Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors

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The cellular sites and mechanisms of human cytomegalovirus (HCMV) latency are still poorly defined. Although evidence suggests that peripheral blood monocytes are one site of latency in the healthy carrier, it is unlikely that monocytes represent a site of primary HCMV infection. Consequently, we have analysed CD34+ bone marrow progenitors, precursors of monocytes, to determine whether they are a site of HCMV carriage in normal virus carriers. For the first time, we demonstrate the presence of endogenous HCMV within bone marrow progenitors in the absence of HCMV lytic gene expression. These findings are consistent with previous evidence showing that the permissiveness of myeloid cells for HCMV is critically dependent on the differentiation state of the cell.

Following primary infection, human cytomegalovirus (HCMV), establishes a life-long latent infection in the host. In contrast to knowledge of the cellular sites of lytic infection during active disease (Myerson et al., 1984; Sinzger et al., 1995; Wiley et al., 1986), cellular sites of latency are poorly defined. Our previous in vitro studies identified monocytes as a major site of persistence in normal virus carriers (Taylor-Wiedeman et al., 1991). Subsequent studies showed no evidence of endogenous HCMV lytic gene expression in monocytes, although immediate early (IE) gene expression could be induced on differentiation of these cells to monocyte-derived macrophages (MDM) (Taylor-Wiedeman et al., 1994). These results are consistent with in vitro studies demonstrating that HCMV lytic gene expression was dependent on the differentiation state of the cell (Gonczol et al., 1984; Shelbourn et al., 1989; Sinclair et al., 1992). Monocytes only circulate in the peripheral blood for 1–3 days and HCMV cannot be reproducibly isolated from the peripheral blood of healthy virus carriers. In addition, monocytes are unable to be efficiently infected with HCMV in vitro (Einhorn & Ost, 1984). It is therefore difficult to postulate that the carriage of virus by monocytes results from direct primary infection. We have previously suggested that HCMV infects myeloid cells earlier in their lineage, as bone marrow progenitors (Sinclair & Sissons, 1994), a hypothesis supported by experiments showing that bone marrow progenitors could be infected by HCMV in vitro and, with the exception of one report (Maciejewski et al., 1992), could carry the virus in the absence of lytic virus gene expression (Kondo et al., 1994; Minton et al., 1994). In addition, monocytes grown out from bone marrow mononuclear cells (BMMC) in long-term culture were shown to carry HCMV DNA, implying that HCMV was present in bone marrow progenitors (Minton et al., 1994). However, to date, endogenous HCMV DNA has not been directly demonstrated in bone marrow progenitors. This work describes the isolation of pure populations of peripheral blood monocytes from normal virus carriers by fluorescence-activated cell sorting (FACS) and confirms the presence of HCMV DNA in these cells. Furthermore, we have isolated bone marrow progenitors carrying the CD34 antigen on their cell surface, a marker which is lost early in bone marrow cell differentiation, and have determined whether endogenous HCMV DNA and RNA is present in these cells.

Venous blood (60 ml) was venesected from six healthy laboratory volunteers, five of whom were seropositive and one seronegative by ELISA (CAPTIA CMV-TIA; Mercia Diagnostics). Blood was diluted 1:2 with sterile PBS (Sigma) and layered onto Ficoll–Hypaque gradients (Lymphoprep; Nycomed). Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation at 2000 r.p.m. for 30 min without brake on deceleration, washed twice in sterile PBS and stained with directly FITC-conjugated antibody to CD14 (Sigma), specific for monocytes in peripheral blood. CD14+ and CD14− cells were sorted using a FACS Vantage cell sorter (Becton Dickinson), CD14+ purity ranging from 97–99%. DNA was isolated from both populations using a modified sodium perchlorate technique (Taylor-Wiedeman et al., 1991).

CD14+ or CD14− cell DNA (1 µg) was amplified using nested PCR targeting exon 4 of the major IE region of HCMV as previously described (Taylor-Wiedeman et al., 1991). The amplified product after a total of 65 cycles was a 293 bp fragment. In duplicate experiments, IE-1 exon 4 PCR was positive in CD14+–sorted populations from 3/5 seropositive subjects, confirming the presence of HCMV DNA in these cells.
Fig. 1. HCMV DNA is present in CD14+ peripheral blood monocytes. An ethidium bromide-stained agarose gel (a) showing IE-1 exon 4-specific PCR amplification from CD14+ (+) and CD14- (-) cell DNA isolated from five healthy seropositive virus carriers (lanes 1–5) and one healthy seronegative volunteer (lane 6). Results of β-globin PCR on the same DNA samples, amplifying a 247 bp fragment, are shown in (b). Lanes M, 1 kb DNA ladder.

Having confirmed the presence of HCMV DNA in CD14+ cell populations, we isolated pure populations of CD34+ bone marrow progenitors to determine whether HCMV DNA and RNA were present. Human bone marrow was obtained from eight subjects undergoing routine total hip replacement along with venous blood for serotesting (five seropositive, three seronegative). Bone marrow was diluted 1:4 in sterile PBS and layered onto Ficoll-Hypaque gradients. BMMC were extracted following centrifugation at 2000 r.p.m. for 30 min without brake on deceleration, washed twice in PBS and stained with directly FITC-conjugated antibody to CD34 (Becton Dickinson). The resulting yield of CD34+ populations ranged from 9 x 10⁵–2 x 10⁶ cells and in purity from 95–99% for seropositive and 92–99% for seronegative subjects. DNA was isolated as described above and total RNA was isolated from four of the subjects’ CD34+ populations using an RNA extraction kit (Promega) based on the method of Chomczynski & Sacchi (1987).

CD34+ cell DNA (1 μg) was amplified using the IE-1 PCR described above. PCR products were Southern blotted and hybridized with end-labelled [γ-32P]ATP IE-1 probe by a standard method (Sambrook et al., 1989). In duplicate experiments, PCR gave a positive band at 293 bp with a corresponding positive signal on the Southern blot for all five of the seropositive subjects (Fig. 2a). Two out of three seronegative subjects were PCR-negative although one gave a reproducibly weak positive band at 293 bp despite stringent negative controls, which were all negative. This phenomenon of PCR-positive, seronegative individuals has been previously documented although, to date, its relevance to latency and reactivation is undetermined (Stanier et al., 1989; Taylor-Wiedeman et al., 1991). DNA from all eight subjects gave a positive result for the β-globin gene PCR (Fig. 2b). In order to control for the potential presence of HCMV-containing CD14+ cell contamination of our CD34+ populations, representative aliquots of CD34+ sorted cells were stained with directly phycoerythrin-conjugated CD14 antibody (Sigma) and analysed by flow cytometry. Between 0–6–1% of cells from the CD34+ populations stained with anti-CD14 antibody. This level of contamination by potential HCMV-containing monocytes is not great enough to account for the positive PCR signal seen in our experiments, as previous experience has shown that a minimum of 1 μg of CD14+ DNA, equivalent to approximately 1 x 10⁶ cells, is required to give a positive signal with the IE-1 exon 4 PCR (M. Mendelson & J. Sinclair, unpublished results; Taylor-Wiedeman, 1992).
which predominantly used adherent PBMC (Taylor-Wiedeman et al., 1991). In addition, we have shown that no virus DNA can be detected in CD14⁺ populations in vivo, a population representing mainly lymphocytes, a site previously implicated in latency (Einhorn & Ost, 1984; Rice et al., 1985; Schrier et al., 1985). We have proceeded to demonstrate the presence of endogenous HCMV DNA in CD34⁺ bone marrow progenitors from normal virus carriers in vivo, which cannot be due to contamination by monocytes. We are aware that, apart from bone marrow progenitors, CD34 antigen is also expressed by endothelial cells, a cell type infected by HCMV during active disease in vivo. Using an endothelium-specific monoclonal antibody (EN-4, Harlan-Seralab) and an indirect immunofluorescence assay, we found no evidence of contamination by endothelial cells in our pure sorted populations of CD34⁺ progenitors (data not shown). Interestingly, Van Laer et al. (1995) have recently been unable to demonstrate HCMV DNA in CD34⁺ populations from healthy bone marrow donors by PCR using DNA template from 10⁶ cells. This may be explained in part by the small numbers of cells used to isolate DNA and also by different DNA extraction and PCR protocols. It is clear that the frequency of HCMV-infected cells in peripheral blood is extremely low (probably less than 1 in 10000 PBMC) and therefore differences in sensitivity of PCR caused by different techniques may be the reason that HCMV is not consistently detectable in peripheral blood or bone marrow.

In addition to our PCR results, we have found no evidence of lytic HCMV major IE gene expression in CD34⁺ cells by RT–PCR. Kondo et al. (1994) recently demonstrated the presence of predominantly unspliced antisense IE-1 transcripts in cells co-expressing CD33, CD15 and CD14 antigens, obtained by in vitro infection of human fetal liver or bone marrow cells with the latZ derivative of HCMV strain Towne RC256 or low passage isolate Toledo and subsequent propagation in suspension culture in the absence of virus production. These transcripts were estimated to be present in no less than 2–5% of these in vitro-infected cells. Interestingly, endogenous novel sense and antisense transcripts from the same major IE region have also been found in healthy carriers (Kondo & Mocarski, 1995). Co-expression of CD33, CD15 and CD14 first occurs during monocyte development on the cell surface of bone marrow monoblasts, cells which are already committed to the monocyte–macrophage lineage. Although in the experiments of Kondo and coworkers a small number of cells used to initiate the cell cultures expressed CD34 antigen, at the time of sampling for RT–PCR the cells were uniformly CD34⁺. The cells studied in our experiments represent a more primitive non-committed population, distinct from the committed cells studied by Kondo and coworkers. An alternative explanation may be that our methods and those used by Kondo and coworkers for analyzing IE gene transcripts were not comparable, and that our inability to detect their latent transcripts was due to suboptimum sensitivity. To address this
question, we have analysed unsorted BMMC and sorted CD34+ populations from further seropositive and seronegative individuals, using identical methods to those used by Kondo and coworkers (Kondo et al., 1994; Kondo & Mocarski, 1995), and have been unable to detect any sense or antisense HCMV major IE transcripts despite the ability to detect endogenous cellular RNA from the histidyl L-RNA synthetase gene by this RT–PCR protocol (data not shown). However, we have not yet determined the sensitivity of the PCRs used to identify latent transcripts, which may be the critical factor determining detection.

In summary, we have confirmed that CD14+ monocytes are a site of persistence of HCMV DNA and have shown that CD34+ bone marrow progenitors carry endogenous HCMV DNA in vivo. Furthermore, we have been unable to detect any evidence of HCMV lytic gene expression in these cells. These findings are consistent with the hypothesis that HCMV infects bone marrow progenitors and persists through monocyte–macrophage cell lineage differentiation and division in the absence of lytic gene expression, by an as yet unknown mechanism.

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References


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