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PCR WITH DEGENERATE PRIMERS FOR HIGHLY CONSERVED DNA POLYMERASE GENE OF THE HERPESVIRUS FAMILY SHOWS NEITHER HUMAN HERPESVIRUS 8 NOR A RELATED VARIANT IN BONE MARROW STROMAL CELLS FROM MULTIPLE MYELOMA PATIENTS

Massimo DOMINICI[1], Mario LUPPI[2]*, Diana CAMPIONI[1], Francesco LANZA[1], Patrizia BAROZZI[2], Raffaella MILANI[1], Sabrina MORETTI[1], Gianpaolo NADALI[3], Romedio SPANEDDA[1], Raffaella TROVATO[2], Giuseppe TORELLI[2] and Gianluigi CASTOLDI[1]

[1]*Section of Hematology, Department of Biomedical Sciences and Advanced Therapies, University of Ferrara, Ferrara, Italy*
[2]*Section of Hematology, Department of Medical Sciences, University of Modena, Modena, Italy*
[3]*Institute of Hematology, University of Verona, Verona, Italy*

The possibility has been raised that either a human herpesvirus-8 (HHV-8) variant or a novel, unidentified, γ-herpesvirus related to HHV-8 is frequently associated with multiple myeloma (MM), which could explain the lack of antibodies to HHV-8 antigens and the discordant results from polymerase chain reaction (PCR) studies of HHV-8-specific sequences in MM patients. Thus, we used a sensitive PCR assay with degenerate primers targeting the highly conserved DNA polymerase gene of the herpesvirus family to examine the long-term cultures of bone marrow stromal cells (BMSCs) from 19 MM, 3 monoclonal gammopathies of undetermined significance and 6 control patients. Both the culture supernatant and the adherent stromal layer were examined from the 2nd until the 8th week of culture to assess the immunophenotype of the various cell types harvested for the molecular analysis. BMSCs consisted of a mixed population of fibroblast, macrophage, dendritic and endothelial cells. An amplified product of the expected size was obtained only in 3 MM cases, both in the adherent and nonadherent fractions. Direct sequencing and alignment of the nucleotide and amino acid sequences showed that the DNA sequences were 100% identical to Epstein-Barr virus (EBV) DNA. The PCR positivity was due to the presence of EBV-infected lymphoblastoid cells with plasmacytoid features, expressing the EBV-encoded latent membrane protein-1 and detectable either in the stromal cells or in the culture supernatant. Our data do not support a causal role of either HHV-8 or a novel herpesviral variant related to HHV-8 in MM. *Int. J. Cancer* 86:76–82, 2000.
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Human herpesvirus 8 (HHV-8) is the last discovered member of the herpes virus family, with close homology to Herpes virus saimiri and Epstein-Barr virus (EBV). Several serological and molecular studies have shown a clear pathogenetic association between HHV-8 infection and Kaposi sarcoma (KS) (Schulz, 1998). HHV-8 infection has been also documented in rare lymphoproliferative diseases, mainly associated with acquired immunodeficiency syndrome (AIDS), namely primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Schulz, 1998). Conversely, the detection of HHV-8 sequences in human immunodeficiency virus (HIV) negative lymphoproliferative conditions is an uncommon finding (Schulz, 1998; Luppi *et al.,* 1996). Rettig *et al.* (1997) have reported the detection of human HHV-8 sequences in long-term cultures of bone marrow stromal cells (BMSCs) by polymerase chain reaction (PCR) as well as in bone marrow core biopsies by *in situ* hybridization (Said *et al.,* 1997) from the vast majority of patients with multiple myeloma (MM) and from a subset of patients with monoclonal gammopathy of undetermined significance (MGUS). As HHV-8 genome encodes a homolog of one of the cytokines, interleukin-6, that is involved in MM pathogenesis, it has been proposed that the virus may be involved in the development from MGUS to MM by infecting normal BMSCs and inducing the proliferation of myeloma cells in an indirect manner, without the need to directly infect the neoplastic plasma cells (Rettig *et al.,* 1997). These findings have been confirmed by 4 further PCR studies, also showing the presence of HHV-8 sequences in patients with Waldenström macroglobulinemia (Tarte *et al.,* 1999). However, at least 12 independent molecular studies have not been able to detect HHV-8 sequences in MM patients, either in bone marrow biopsies or in short- and long-term cultures of bone marrow stromal cells or in leukapheresis cells collected after mobilization with chemotherapy and granulocyte-colony stimulating factor (G-CSF) or in dendritic cells (DC) cultured from bone marrow and $CD34^+$ selected blood progenitor cells and not even in conditions of severe immunosuppression (Tarte *et al.,* 1999). The association between HHV-8 infection and MM has also been questioned because at least 13 studies have failed to detect antibodies against HHV-8 antigens in MM patients, despite a normal humoral response to other herpesviruses, while only 1 study has reported a higher HHV-8 seroprevalence in MM patients than in blood donors (Tarte *et al.,* 1999). One possible explanation for such discrepancies may be that MM patients are infected with a variant of HHV-8 that encodes for antigens not recognized by available serological assays and with such a high nucleotide sequence variation that cannot be detected by PCR assays with specific primers. This possibility has been strengthened by the detection of one HHV-8 genomic sequence (orf26) but not of other regions of the genome (orf72 and orf 75) in the bone marrow stromal cells obtained not only from MM patients but also from control, healthy subjects, in absence of a detectable serologic response (Tisdale *et al.,* 1998). On the basis of these findings, it has been proposed that a related human herpesvirus does exist that retains sequence homology in the orf26 region but not in the other genomic regions tested. Relevant to this, new herpesviruses closely related to HHV-8 has been identified in non-human primates (Rose *et al.,* 1997; Desrosiers *et al.,* 1997). Of interest, orf26 sequences have been detected by PCR in bone marrow stromal cell cultures and also antibodies to HHV-8 antigens has been detected by immunofluorescence assay (IFA) in normal Rhesus macaques (Desrosiers *et al.,* 1997).

To test the possibility that either HHV-8 or a new herpesvirus, related to HHV-8, is associated with MM, we have assayed the long-term cultures of BMSC of MM and MGUS patients for the presence of an unknown herpesvirus, using degenerate PCR primers targeting the highly conserved DNA polymerase gene of the herpesvirus family (Rose *et al.,* 1997).

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Massimo Dominici and Mario Luppi contributed equally to this work.

*Correspondence to: Section of Hematology, Department of Medical Sciences, University of Modena, Via Del Pozzo 71, I-41100 Modena, Italy. Fax: +39.059.424549. E-mail: mluppi@unimo.it

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MATERIAL AND METHODS

Bone marrow cultures

EDTA-treated bone marrow was collected from 19 patients with MM, from 3 patients with MGUS and from 6 control subjects (1 with B-cell chronic lymphocytic leukemia, 1 with B-cell non-Hodgkin's lymphoma, centrocytic type, 1 with myelodysplastic syndrome and 3 healthy subjects, after informed consent, according to local ethics committee). MM patients were classified according to Durie and Salmon (1975) criteria. All patients were not infected with HIV, and their clinicopathological characteristics are summarized in Table I. Of note, 1 patient (P12) was affected with cutaneous, classic KS.

Bone marrow mononucleated cells (BMMNCs) were fractionated on Lympholyte-H (Cedarlane, Hornby, Canada) by centrifugation at 450 g for 20 min. After 2 washings with Dulbecco PBS 1× (Life Technologies, Renfrewshire, UK), stromal cell cultures were established with long-term culture medium (LTC-m), slightly modified from the original report by Gartner and Kaplan (1980) by plating BMMNCs at a density of 2 to 3 $\times$ 10^6 cells/ml in Iscove modified Dulbecco medium (Euroclone, Paignton, UK) supplemented with 12.5% FCS, 12.5% horse serum (StemCell Technologies, Vancouver, Canada), 1% penicillin/streptomycin and 1% L-glutamine (Hyclone, Cramlington,UK) and incubated at 37°C in 5% CO_2. Fifty percent of the medium was changed weekly until the adherent stroma was confluent. PCR analysis was performed as described (Luppi *et al.,* 1996) on crude extracts obtained from both culture supernatants and BMSCs either after 2 weeks of culture or when a confluent layer of adherent cells was observed, *i.e.,* after 3 to 8 weeks of culture. BMMNCs were also plated in LTC medium at a density of 2 $\times$ 10^6 cells/ml in TC chamber slides (Nunc, Life Technologies, Roskilde, Denmark) for immunophenotype characterization.

Clonogenic stromal assays

To assess the proliferation capabilities of the samples, 1 $\times$ 10^6 BMMNCs/ml were also seeded with LTC medium in Collagen I Petri dishes (35 mm) (Biocoat, Becton-Dickinson, Bedford, MA), and fibroblast colony-forming cell (CFU-F) assays were performed (Castro-Malaspina *et al.,* 1980). Colonies with more than 50 fibroblasts were scored in the prevalent mononucleated cell population of the adherent layer. The same method was used for the detection of endothelial colonies (ECs). Both the CFU-F assay and ECs detection were carried out after 15 days using an inverted microscope (10×, Wilovert-Will Wetzlar, Germany).

Immunophenotypic characteristics of cultured cells

Intact adherent cell layers at different times (ranging from 2 to 8 weeks) of culture were studied using an immunohistochemical technique in combination with a wide range of polyclonal and monoclonal antibodies (MAbs). After LTC-m removal, the TC chamber slides were washed in pre-warmed TBS (pH 7.6), air-dried and fixed in acetone and methanol 1:1 for 1 min. Negative controls with isotype matched nonrelevant MAbs (mouse IgG_1, $IgG2_a$, IgM) were tested in all experiments. Fixed slides were incubated for 10 min with normal rabbit serum (DAKO, Milan, Italy) and then washed in TBS. The slides with primary MAb were incubated for 30 min. Rabbit anti-mouse immunoglobulins (DAKO) and alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO) were added, each step for 30 min incubation. Alkaline phosphatase substrate was added and incubated for 15 min. The slides were washed in TBS, contrasted with haematoxylin, mounted in a suitable acqueous mounting medium, and microscopical examinations were carried out (Diaplan microscope; Leitz, Wetzlar, Germany).

In EBV-positive cells, a multiparametric flow cytometry analysis was performed according to a previously described method (Bi *et al.,* 1994).

Monoclonal antibodies

MAbs used to test APAAP staining were as follows: anti-human alpha-smooth muscle actin MAb (dilution 1:25 to 1:50; clone 1A4, subclass IgG_2), CD68 MAb (dilution 1:50 to 1:100; clone KP1, subclass IgG_1), Vimentin MAb (dilution 1:5 to 1:10; clone V9, IgG_1), factor VIII-related-antigen MAb (dilution 1:200; clone F8/86, subclass IgG_1), CD11c MAb (dilution 1:25 to 1:50; clone KB90, subclass IgG_1) and anti-EBV MAb to LMP-1 (dilution 1:100; clone CS1-4) were provided by DAKO. CD1a MAb (dilution 1:20; clone OKT-6, IgG_1) was from Ortho (Milan, Italy). CD14 MAb (dilution 1:50; clone MY4, IgG_{2b}) was from Coulter (Miami, FL). Anti-collagen I-II-III MAb (dilution 1:20; clone

TABLE I – CLINICOPATHOLOGICAL CHARACTERISTICS AND DETECTION OF HERPESVIRUS DNA POLYMERASE GENE IN MM AND MGUS PATIENTS

Patient	Age (years)	Sex	Ig class	Stage	MC (gr/L)	Therapy	PCR for herpesviral DNA polymerase gene
MGUS							
P1	78	M	G/l	—	18,3	no	neg
P2	61	F	G/l	—	16,1	no	neg
P3	45	M	G/k	—	16,0	no	neg
Not treated MM							
P4	74	M	G/l	II B	24,3	no	neg
P5	76	M	D/l	IIIB	28,4	no	neg
P6	34	M	G/l	IA	32,5	no	neg
P7	67	F	G/k	IIIB	42,4	no	neg
P8	77	F	G/l	IA	21,3	no	pos
P9	74	M	G/l	IIIB	37,1	no	neg
P10	64	F	G/k	IIA	22,4	no	neg
Treated MM							
P11	62	M	A/l	IIIB	7,0	DAV	neg
P12	70	M	G/l	IIIB	24,3	MD	neg
P13	51	F	A/k	IA	20,5	MD	neg
P14	74	F	G/k	IIA	28,4	MD	neg
P15	62	M	G/k	IIIB	6,1	DAV	neg
P16	52	F	G/l	IA	14,0	M+D/DAV	neg
P17	51	M	G/l	IA	37,0	M+D/DAV	neg
P18	57	F	G/k	IA	15,1	DAV/ABMT	neg
P19	62	F	G/l	IIA	35,0	MD	pos
P20	80	F	A/l	IIB	21,4	MD	neg
P21	60	F	A/k	IIIB	22,0	DAV	pos
P22	51	M	G/l	IIIA	37,2	MD	neg

MC: monoclonal component; DAV: dexamethasone, adriamycine, vincristine; MD: Melphalan and prednisone; ABMT: autologous bone marrow transplantation.

MC1-HA) was from Sanbio-Monosan (Uden, The Netherlands). The following MAbs were also used in combination with a flow cytometer (Facscan; Becton Dickinson, Milan, Italy): CD83 MAb (clone HB15a, IgG_{2b} from Immunotech, Marseille, France); CD10 MAb (clone SS2/36, IgG_1 from DAKO); CD 34 (Q-BEND/10, IgG_1 from Serotec, Oxford, UK). HLA-DR (clone L243, IgG_{2a}), CD19 (clone SJ25C1,IgG_1), CD20 (clone L27,IgG_1) and CD38 (clone HB-7, IgG_1) MAbs from Becton-Dickinson (Milan, Italy); B-B4 MAb (clone B-B4, IgG_1 from Immuno Quality Product, Groningen, The Netherlands).

PCR analysis

Crude extracts, obtained from aliquots of 2×10^5 bone marrow adherent cells and from culture supernatants (Gartner and Kaplan, 1980), were analyzed using a nested PCR assay with the degenerate primer pools DFASA/GDTD1B and VYGA/GDTD1B described by Rose *et al.* (1997), targeting a conserved region in the herpesvirus family that codes for substrate binding sites within the DNA polymerases. The sensitivity of this PCR assay was assessed by diluting 1 μg to 0.1 pg of BCBL-1 DNA in 1 μg of the DNA of the HHV-8-negative HSB-2 cell line (corresponding to approximately 150,000 cells). The products of secondary nested PCR were subjected to direct sequence analysis by "cycling sequencing," a variation of the classic dideoxy chain termination technique (Luppi *et al.*, 1996). Searches of the GenBank database using the BLAST Web server were performed, and alignment of nucleotide sequences as well as amino acid translations of sequences of the amplified DNA fragments compared with that of other known human and non-human herpesviruses has been performed as reported elsewhere (Rose *et al.*, 1997). Furthermore, a nested PCR assay for EBV-latent membrane protein-1 (LMP-1) gene sequences (Knecht *et al.*, 1997) was also performed on the same bone marrow adherent cell samples. To test for integrity of the DNA and to exclude the presence of PCR inhibitors, PCR amplification with DQα primers was performed on all samples (Luppi *et al.*, 1996). To reduce the risk of PCR contamination, all necessary precautions were taken (Luppi *et al.*, 1996). The positive controls consisted of DNA extracted from 3 KS biopsy samples, 1 lymph node with MCD and from 1 EBV-negative PEL specimen previously found to be positive for HHV-8 genome (Luppi *et al.*, 1996 and data not shown) and from the EBV-positive B95.8 cell line (kindly provided by Dr. R.I. Fox, La Jolla, CA). The negative controls consisted of placental DNA and the HHV-8-negative and EBV-negative HSB-2 and Molt 3 cell lines.

RESULTS

Clonogenic stromal assays

No significant differences in the number of CFU-F between MM and control samples were observed (mean 19 ± 8 SE/10^6 BMMNCs). However in 5 patients (P7, P8, P14, P19, P21), few CFU-F (mean 3 ± 2 SE/10^6 BMMNCs) were detected and fibroblasts never reached the confluence even after 4 months of culture. ECs were spontaneously present in 12 of 22 (54.5%) stroma studied. They appeared as small polygonal-shaped clusters, which were sometimes constituted by binucleated cells (Fig. 1*a*). Interestingly, in 4 patients (P1, P5, P11, P12), this population represented up to 30% of the confluent adherent cell layer in the absence of any specific culture system usually performed to obtain ECs. The ECs often anticipated the CFU-F development, becoming undetectable after 35–40 days of culture because of the huge fibroblast proliferation.

Immunophenotypic features of stromal cells

As HHV-8 sequences were originally detected only in DCs (Rettig *et al.*, 1997; Said *et al.*, 1997), the presence of these cells was investigated in our LTC using a variety of reagents, including 2 typical dendritic markers CD1a and CD83, along with HLA-DR, CD11c and CD68 (Table II). During the first 2 weeks of culture, the adherent layer mainly consisted of a mixed population of mononucleated cells (Fig. 1*b*). In several samples, DCs represented about 20%–40% of the adherent cells, while later on, when fibroblast proliferation took place, their number suddenly decreased. At confluence, the greatest part (80%–85%) of the BMSC consisted of fibroblast cells (Fig. 1*c*), which resulted strongly positive for CD68 MAb, as well as for mesenchymal-associated markers, such as smooth muscle-alpha-actin, vimentin and anti-collagen I-II-III as previously reported (Wilkins and Jones, 1995) (Table II). CD14 and CD68 positive macrophages were scattered upon the fibroblast layer, accounting for 8%–12% of the adherent layer. Endothelial cells appeared as small colonies, formed by mononucleated and binucleated cells that were von Willebrand factor-positive (Fig. 1*d*). At confluence, the proportion of cell populations remained almost the same until the 12th week, except for the appearance of adipocytes, as it normally occurs in LTC, and a concomitant decrease of the ECs. Immunophenotypic features of the adherent layer from the MM, MGUS and control samples did not exhibit significant discrepancies (Table II). In P8, P19 and P21, cluster of hematopoietic cells was observed in association with the stromal fibroblasts (Fig. 1*e*). These hematopoietic cells remained present over time and increased in number, while in the other MM, MGUS and control long-term cultures hematopoietic cells markedly diminished after 5 to 7 weeks. In these samples, as culture progressed, a proportion of the growing cells detached from the stromal layer, becoming detectable in the culture supernatant. Growth of these cells could also be maintained independently from stroma. Morphological analysis at 8 weeks of culture revealed that most of these cells were plasmacytoid (Fig. 1*f*). Multiparametric flow cytometry showed the presence of 2 populations, 1 of small (about 20%) and 1 of large (about 80%) size, both negative for B-B4, CD34 and CD10 but expressing abundant CD19, CD20 and HLA-DR. Expression of CD38 was documented in most of the large but only in a minority of the small cells.

Characterization of herpesvirus infection

The amplification of *DQα* gene was positive in all samples, suggesting the absence of major PCR inhibitors. Using a nested PCR with degenerate primers for the herpesvirus DNA polymerase gene, we were able to detect the presence of HHV-8 DNA in 1 pg of genomic DNA of the HHV-8-infected BCBL-1 cell line diluted in 1 μg of genomic DNA of the HHV-8-negative HSB-2 cell line. Since every BCBL-1 cell contained an average of 30 copies of the HHV-8 genome, 1 pg of BCBL-1 DNA corresponded to about 5 HHV-8 copies (Tarte *et al.*, 1999). Using this sensitive PCR assay, amplification products of the predicted size of about 236 bp were detected in the bone marrow stromal cell fractions, obtained from 3 patients with MM (P8, P19, P21), either after 2 weeks or after 3 months of culture. Amplification products of a similar size were obtained in the 3 KS, the 1 MCD as well as in the PEL specimens but not in negative controls. The resulting PCR fragments were sequenced, and the alignment of the nucleotide as well as amino acid sequences revealed that the DNA sequences identified in the BMSCs from all 3 MM patients were 100% identical to the EBV DNA polymerase gene across the 236 alignment (Fig. 2). The DNA sequence identified in the 3 KS biopsies, in the lymph node with MCD and in the PEL sample was 100% identical to HHV-8 DNA polymerase gene as expected (Rose *et al.*, 1997). The presence of EBV genome was further confirmed by PCR amplification of specific LMP-1 sequences of EBV not only in the adherent cell fractions but also in the culture supernatants from the same patients. Shorter LMP-1 PCR products, compared with the wild-type LMP-1 sequence of the prototypic EBV strain B95.8, were identified in 2 of the 3 MM patients. DNA sequencing of the shorter PCR products showed a deleted segment spanning 30 bp (at positions 168285 to 168256) and a deleted segment spanning 69 bp (at positions 168325 to 168255) (Knecht *et al.*, 1997) in the 2 MM cases, respectively (Fig. 3). In the remaining MM case, a wild-type LMP-1 sequence was identified. To further characterize the EBV infection in the 3 MM cases, both the adherent and the non-adherent cell fractions of the long-term cultures were examined with anti-EBV MAb to LMP-1. Positive reactivity was observed in

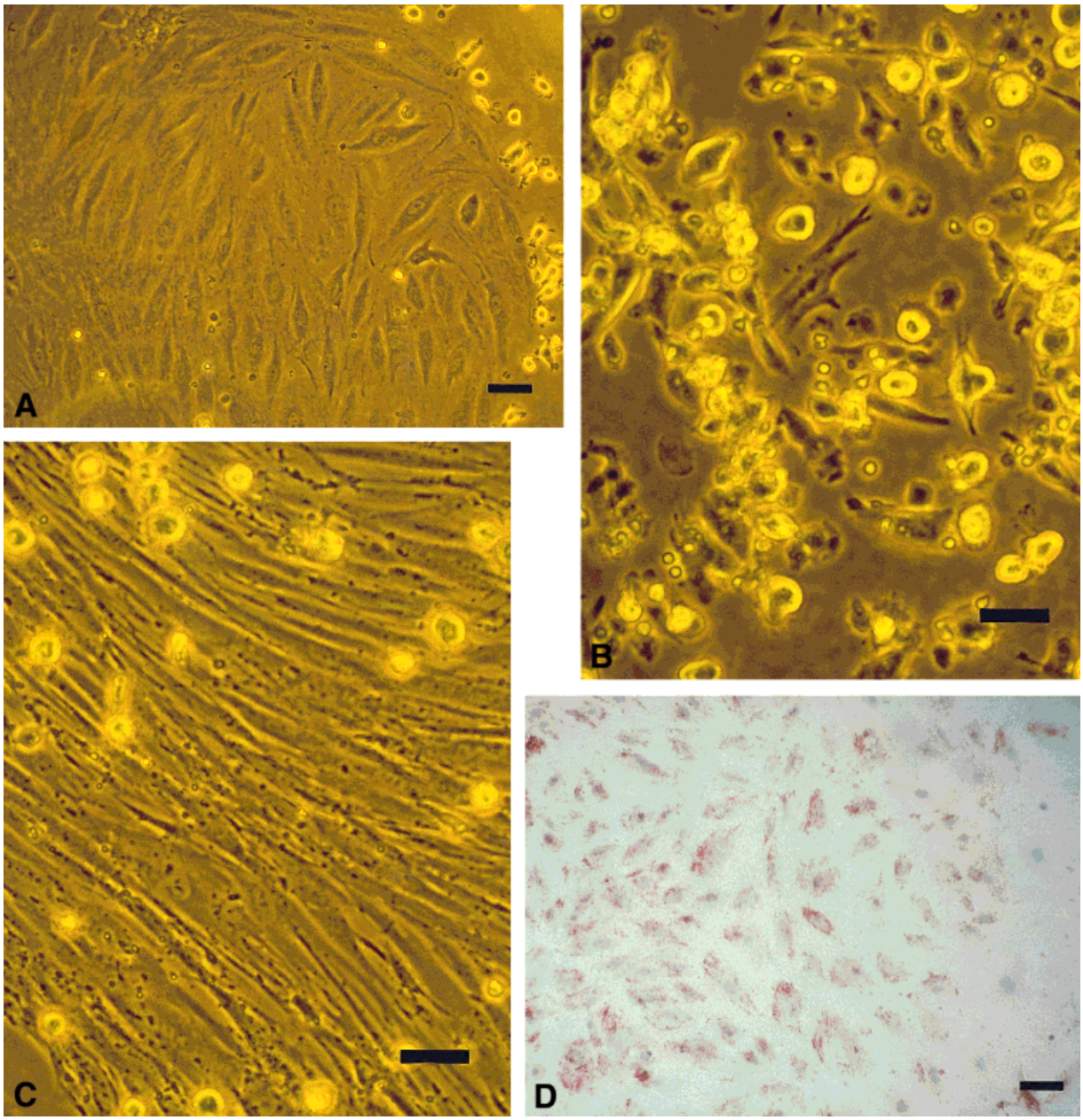

FIGURE 1.

all 3 cases and restricted to a proportion of lymphoblastoid cell line (LCL) with plasmacytoid features, whereas fibroblast and other stromal cells were consistently negative (Fig. 1*g*).

DISCUSSION

So far, the presence of HHV-8 genome has been investigated in MM patients using PCR assays with specific primers, mainly for the orf26 sequences, but discordant results have been obtained (Rettig *et al.*, 1997; Said *et al.*, 1998; Tarte *et al.*, 1999). Thus, we took advantage of a PCR assay with generic primers, which has been previously demonstrated to amplify efficiently several variants of the herpesvirus family. This assay has allowed the identification of 2 new herpesviruses closely related to HHV-8 in the pathologic tissues from 2 macaque species affected with retroperitoneal fibromatosis, a type of simian vascular fibroproliferative neoplasm with striking similarities to KS (Rose *et al.*, 1997). We assumed that this PCR approach could potentially reduce the risk of false-positive results linked to the use of the PCR assay with primers specific for orf26, a test that has been widely used on plenty of specimens in our as well as in several other laboratories immediately after the discovery of HHV-8 (Schulz, 1998). Moreover, a PCR assay with degenerate primers targeting a conserved herpesvirus gene, like the DNA polymerase gene, would be ideal in the attempt to investigate the presence of an HHV-8 variant or an unknown herpesvirus closely related to HHV-8.

The culture conditions may influence the detection of HHV-8 and give rise to false-negative results because either the medium used for the establishment of BMSC cultures could not favor the proliferation of infected dendritic cells (Tisdale *et al.*, 1998) or LTC conditions could induce cell detaching from the adherent layer, as it has been reported to occur with cultured animal DCs (Hart, 1997). Furthermore, HHV-8 may be lost during early pas-

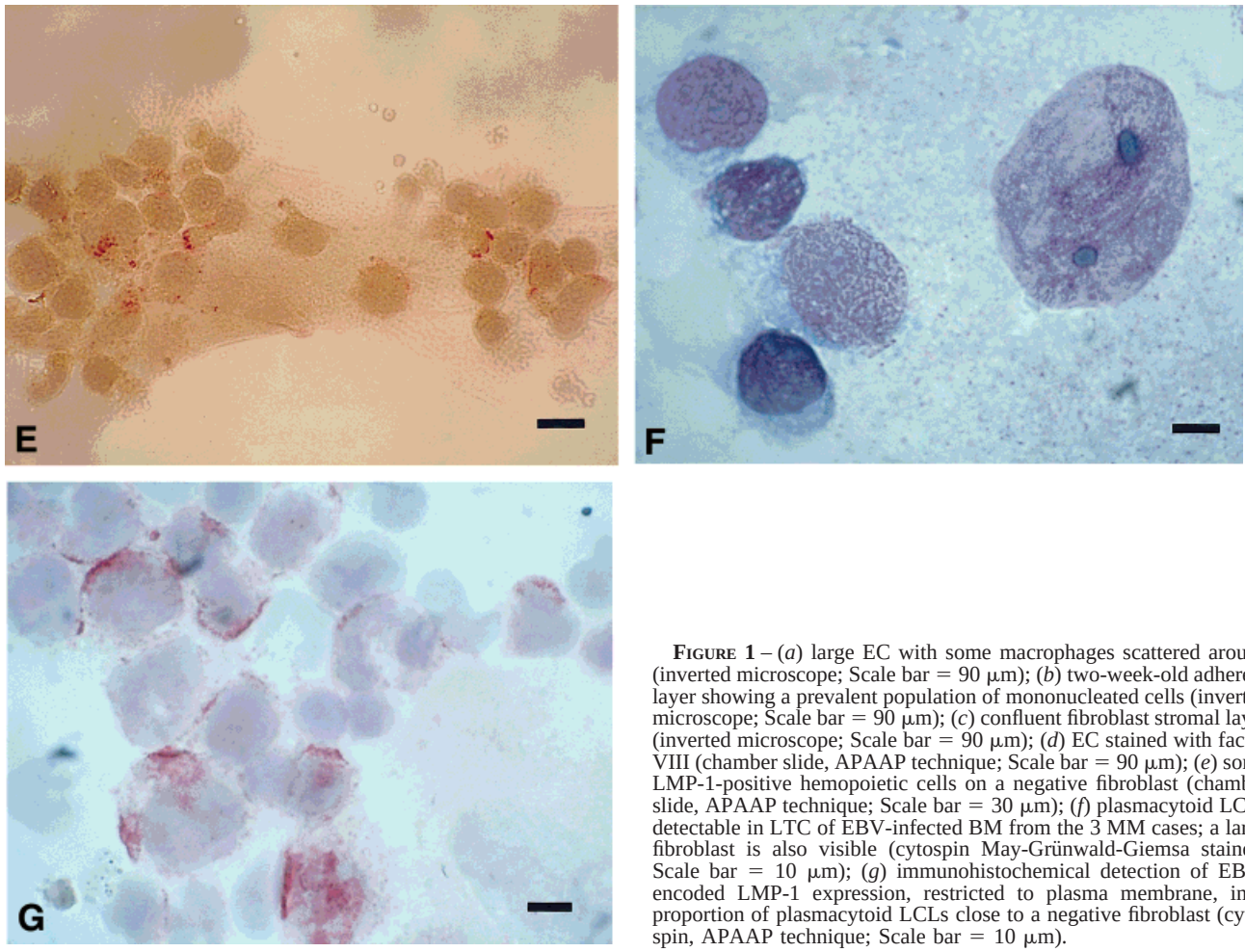

FIGURE 1 – (*a*) large EC with some macrophages scattered around (inverted microscope; Scale bar = 90 μm); (*b*) two-week-old adherent layer showing a prevalent population of mononucleated cells (inverted microscope; Scale bar = 90 μm); (*c*) confluent fibroblast stromal layer (inverted microscope; Scale bar = 90 μm); (*d*) EC stained with factor VIII (chamber slide, APAAP technique; Scale bar = 90 μm); (*e*) some LMP-1-positive hemopoietic cells on a negative fibroblast (chamber slide, APAAP technique; Scale bar = 30 μm); (*f*) plasmacytoid LCLs detectable in LTC of EBV-infected BM from the 3 MM cases; a large fibroblast is also visible (cytospin May-Grünwald-Giemsa stained; Scale bar = 10 μm); (*g*) immunohistochemical detection of EBV-encoded LMP-1 expression, restricted to plasma membrane, in a proportion of plasmacytoid LCLs close to a negative fibroblast (cytospin, APAAP technique; Scale bar = 10 μm).

TABLE II – PHENOTYPIC CHARACTERISTICS OF BONE MARROW STROMAL CELL CULTURE INVESTIGATED IN MM PATIENTS

Markers	Fibroblasts	Endothelial cells	Macrophages	Dendritic cells
CD68	+++[1]/S[2]	–	+++/S	++/I
Alfa-actin-SM	+++/I	–	–	–
Vimentin	+++/S	–	++/I	++/W
Collagen I-II-III	+++/I	–	–	–
Factor VIII (vWF)	–	+++/I	–	–
CD83	–	–	–	+++/I
CD1a	–	–	–	++/W
HLA-DR	–	–	+++/S	+++/S
CD14	–	–	+++/I	–
CD11c	–	–	+++/I	+++/W

[1] Percent of positive cells: +++ (61%–100% positivity), ++ (31%–60% positivity), + (5%–30% positivity), – (negative cells).–[2] Intensity of staining: W, weak; I, intermediate; S, strong.

sages in culture during the establishment of KS cell lines (Lebbè *et al.*, 1995), and this might occur in the bone marrow stromal culture from MM patients. To circumvent these problems, we chose to establish long-term cultures according to the protocol of Gartner and Kaplan (1980), obtaining at different times of culture, a mixed population of fibroblastic, macrophage, dendritic and endothelial cells. The same protocol has been followed by Rettig *et al.* (1997), who reported the presence of HHV-8 orf26 sequences in the bone marrow stromal cells of MM patients. Moreover, we have analyzed both the culture supernatant and the adherent stromal layer, starting from the 2nd until the 8th week of culture, to ascertain that our culture conditions did not affect the sensitivity of HHV-8 detection by PCR. The search for herpesviral sequences in LTC at confluence, mainly composed of fibroblasts, is also important, since BMSC cultures that support the growth of myeloma cells by producing human interleukin 6 are indeed an adherent layer at confluence, composed mainly of fibroblasts and rare macrophages (Calligaris-Cappio *et al.*, 1991). The possibility to maintain the same culture condition in TC-chamber slides for a parallel immunophenotyping allowed us to assess the immunophenotype of the various cell types harvested for the molecular analysis, thus excluding the possibility that negative results were caused by the absence of specific cell types infected by the virus.

Rather unexpectedly, our PCR study allowed the identification of EBV sequences in the long-term cultures of 3 of 21 MM cases. The PCR positivity for EBV is due to the presence of EBV-infected LCLs with plasmacytoid features identifiable either in the

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MM Pt8          NGLFPCLSIAETVTLQGRTMLERAKAFVEALSPANLQALAPSPDAWAPLNPE
MM Pt19         ..................................................
MM Pt21         ..................................................
HHV-8           S.IL...N.........K....SQ.....I..ER.AG.LRR.---VDVS.D
RFHVMn          S.IL...N.........K...TSQ....GI..TA.AD.LQR.---IEAS..
RFHVMm          S.IL...N.........K...TSQ....GI..KD.SD.IQR.---IDAS.D
RRV             S..L..IN.......R.....MS.SY....TTED.RTRLGRE---VTARHG
EBV             ..................................................
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FIGURE 2 – Alignment of amino acid translations of sequences from the herpesvirus DNA polymerase gene obtained from the MM patients with analogous regions from the DNA polymerase sequences of other γ-herpesviruses: EBV (GeneBank accession number V01555), HHV-8 and the 2 HHV-8 homologs in monkeys (RFHVMn and RFHVMm). Dots indicate identity with the first sequence in the alignment.

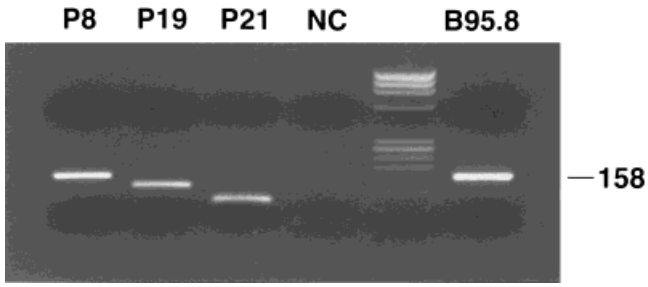

FIGURE 3 – Ethidium bromide-stained gel showing LMP-1 PCR products. In the EBV-infected B95.8 cell line and in one MM case (P8), the LMP-1 product has the expected size of 158 bp (wild-type variant) compared with the Hae III digested molecular marker (Boehringer Mannheim, Germany). The LMP-1 products were shorter compared with the wild-type LMP-1 sequence in the 2 other MM cases, showing the 30 bp deletion in case P19 and the 69 bp deletion in case P21, respectively.

stroma or in the supernatant. The expression of LMP-1 was also consistent with the type of latency characteristic of EBV-infected LCLs (Knecht *et al.,* 1997). Establishment of EBV-infected LCL resulting from the immortalization of non-malignant B cells from the bone marrow or the peripheral blood of MM patients is well known (Pellat-Deceunynk *et al.,* 1995). In some cases, EBV-infected LCLs may show morphologic and phenotypic features of plasma cells, although being not related to the myelomatous clone. The novel aspect of our results is that the evaluation of clonogenic stromal assays showed a strong reduction of CFU-F number, so that the adherent layer never reached a complete confluence. The possible involvement of EBV infection in affecting stromal growth in MM or other diseases is currently under investigation. In 2 MM patients, EBV genomes with deleted variants of the *LMP-1* gene were observed. These variants have a higher oncogenic potential compared with the wild-type variants *in vitro*, although their clinical relevance *in vivo* has not been established (Knecht *et al.,* 1997). Anyway, LCLs from the normal B lymphocytes from healthy subjects carrying either the 30 bp or the 69 bp deleted variants have been described by Knecht *et al.* (1997).

The results of our PCR study indicate that neither HHV-8 nor a herpesvirus closely related to HHV-8 can be consistently detected in LTC from MM patients. These negative results have also been obtained in the 4 MM cases showing an unusually high percentage of endothelial cells, spontaneously grown in bone marrow stromal cultures. This finding is of interest, as the possibility exists that endothelial cells in bone marrow stroma may be a target of HHV-8 infection in MM, given that HHV-8 is found in the microvascular endothelial cells in KS lesions (Schulz, 1998) and apparent immortalization of primary bone marrow endothelial cells has been achieved *in vitro* by infecting these cells with purified HHV-8 particles in the presence of vascular endothelial growth factor (Flore *et al.,* 1998). The BMSC culture established from one MM patient (P12) affected with classic KS was also negative. The efficiency of the PCR with degenerate primers used in our study has been validated by the sensitivity assay on the EBV-negative, HHV-8-positive BCBL-1 cell line and, importantly, by the successful detection of HHV-8 DNA polymerase gene in all the examined cases of KS, MCD and PEL already found to harbor HHV-8 sequences. Our PCR findings are in agreement with the results of serologic studies performed by our and other groups, which have failed to find evidence for an increased detection rate of antibodies against HHV-8 in MM and MGUS patients (Whitby *et al.,* 1997; Tarte *et al.,* 1999). Anti-HHV-8 antibodies have not been documented in MM patients, not even using for the IFA a new HHV-8-positive myeloma cell line established from a myelomatous effusion (Hyjek *et al.,* 1998). We cannot rule out the possibility that very rare cells in our bone marrow stromal cell cultures might be infected with either HHV-8 or a related variant. However, if herpesviral sequences were present at a level below the lowest limit of detection of our assay, the proposed causal role of either HHV-8 (Rettig *et al.,* 1997) or a related variant (Tisdale *et al.,* 1998) in the pathogenesis of MM would be unlikely. It remains possible that a very rare bone marrow cell, which can be expanded under very specific culture conditions, can be infected by HHV-8 in MM and, perhaps, also in other conditions (Tarte *et al.,* 1999), but that for unknown reasons, no detectable immune response is elicited.

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