Human herpesvirus 6 (HHV-6), like human cytomegalovirus (hCMV), belongs to the subfamily \textit{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., 1999). 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 \textit{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 \textit{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 \textit{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 \(\mu\)g granulocyte colony-stimulating factor (G-CSF) 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).

\textit{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 $5 \times 10^5$–$1 \times 10^6$ cells were kept at $-80^\circ C$ for subsequent molecular analysis. When available, additional aliquots were removed at the end of the culture period for cytopsins (with May–Grünewald–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 during expansion. To increase sensitivity, a nested-PCR was then performed on $2 \mu l$ first-round PCR product with $0.625 U$ Taq polymerase and the newly designed primers PE1n (5’-GTGGTTTCTAGCCGCYCATAG-3’) and PE2n (5’-GCGATGAYAYAGCTGCGGTTC-3’) (Y is T or C). Annealing temperature was $65^\circ C$. 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 $10 \mu g$ 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/sex</th>
<th>Pathology</th>
<th>HHV-6 serostatus</th>
<th>Duration of culture (days)</th>
<th>Fold increase in TNCs at end of culture</th>
<th>Cell differentiation at end of culture* (%)</th>
<th>Detection of HHV-6 U100 gene</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DNA</td>
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<tr>
<td>0</td>
<td>62/M</td>
<td>MM</td>
<td>−</td>
<td>21</td>
<td>180</td>
<td>0 Blasts 62 immature neutrophils 36 monocytes</td>
<td>−</td>
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<tr>
<td>1</td>
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<td>+</td>
<td>14</td>
<td>1</td>
<td>79 0 20</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
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<td>NHL</td>
<td>+</td>
<td>21</td>
<td>3</td>
<td>64 3 36</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>54/F</td>
<td>NHL</td>
<td>+</td>
<td>14</td>
<td>6</td>
<td>ND ND ND</td>
<td>ND</td>
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<td>72</td>
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<td>216</td>
<td>54 1 45</td>
<td>−</td>
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<tr>
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<tr>
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<td>71 0 19</td>
<td>ND + (d14)</td>
</tr>
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<td>+</td>
<td>14</td>
<td>4</td>
<td>69 0 28</td>
<td>+ (d14)‡</td>
</tr>
<tr>
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<td>18</td>
<td>76 0 23</td>
<td>+ (d14, d21)</td>
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<tr>
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<td>MM</td>
<td>+</td>
<td>21</td>
<td>64</td>
<td>68 0 30</td>
<td>+ (d14, d21)</td>
</tr>
</tbody>
</table>

*May–Grünewald–Giemsa-stained cytopsins 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.
estimation of the detection limit at 4 viral genomes in $10^4$ cells for nested PCR and at 1 infected cell in $10^6$ 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 infection of cells, to HHV-6 reactivation.

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. 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.

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HHV-6 reactivation during CD34$^+$ cell differentiation

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


