics at Bilkent University in a double-blind fashion. Since the remaining 15 DNA samples were sent to the US without a back-up aliquot they could not be studied in Turkey.

HHV-8 detection by DNA-PCR

Extraction of nucleic acids: DNA was extracted by using the Easy DNA kit (Invitrogen, La Jolla, CA, USA), and the concentration was measured by a fluorometer (Hoefer Scientific, San Francisco, CA, USA). Concentration of the DNA was adjusted to 50 ng/ μ l. RNA was prepared with RNazol (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions, and measured by a spectrophotometer.

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DNA-PCR

For detection of KS 330₂₃₃ (ORF 26), a forward primer (5'-AGC CGA AAG GAT TCC ACC AT-3') and a reverse primer (5'-TCC GTG TTG TCT ACG TCC AG-3') was used. We evaluated 200 ng of DNA (equivalent to the amount of DNA from 30000 cells). A total of 45 cycles of PCR amplification was performed on each sample. The PCR mixture consisted of Buffer-B (PCR Optimizer Kit; Invitrogen, San Diego, CA, USA), Taq polymerase (Perkin Elmer, Norwalk, CT, USA), and Taq antibody (Clontech, Palo Alto, CA, USA). Each PCR cycle consisted of 1 min at 95°C, 90 s at 55°C, 2 min at 72°C, after an initial denaturation of 5 min at 95°C. The positive control was genomic DNA from the HHV-8-containing pleural effusion lymphoma cell line KS-1. To evaluate whether genomic DNA was intact and in adequate amounts, PCR was performed for all the specimens using either beta actin primers (Stratagene, La Jolla, CA, USA) (forward primer, 5'-TGA CCG GGT CAC CCA CAC TGT GCC CAT CTA-3'; reverse primer, 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'), or *hMLH1* (human mut-L homologue 1, a DNA mismatch repair gene) exon 14 primers (forward primer, 5'-TGGTG TCTCTAGTCTCTGG-3'; reverse primer, 5'-CATTGTTGTAG-TAGCTCTGC-3'). Two sets of primers were used to amplify ORF74 (vIL-8R) (forward primer, 5'-CAGCACTAGGTTA GGTTGAA-3'; reverse primer, 5'-GAACGGGAGGCTAGAT-TAAA-3') to generate a 533 base pair product and ORFK9 (vIRF) (forward primer, 5'-TGT GGT GGC TGC GAT AAC-3'; reverse primer, 5'-TCT TCG TCT TCC CAC TCT A-3') to give a PCR product of 147 bp. To eliminate bias in our results, the PCR assays were performed randomly and in a blinded fashion in both institutions. In Turkey, ORFK9 and *hMLH1* primers were used for confirmation. Negative controls were either a PCR mix without DNA or with placental DNA (Sigma, St Louis, MO, USA). Amplified PCR products (10 µl) were electrophoresed on a 1% agarose gel impregnated with ethidium bromide and then photographed. In order to determine the sensitivity of our PCR assay to detect HHV-8, plasmid containing these HHV-8 primers was serially diluted with normal human granulocyte DNA, and PCR amplification was performed. The results showed that the sensitivity of the PCR assay could detect approximately one to two copies of vIRF or ORF26.¹²

RT-PCR

One µg of total RNA was used in reverse transcription. RNA was extracted from BM biopsies of MM and MGUS patients as well as controls. First-strand synthesis was performed at 42°C for 1 h with 1 µg of total RNA in 0.1 mM DTT, and 50 µM downstream primer after an initial incubation at 65°C for 10 min. Following denaturation at 95°C for 5 min, cDNA was used as template to run PCR in the presence of Taq buffer, upstream primer and Taq polymerase. The PCR protocol was as follows: initial denaturation at 95°C for 5 min, 44 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, with a final 5 min extension step at 72°C. The ORFK9 primers were used in RT-PCR. To ensure that the samples were not contaminated with DNA, they were treated with RNAase-free DNAase before performing RT-PCR.

Sequence analysis

The KS330₂₃₃ PCR product was cloned into the pCR 2.1 vector (Invitrogen, San Diego, CA, USA) and sequenced with the Sequenase Version 2.0 Sequencing Kit (Amersham Life Science, Cleveland, OH, USA) according to the manufacturer's instructions, and developed on X-ray film.

Data were analyzed using the DNAsis program (Hitachi, San Bruno, CA, USA), and compared with the GenBank library data for HHV-8. For comparison we used two HHV-8-containing cell lines, KS-1 and BC-1.¹³

Results

In this study, BM biopsy samples from patients with MM ($n = 21$), MGUS ($n = 2$), plasmacytoma ($n = 1$), and other hematological disorders ($n = 6$), in addition to five healthy control subjects, were evaluated for the presence of HHV-8. An amplified product specific for KS330₂₃₃ primers was observed in 17 of 21 MM patients. Specifically, four of the six previously treated patients, and 13 of the 15 newly diagnosed MM samples revealed viral presence (Figures 1 and 2) (Table 1). Results with three sets of primers (ORF26, ORFK9, and

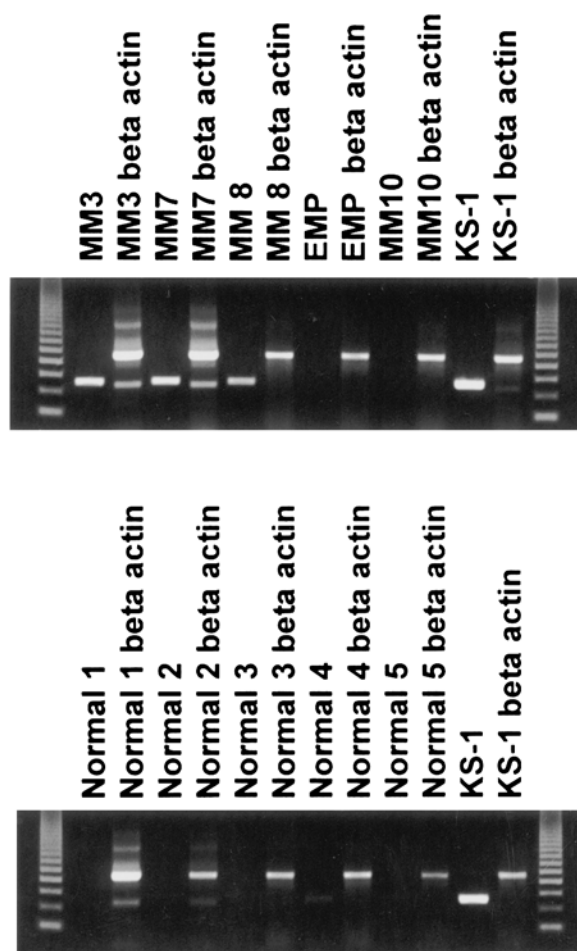


Figure 1 Detection of KSHV gene sequences in DNA isolated from bone marrow core biopsy of patients (upper lane: MM 3, 7, 8 and 10, and extramedullary plasmacytoma (EMP)), normal controls (lower lane) and as a positive control KS-1 cell line by DNA-PCR using ORF26 (KS330₂₃₃) and beta-actin primers.

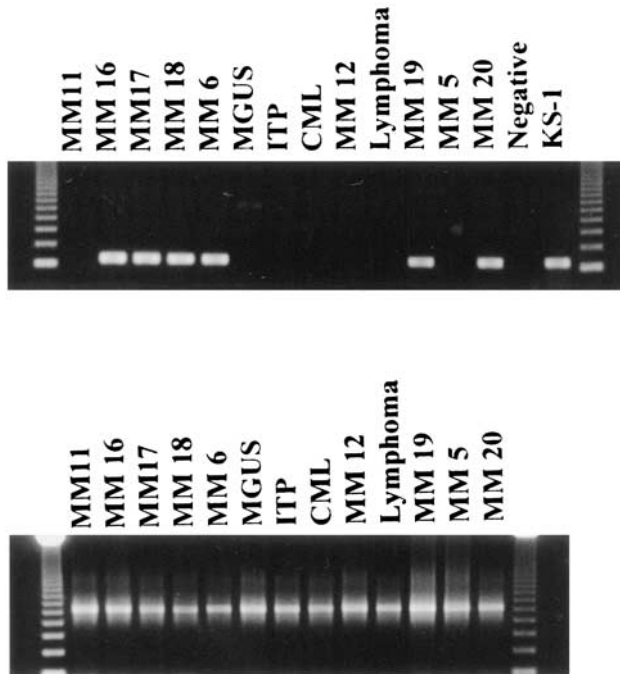


Figure 2 DNA-PCR amplification results on DNA samples obtained from BM core biopsy from patients with MM (MM 11, 16, 17, 18 and 6; MGUS: MM 12, 19, 5 and 20), other hematological disorders such as ITP, CML and lymphoma and DNA from KS-1 cell line (positive control) using the vIRF primer (ORF K9) (upper lane) and beta actin control (lower lane).

ORF74) were in agreement as shown in Figure 3 and Table 1. Fourteen of the 29 samples including patients with plasma cell disorders, and controls were independently analyzed in Turkey. These analyses were done blindly and results were completely in agreement with the results obtained in the US Laboratory (Figure 4). Interestingly, the MM patient (patient 2) who was in remission following autologous PBSCT showed the absence of the virus. In addition, using these same three sets of HHV-8 primers, no amplified product was obtained from the patients with CML, ITP, lymphoma, and polyclonal gammopathy (Figure 2). BM biopsy samples from the five normal subjects were also negative for the presence of HHV-8 in both laboratories (Figures 1 and 4).

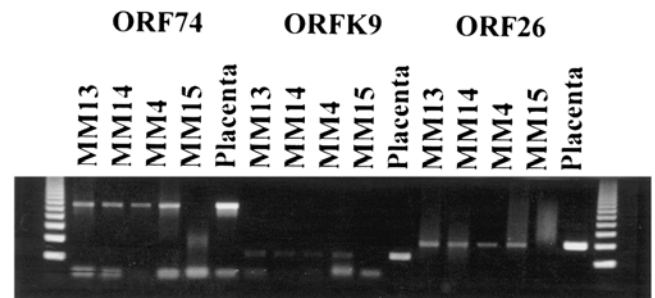


Figure 3 Comparison of three different primers: ORF 74 (vIL-8R), ORFK9 (vIRF) and ORF 26 (KS330₂₃₃) on the same samples (MM 13, 14, 4 and 15), negative and positive controls.

Table 1 Patient characteristics and the results of the DNA PCR with three pairs of primers

Patient No.	Stage	Age	M type	Year of dx	KS330 ₂₃₃ (ORF26)	v IRF (ORF K9)	v IL-8R (ORF74)
<i>Previously treated patients</i>							
1	IA	55	Lambda	1996	+	+	+
2	IIA	54	IgA lambda	1996	-	ND	ND
3	IIIB	58	IgG lambda	1998	+	+	ND
4	IIIA	47	IgG kappa	1996	+	+	+
5	IIIA	50	Kappa	1998	-	-	ND
6	IIIA	46	IgG	1999	+	+	ND
<i>Untreated patients</i>							
7	IIA	65	IgG kappa	1998	+	+	+
8	IIIB	72	IgA	1998	+	+	+
9		66	Plasmacytoma	1998	-	-	-
10	IIIA	56	IgG	1998	-	-	-
11	MGUS	43	IgG kappa	1998	-	-	-
12	MGUS	60	IgG	1998	-	-	ND
13	IIIA	78	IgG	1998	+	+	+
14	IIA	62	Kappa	1998	+	+	+
15	IIIB	58	IgA	1998	+	+	+
16	IIIA	38	Lambda	1998	+	+	-
17	IIA	70	IgA kappa	1999	+	+	+
18	IIA	51	IgG lambda	1999	+	+	+
19	IIA	69	IgG kappa	1999	+	+	+
20	IIA	66	IgG lambda	1999	+	+	+
21	IIIB	79	IgG	1998	+	+	ND
22	IIIA	71	IgG kappa	2000	ND	+	ND
23	IIA	50	Lambda	2000	ND	+	ND
24	IA	61	Lambda	1998	ND	+	ND

ND, not done.

Table 2 DNA and RT-PCR results using primer ORFK9 (vIRF)

Patient No.	DNA-PCR	RT-PCR
1 (MM)	+	+
8 (MM)	+	+
10 (MM)	-	-
12 (MGUS)	-	-
Control 1	-	-
Control 2	-	-
Control 3	-	-

HHV-8 strain

Sequence analysis of ORF26 showed an HHV-8 strain pattern consistent with a C3 subtype (Table 3) which has also been found in MM patients from the United States.¹⁴

Discussion

Following the detection of HHV-8 in Kaposi's sarcoma, its association with AIDS-related body cavity-based lymphoma, and multicentric Castleman's disease has been reported.^{15,16} Since the initial observation demonstrating HHV-8 in nonmalignant stromal cells from patients with MM,³ conflicting reports have been published.^{3-7,10,11,16-24} The HHV-8 genome encodes homologues of IL-6, IL-8R, viral interferon regulatory factor (vIRF), macrophage inflammatory proteins, bcl-2, and cyclin D1.²⁵ These molecules may be involved in both promoting HHV-8 infection as well as directly or indirectly contributing to malignant transformation and growth. Since HHV-8 infects the supportive nonmalignant cells in the myeloma bone marrow, the role of this virus is probably due to an indirect influence in this malignant disease.

This study provides further evidence for the presence of this virus in myeloma patients from another part of the world, supporting its importance in myeloma pathogenesis. In this study, we observed HHV-8 presence in 17 of the 21 Turkish patients with MM. In contrast, none of the healthy subjects or patients with other hematological disorders showed the presence of HHV-8. Patients with solitary plasmacytoma (uninvolved marrow) or MGUS also did not show evidence of the virus in this study. One of the treated MM patients in this study without HHV-8 was observed in a patient in remission following autologous PBSCT. This type of treatment may reduce the viral load below the sensitivity of this PCR assay. The inability to detect virus following PBSCT was reported previously and was regarded as evidence against the role of the virus in MM.^{18,23,26} However, as demonstrated in this study, the lack of detectable HHV-8 in this group of patients may not be uncommon, especially in responding patients. In addition, the absence of the virus in circulating cells is not sufficient to rule out the presence of HHV-8 in MM patients.^{11,24,26} In support of this, the phenotype of cells examined for viral presence by other groups differed from the cells that have been shown by our group and others to contain HHV-8 in myeloma patients' blood samples. Certainly, the frequency of virally infected circulating cells may be less than the sensitivity of the method of detection which can also vary greatly between laboratories. Thirteen of the 15 previously untreated MM patients demonstrated the presence of the virus. BM biopsy material was essential for the demonstration of the virus. In contrast, BM aspirates showed viral presence in only one sample in this

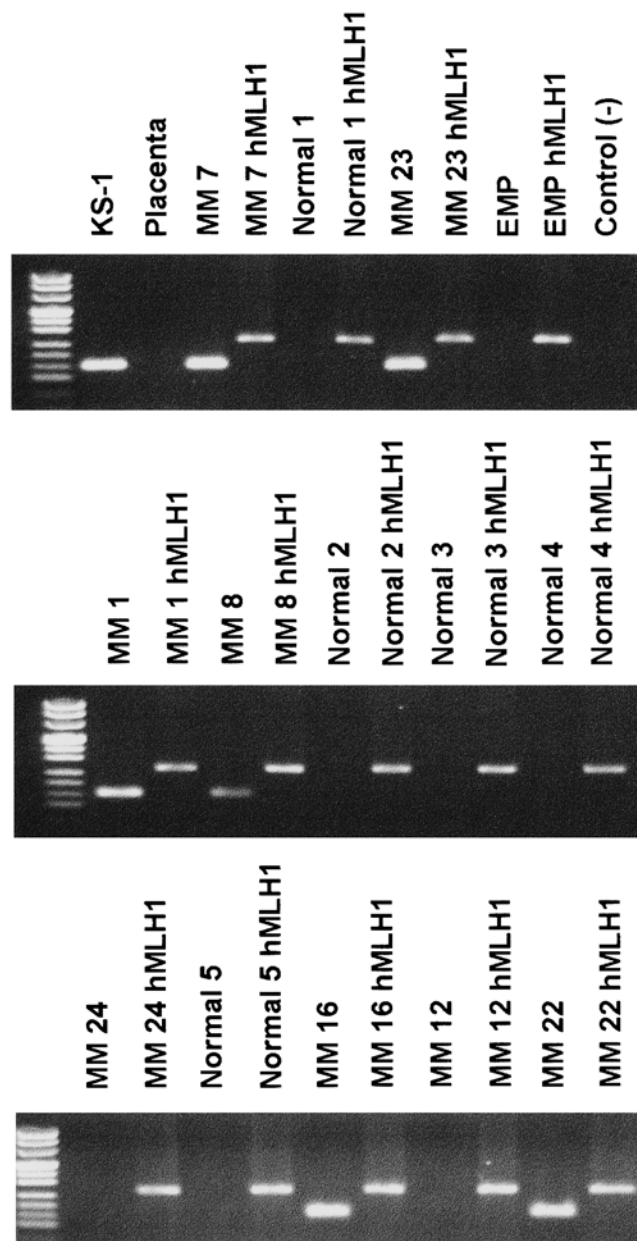


Figure 4 DNA-PCR amplification results on samples from patients with MM (MM 1, 7, 8, 12, 16, 22, 23, 24); EMP and five normal controls performed in an independent laboratory in Turkey using the vIRF(ORFK9) and hMLH1(human mismatch repair gene) primers.

RT-PCR results

As demonstrated by amplification with beta actin primers, intact RNA was obtained from seven individuals (Table 2). Four patients with monoclonal gammopathies (myeloma, $n = 3$; MGUS, $n = 1$), and three controls were analyzed. Using the ORFK9 primer pair, the two MM patients showing viral RNA also contained HHV-8 DNA (Table 2). Importantly, in two patients (MM and MGUS) without DNA evidence of HHV-8, there was also no amplified product with RT-PCR. In the three normal subjects' BM biopsies, no viral RNA was found consistent with the DNA results.

Table 3 Sequence from 233 base pair fragment within ORF26

DNA source	Country	Dx	475899	595	614	621	631	632	647	653	654	664	668	688	691	705	719	731	738	750	756	757	798	47788	Sub-group
Chang et al		KS	C	G	A	C	C	C	T	C	G	G	A	T	G	C	C	A	A	A	T	C	T	G	A
KS	USA	KS					C	C										A	A						A
KS-1		C-L					A	T										A	C						B
BC-1		C-L					C	T										A	A						A
MM	USA	MM					A	T										G	C						C3
MM 1	TR	MM					A	T			T							G	C			C			C3
MM 4	TR	MM					A	T			T			C				G	C						C3
MM 7	TR	MM					A	T			T							G	C						C3
MM 18	TR	MM					A	T			T							G	C						C3
MM 19	TR	MM					A	T			T							G	C						C3

Tr, Turkish.

study (data not shown) consistent with our previous findings in US MM specimens.³ Since fresh BM aspirate is likely to contain a lower proportion of dendritic cells than biopsy material, a reduced ability to detect HHV-8 in these samples is not surprising.¹⁷

Previous reports on the presence of HHV-8 in MM have yielded conflicting results. Investigators have focused on the detection of molecular evidence in a variety of tissues, including BM aspiration or biopsy material, cultured dendritic cells from leukapheresis products or BM samples.^{3-11,17,18,23-30} Others searched for serological evidence by antibodies against the virus.¹⁹⁻²² All of these studies have been reviewed recently.^{17,23} The most frequent method used in the studies that failed to demonstrate viral presence in MM were PCR assays with peripheral blood dendritic cells obtained after long-term culture.^{23,24,26} The types of cells obtained in these negative studies were highly variable, and contained different cell populations than those studies showing HHV-8 in these patients.^{3,7} In addition, the PCR assays including the types of primers and positive and negative controls for detecting HHV-8 were highly variable in these studies.^{22-24,27} Other groups from the United States and Europe have identified HHV-8 DNA and RNA in MM patients consistent with this study.^{3-5,7} With an attempt to eliminate this uncertainty, we used the same primer sets in two independent laboratories in a blind fashion, and the results were completely concordant. In addition, the presence of the same strain, C3, in both US and Turkish myeloma patients further supports the presence of a specific HHV-8 subtype in this B cell malignancy.¹⁴

In conclusion, this study demonstrates the frequent presence of HHV-8, confirmed by two independent laboratories, in MM patients with a specific viral strain in another geographic location; and, thus, provides further support for the important role of this type of HHV-8 in the pathogenesis of multiple myeloma.

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