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Short Communication

Reactivation of human herpesvirus 6 during *ex vivo* expansion of circulating CD34$^+$ haematopoietic stem cells

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Human herpesvirus 6 (HHV-6) replication was evaluated during *in vitro* expansion of CD34-positive cells that were selected from 11 peripheral blood progenitor cell (PBPC) samples. In order to permit cellular differentiation towards the myeloid lineage, PBPCs were cultured for 14–21 days in a liquid, serum-free medium supplemented with interleukin 1 (IL1), IL3, IL6, granulocyte–macrophage colony-stimulating factor, granulocyte colony-stimulating factor and stem-cell factor. Among the 10 cultures from HHV-6-seropositive patients, the late, alternatively spliced U100 viral mRNA was detected in five of them after PBPC culture for 14 or 21 days. Recovery of infectious virus from one of the expansions, associated with an increase of HHV-6 viral load and detection of the U100 spliced messenger, confirmed the occurrence of a complete replicative cycle. These data thus demonstrate for the first time that haematopoietic differentiation can lead to HHV-6 reactivation.

Human herpesvirus 6 (HHV-6), like human cytomegalovirus (hCMV), belongs to the subfamily *Betaherpesvirinae* and presents a strong tropism for haematopoietic cells. Significantly, active HHV-6 infections are associated with cytopenia, particularly in recipients of allogeneic haematopoietic stem-cell transplants (Carrigan & Knox, 1994; Imbert-Marcille *et al.*, 2000). Various haematopoietic cells have been identified as cellular targets for this virus, including CD34$^+$ progenitor stem cells, which are one of its sites of latency (Isomura *et al.*, 2000; Luppi *et al.*, 1999). Upon *in vitro* infection with HHV-6, mature, differentiated haematopoietic cells become permissive for virus replication and inhibition of haematopoietic colony formation is observed (Isomura *et al.*, 1997, 2003). Monocytes have also been identified as sites of viral latency (Kondo *et al.*, 2003) and macrophages are able to support the viral lytic cycle (Kondo *et al.*, 1991). Moreover, cells in the monocyte/macrophage lineage are recognized as the most permissive cell population that is primarily responsible for HHV-6 viraemia (Kondo *et al.*, 2002).

The origin of monocyte infection has not yet been described and previous attempts to induce HHV-6 reactivation in *in vitro* semi-solid cultures of CD34$^+$ cells have failed (Luppi *et al.*, 1999). The use of liquid media to perform stem-cell culture has been described to favour virus replication in cells infected with high titres of hCMV strains (Maciejewski *et al.*, 1992; Movassagh *et al.*, 1996). In order to further our understanding of herpesvirus reactivation, we thus assessed whether HHV-6 infection occurs naturally (e.g. in the absence of exogenous superinfection) during *in vitro* culture of myeloid progenitor cells in liquid media supplemented with a combination of cytokines that are involved in haematopoiesis.

After informed and written consent, peripheral blood progenitor cells (PBPCs) were collected from ten patients undergoing leukapheresis after stem-cell mobilization with 5 μg granulocyte colony-stimulating factor (G-CSF) kg^{-1} day^{-1} (Neupogen; Amgen) for 5 days. Highly purified peripheral CD34$^+$ cells were isolated by using the CliniMACS cell-selection system (Miltenyi Biotec) (Schumm *et al.*, 1999). Purity was 99 %, except for culture no. 2 (96 %), as determined by flow cytometry. HHV-6 serostatus was determined by ELISA (HHV-6 IgG EIA; Biotrin).

Ex vivo expansion of 10^6 CD34$^+$ PBPCs was carried out for 14–21 days in 10 ml STEM ALPHA.AG medium (Stem Alpha) supplemented with 10 ng interleukin 1 (IL1), IL3, IL6, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF and stem-cell factor (SCF) ml^{-1}. Every 7 days, non-adherent cells were removed and counted: 10^6

cells were replated in fresh medium and aliquots of 5×10^5–1×10^6 cells were kept at −80 °C for subsequent molecular analysis. When available, additional aliquots were removed at the end of the culture period for cytospins (with May–Grünwald–Giemsa staining) and co-cultivation with peripheral blood mononuclear cells from an HHV-6-seronegative donor. Among the 11 *ex vivo* expansions (Table 1), six (cultures 0, 2, 4, 5, 9 and 10) were maintained for 21 days and exhibited an increase in total nucleated cells (TNCs) at the end of the culture period that ranged from three- to 216-fold (median was 68-fold). The others could not be expanded beyond 2 weeks (cultures 1, 3, 6, 7 and 8) and showed a 0·5- to sixfold increase in TNCs at the end of the culture period (median was onefold). As expected, a higher number of mature cells was obtained after 21 days; as cytokines were added to the media, these mature cells were mostly monocytes and immature granulocytes. These results are in accordance with those of a previous study that was conducted in liquid medium without serum (Mahe *et al.*, 1996). The fact that there was little or no expansion in some of the cultures was not related to age, sex or underlying disease of patients.

HHV-6 DNA and the late, alternatively spliced U100 viral mRNA were amplified from aliquots obtained before and during expansion. To increase sensitivity, a nested-PCR procedure was used and rigorous separation of all steps was applied to avoid false-positive results. DNA and RNA were extracted from 5×10^5 and 1×10^6 cells, respectively, by using QIAamp DNA extraction and QIAamp viral RNeasy extraction kits (Qiagen). The latter included a DNase treatment. All amplifications consisted of 35 cycles and were carried out in a total volume of 25 µl. First-round PCR and RT-PCR were performed by adding 4 µl extract and using 0·625 U *Taq* polymerase (Promega) for U100 DNA amplifications and an mRNA-selective PCR kit (AMV) (TaKaRa Bio) for the U100 mRNA detection, according to our previously published procedure (Andre-Garnier *et al.*, 2003). A nested PCR was then performed on 2 µl first-round PCR product with 0·625 U *Taq* polymerase and the newly designed primers PE1n (5′-GTGGTTTC-AGGCGCYYCATAG-3′) and PE2n (5′-GCGATGAYAAY-AGCTGCGGTTC-3′) (Y is T or C). Annealing temperature was 65 °C. After nested PCR, expected sizes of DNA or unspliced cDNA amplified products were 339 and 371 bp for the A and B variants, respectively. A 238 bp fragment was expected for the cDNA U100 spliced form of both variants. Amplification specificity was assessed as described previously (Andre-Garnier *et al.*, 2003) with 10 ng biotinylated S1 (HHV-6-A) or S2 (HHV-6-B) probes ml^{-1}, using the GEN-ETI-K DNA detection system (DiaSorin) (Ferre-Aubineau *et al.*, 1995). As controls, detection of human glucose-6-phosphate dehydrogenase mRNA in all samples and amplification of viral genes in extracts obtained from cultures of the HST strain (HHV-6-B) were performed. Sensitivities of nested procedures have been evaluated by amplifying serial dilutions of positive controls that were obtained from HHV-6-infected cultures. This allows

Table 1. *Ex vivo* expansion of 11 samples of peripheral CD34$^+$ cells and assessment of HHV-6 replication

Abbrevations: d, day; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; ND, not determined (insufficient number of cells); −, negative; +, positive.

Patient characteristics				*Ex vivo* expansions						Detection of HHV-6 U100 gene	
No.	Age/sex	Pathology	HHV-6 serostatus	Duration of culture (days)	Fold increase in TNCs at end of culture period	Cell differentiation at end of culture* (%)				DNA	mRNA†
						Blasts	Immature granulocytes	Neutrophils	Monocytes		
0	62/M	MM	−	21	180	0	62	2	36	−	−
1	41/M	NHL	+	14	1	1	79	0	20	−	−
2	55/F	NHL	+	21	3	2	64	3	36	−	−
3	54/F	NHL	+	14	6	ND	ND	ND	ND	−	−
4	61/M	MM	+	21	72	2	61	0	37	−	−
5	47/M	NHL	+	21	216	0	54	1	45	−	−
6	47/M	NHL	+	14	0·5	ND	ND	ND	ND	ND	+ (d14)
7	63/M	MM	+	14	0·6	10	71	0	19	ND	+ (d14)
8	54/M	NHL	+	14	4	2	69	0	28	+ (d14)	+ (d14)‡
9	45/M	NHL	+	21	18	1	76	0	23	+ (d14, d21)	+ (d21)‡
10	61/F	MM	+	21	64	1	68	0	30	+ (d14, d21)	+ (d14)†

*May–Grünwald–Giemsa-stained cytospins of expanded CD34$^+$ progenitor cells at the end of the culture period. Cell counting was done in triplicate; data are expressed as mean percentages.

†Glucose-6-phosphate dehydrogenase mRNA was positive in all cDNA samples except for culture 10 on day 21.

‡Spliced form of U100 mRNA.

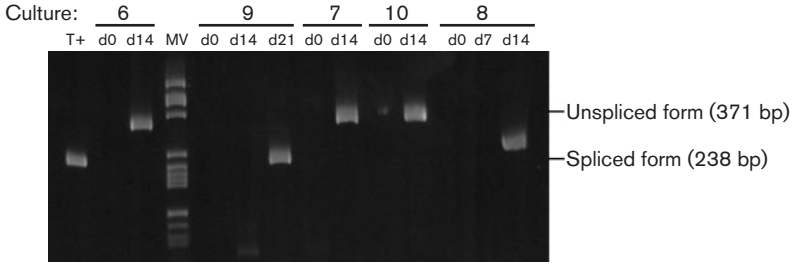

Fig. 1. PAGE and detection of HHV-6 U100 RT-PCR products of five samples from *ex vivo* CD34$^+$ progenitor cell expansions in the presence of IL1, IL3, IL6, SCF, GM-CSF and G-CSF. T+, Positive-control spliced mRNA; MV, size marker.

estimation of the detection limit at 4 viral genomes in 10^4 cells for nested PCR and at 1 infected cell in 10^4 cells for nested RT-PCR.

Among the 10 cultures from HHV-6-seropositive patients, half expressed the spliced HHV-6 U100 mRNA (Fig. 1; Table 1). The early, 371 bp, unspliced form was amplified at day 14 in three cultures: two of these cultures were stopped on that day (cultures 6 and 7) and a PCR inhibitor was detected at day 21 in the other culture (culture 10). The late, 238 bp, spliced form, which is indicative of a complete replication cycle, was detected at the end of the culture period in cultures 8 and 9. These data were confirmed in a second set of experiments and, because cultures were not performed at the same time, the possibility of viral cross-contamination can be excluded. HHV-6 DNA was not amplified from unexpanded CD34$^+$ cells, as reported in another study for one-third of CD34$^+$ samples (Luppi *et al.*, 1999). HHV-6 DNA was, however, detected in all mRNA-positive samples that were also evaluated for DNA amplification (cultures 8–10). This suggests indirectly that viral DNA was present in small amounts and became detectable with our PCR method during the differentiation process only when the DNA load increased. As our PCR methodology has previously been used in a clinical study to assess active infection (Imbert-Marcille *et al.*, 2000), this shows that samples were obtained from patients who were not actively infected at the time of PBPC collection. The length of amplified DNA fragments allowed us to confirm that HHV-6 strains were B variants in all cases. Our data thus demonstrate for the first time, and in the absence of *in vitro* cell infection, that HHV-6 can enter a replication cycle during haematopoietic differentiation. This last point was confirmed in culture 10, the only one for which a sufficient amount of cells was obtained to perform two complementary tests. The first one was a co-culture of a sample from day 21 with fresh peripheral blood mononuclear cells from an HHV-6-seronegative donor, which led to the recovery of infectious virus. This was confirmed by the appearance of the characteristic cytopathic effect and expression of a late HHV-6 antigen after 2 weeks co-culture (Fig. 2). The second test was a real-time PCR that was performed as described previously (sensitivity, 10 copies per reaction) (Gautheret-Dejean *et al.*, 2002) with each sequential sample of this CD34$^+$ expansion. An 18-fold increase in HHV-6 viral load was observed during week 3 of culture (401 copies in 10^6 cells at day 7, 416 copies in 10^6 cells at day 14 and 7296 copies in 10^6 cells at day 21), which is indicative of genome replication.

Our results confirm that CD34$^+$ haematopoietic progenitors carry latent HHV-6, at least in some seropositive patients, which concurs with previous findings (Luppi *et al.*, 1999). Above all, our data demonstrate for the first time that haematopoietic differentiation can lead, in the absence of *in vitro* infection of cells, to HHV-6 reactivation.

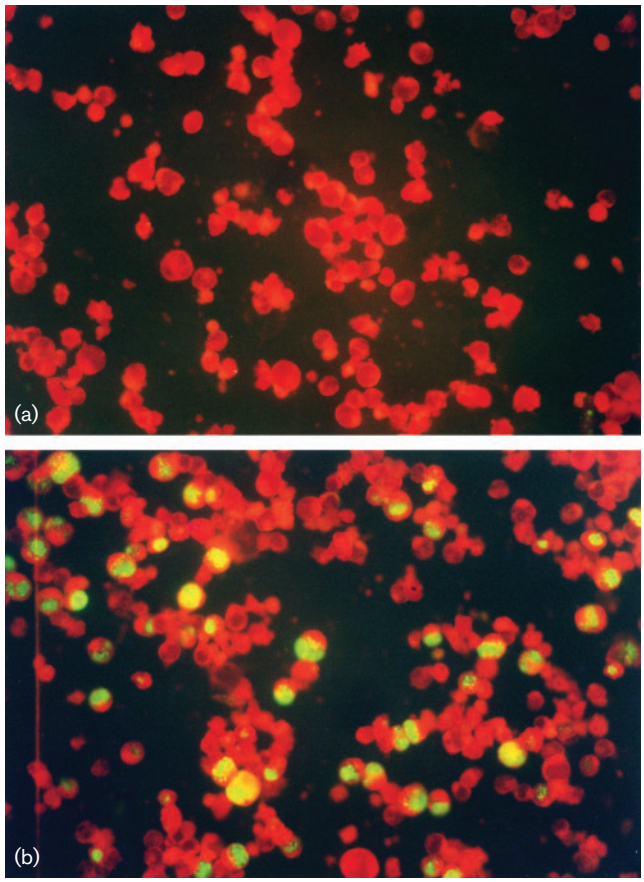

Fig. 2. Results of co-culture of PBMCs from an HHV-6-seronegative donor with the day 21 sample of CD34$^+$ expansion culture 10: expression of the 10G6 late HHV-6 antigen by indirect immunofluorescence. (a) PBMCs cultivated alone for 14 days; (b) co-culture for 14 days of PBMCs with expansion culture 10.

Studies need to be repeated with PBPCs obtained from normal donors. Mechanisms underlying the reactivation are unfortunately ill-defined. Previous studies performed on various models of HHV-6 latency (e.g. macrophages, peripheral blood mononuclear cells and myeloid cell lines) showed that phorbol ester or co-infection with human herpesvirus 7 can induce HHV-6 reactivation (Katsafanas *et al.*, 1996; Kondo *et al.*, 2003; Yasukawa *et al.*, 1999). We speculate that one or many of the cytokines that were used for $CD34^+$ PBPC expansion may have activated HHV-6 immediate–early gene transcription. The mechanism should be investigated further in relevant models of HHV-6 latency.

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References

Andre-Garnier, E., Robillard, N., Costa-Mattioli, M., Besse, B., Billaudel, S. & Imbert-Marcille, B.-M. (2003). A one-step RT-PCR and a flow cytometry method as two specific tools for direct evaluation of human herpesvirus-6 replication. *J Virol Methods* **108**, 213–222.

Carrigan, D. R. & Knox, K. K. (1994). Human herpesvirus 6 (HHV-6) isolation from bone marrow: HHV-6-associated bone marrow suppression in bone marrow transplant patients. *Blood* **84**, 3307–3310.

Ferre-Aubineau, V., Imbert-Marcille, B. M., Raffi, F., Besse, B., Loirat, R. & Billaudel, S. (1995). Colorimetric microtiter plate hybridization assay using monoclonal antibody for detection of an amplified human immunodeficiency virus target. *J Virol Methods* **55**, 145–151.

Gautheret-Dejean, A., Manichanh, C., Thien-Ah-Koon, F., Fillet, A.-M., Mangeney, N., Vidaud, M., Dhedin, N., Vernant, J.-P. & Agut, H. (2002). Development of a real-time polymerase chain reaction assay for the diagnosis of human herpesvirus-6 infection and application to bone marrow transplant patients. *J Virol Methods* **100**, 27–35.

Imbert-Marcille, B. M., Tang, X. W., Lepelletier, D., Besse, B., Moreau, P., Billaudel, S. & Milpied, N. (2000). Human herpesvirus 6 infection after autologous or allogeneic stem cell transplantation: a single-center prospective longitudinal study of 92 patients. *Clin Infect Dis* **31**, 881–886.

Isomura, H., Yamada, M., Yoshida, M., Tanaka, H., Kitamura, T., Oda, M., Nii, S. & Seino, Y. (1997). Suppressive effects of human herpesvirus 6 on in vitro colony formation of hematopoietic progenitor cells. *J Med Virol* **52**, 406–412.

Isomura, H., Yoshida, M., Namba, H., Fujiwara, N., Ohuchi, R., Uno, F., Oda, M., Seino, Y. & Yamada, M. (2000). Suppressive effects of human herpesvirus-6 on thrombopoietin-inducible megakaryocytic colony formation *in vitro*. *J Gen Virol* **81**, 663–673.

Isomura, H., Yoshida, M., Namba, H. & Yamada, M. (2003). Interaction of human herpesvirus 6 with human CD34 positive cells. *J Med Virol* **70**, 444–450.

Katsafanas, G. C., Schirmer, E. C., Wyatt, L. S. & Frenkel, N. (1996). *In vitro* activation of human herpesviruses 6 and 7 from latency. *Proc Natl Acad Sci U S A* **93**, 9788–9792.

Kondo, K., Kondo, T., Okuno, T., Takahashi, M. & Yamanishi, K. (1991). Latent human herpesvirus 6 infection of human monocytes/macrophages. *J Gen Virol* **72**, 1401–1408.

Kondo, K., Kondo, T., Shimada, K., Amo, K., Miyagawa, H. & Yamanishi, K. (2002). Strong interaction between human herpesvirus 6 and peripheral blood monocytes/macrophages during acute infection. *J Med Virol* **67**, 364–369.

Kondo, K., Sashihara, J., Shimada, K., Takemoto, M., Amo, K., Miyagawa, H. & Yamanishi, K. (2003). Recognition of a novel stage of betaherpesvirus latency in human herpesvirus 6. *J Virol* **77**, 2258–2264.

Luppi, M., Barozzi, P., Morris, C. & 7 other authors (1999). Human herpesvirus 6 latently infects early bone marrow progenitors in vivo. *J Virol* **73**, 754–759.

Maciejewski, J. P., Bruening, E. E., Donahue, R. E., Mocarski, E. S., Young, N. S. & St Jeor, S. C. (1992). Infection of hematopoietic progenitor cells by human cytomegalovirus. *Blood* **80**, 170–178.

Mahe, B., Pineau, D., Robillard, N., Accard, F. & Hermouet, S. (1996). Ex vivo expansion of hematopoietic progenitors from CD34+ cells selected from leukapheresis products of lymphoma and myeloma patients: feasibility and enhancement by fibronectin. *J Hematother* **5**, 671–679.

Movassagh, M., Gozlan, J., Senechal, B., Baillou, C., Petit, J. C. & Lemoine, F. M. (1996). Direct infection of CD34+ progenitor cells by human cytomegalovirus: evidence for inhibition of hematopoiesis and viral replication. *Blood* **88**, 1277–1283.

Schumm, M., Lang, P., Taylor, G., Kuci, S., Klingebiel, T., Buhring, H.-J., Geiselhart, A., Niethammer, D. & Handgretinger, R. (1999). Isolation of highly purified autologous and allogeneic peripheral CD34+ cells using the CliniMACS device. *J Hematother* **8**, 209–218.

Yasukawa, M., Ohminami, H., Sada, E., Yakushijin, Y., Kaneko, M., Yanagisawa, K., Kohno, H., Bando, S. & Fujita, S. (1999). Latent infection and reactivation of human herpesvirus 6 in two novel myeloid cell lines. *Blood* **93**, 991–999.