Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals


University of Cambridge Clinical School, Department of Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, U.K.

Polymorphonuclear leukocytes (PMNL) have been shown to harbour human cytomegalovirus (HCMV) in viraemic patients, but to date PMNL of asymptomatic healthy subjects have not been examined directly to determine whether this is a normal site of HCMV persistence. Using the polymerase chain reaction (PCR), paired DNA samples prepared from adherent peripheral blood mononuclear cells (PBMC), which are known to be a site of persistence of HCMV, and PMNL of 10 healthy adults were analysed. All of seven individuals who were HCMV seropositive, and one of three who were seronegative gave a reproducible signal for HCMV DNA in their adherent PBMC, whereas none of the paired PMNL DNA samples gave a positive result. The remaining two seronegative subjects showed no HCMV DNA in either the PMNL or PBMC samples. In every case where PCR for HCMV was negative, PCR amplification of a control human gene was used to show there was no inability to amplify the DNA. We conclude that within the leukocyte population of normal asymptomatic HCMV carriers, PMNL do not appear to harbour persistent HCMV whereas adherent PBMC in the same subjects are a site of persistence.

We and others have previously used the polymerase chain reaction (PCR) to demonstrate the presence of human cytomegalovirus (HCMV) in leukocytes of seropositive and some seronegative healthy carriers (Taylor-Wiedeman et al., 1991; Stainer et al., 1989). In these normal subjects, peripheral blood monocytes (PBMC) were the principal site of persistence (Taylor-Wiedeman et al., 1991). However, only PBMC were studied and analysis of polymorphonuclear leukocytes (PMNL) from healthy carriers was not addressed. Detection of HCMV in the PMNL fraction of peripheral blood has been reported in patients infected with human immunodeficiency virus (HIV), other immunocompromised patients and immunocompetent patients acutely ill with HCMV (Saltzman et al., 1988; Dankner et al., 1990; Fiala et al., 1975; Rinaldo et al., 1977; Revello et al., 1992; Gerna et al., 1991). Consequently, we were interested in determining whether the granulocyte might also represent a normal site of persistence which would be consistent with the clinical observations that HCMV is present in PMNL of viraemic patients. To determine this, DNA from PMNL cells and adherent PBMC from the same blood sample for each of 10 healthy subjects was prepared and examined by PCR. In this report we show that PMNL are not a source of persistent virus in the leukocyte population of normal asymptomatic HCMV carriers.

Subjects were all healthy adult volunteers whose serological status was determined using a competitive ELISA (CompEnz-CMV, Northumbria Biologicals). One subject's serum gave a negative result in ELISA but DNA from adherent PBMC was PCR-positive. A second serum sample, examined 2 months later using an HCMV latex agglutination assay (Becton Dickinson), showed the individual to have remained seronegative.

Lymphoprep (Nycomed) gradient separations yielded PBMC, banding at the plasma interface, and PMNL-enriched fractions lying at the top of the aggregated red blood cell (RBC) pellet. Both were washed twice in phosphate-buffered saline (PBS) and allowed to incubate in PBS on plastic Petri dishes for 1 h at 37 °C. PBMC adherent cells (a monocyte-rich population) and PMNL-enriched non-adherent cells were collected.

To detect the presence of contaminating monocytes in non-adherent PMNL fractions, a staining technique to detect esterase using α-naphthyl butyrate was employed. Slides of non-adherent PMNL were air-dried and fixed for 30 s with cold buffered formalin acetone (pH 6·6). After rinsing with tap water, slides were allowed to incubate for 45 min at room temperature with a substrate of α-naphthyl butyrate (Sigma) in phosphate buffer (pH 7·4) and a coupler of hexazotized new fuchsin (Sigma). Slides were then rinsed in tap water and counterstained with Mayer's haematoxylin (Sigma) for 45 s. Resultant enzyme activity is seen as red-brown granules in monocytes. Differential counts yielded a
Short communication

Fig. 1. (a) Ethidium bromide-stained agarose gel of the HCMV PCR products for all subjects. The 315 bp amplification product is marked with an arrow. + represents amplification of the AD169 virus; - is the non-DNA-containing negative control; lane M is a 1 kb ladder (Gibco BRL). Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, and 13 and 14 contain paired samples from seven seropositive individuals. Lanes 1, 3, 5, 7, 9, 11 and 13 contain amplification products from adherent PBMC DNA and 2, 4, 6, 8, 10, 12 and 14 contain amplification products from PMNL DNA. Lanes 15 and 16 represent the amplification products of the PBMC and PMNL, respectively, for the one seronegative/PCR-positive subject. Lanes 17 and 18, and 19 and 20 contain paired samples from two seronegative/PCR-negative individuals. Lanes 17 and 19 contain amplification products from PBMC DNA, and 18 and 20 contain amplification products from PMNL DNA. (b) Southern blot of the gel in (a) probed with an oligonucleotide internal to the PCR product. Numbering of lanes as in (a).

range of 90 to 99% PMNL purity (mean 95%); all other cells were mononuclear and none were positive for butyrate esterase. The adherent cells were largely monocytes with 99% CD14-positive, as reported previously (Taylor-Wiedeman et al., 1991) and 95% esterase-positive (data not shown).

DNA was extracted from these cells using a modified sodium perchlorate method (Weetman et al., 1990). PBMC and PMNL DNA concentrations were equalized and 1 μg of each was analysed by PCR.

PCR amplified a 315 bp portion of the major early gene (Greenaway & Wilkinson, 1987). The forward primer, reverse primer and internal oligonucleotide probe were 5’ CGTTATCGTTCTCGTAGG 3’, 5’ GTTGTGGTTGTGCCTGTA 3’ and 5’ CCTACCACGA-ATCGCAGATGA 3’, respectively. This area of the HCMV genome has no complementarity to those of other herpesviruses or the human genome. The primers have been used to amplify HCMV DNA successfully from two unrelated clinical HCMV isolates, from the AD169 strain, and from eight healthy subjects for which detailed results are presented. The cycle parameters and the reaction mix were the same as reported previously (Taylor-Wiedeman et al., 1991) except 50 cycles were used and the reaction mix contained 2.5 mM-MgCl₂ and 0.1% Triton X-100 together with 1.25 units DNA polymerase (Promega) per 50 μl of PCR mixture. In addition, a modified hot start technique was used.

Complete PCR mix was placed on ice immediately after adding template for each individual sample, then transferred directly to a hot (94 °C) PCR machine block to begin denaturation. The PCR sensitivity was 10 copies of HCMV-infected cell DNA as determined by comparison with quantitative amplification of a plasmid target.

In seven of seven seropositive subjects analysed, in at least two experiments, the adherent PBMC DNA was HCMV PCR-positive, whereas the PMNL DNA gave a negative result (Fig. 1). The same result was obtained for the one PCR-positive seronegative subject from whom DNA was prepared from two separate samples obtained 2 months apart (Fig. 1). In this latter individual the results were confirmed by another HCMV PCR using primers in a conserved region of the HCMV glycoprotein B gene (data not shown). Five of the eight HCMV PCR-positive subjects were shown to be positive by visualization of bands of the correct size on an ethidium bromide-stained agarose gel, whereas the other three were shown to be positive by Southern blot only (Fig. 1b). The other two seronegative subjects were consistently negative for HCMV DNA in both cell populations, even after Southern blotting and probing (Fig. 1 a and b).

For all DNA samples analysed, equal amounts of DNA (100 ng) were amplified in a histidyl-tRNA synthetase gene PCR (Taylor-Wiedeman et al., 1991; Corrochano,
All the DNA samples were amplifiable in this PCR. Except for one sample (Fig. 2, lane 17), all amplification products from PMNL DNA were at least equally visible on an ethidium bromide-stained gel when compared to their paired adherent PBMC amplification product (Fig. 2). For the sample in lane 17 and its paired PMNL DNA sample in lane 18, previous analysis had shown equal amplification. This report shows that HCMV was not detectable by PCR in the PMNL cell fraction of seven healthy adult seropositive carriers, whereas HCMV was detected in every one of these subjects’ adherent PBMC DNA. At the same time, the PMNL DNA samples were all shown to be amplifiable using a control PCR for a housekeeping gene. Whilst it should be noted that the results from such a small sample size may be due to chance, the trend from all seven volunteers showing no evidence of HCMV DNA in PMNL strongly implicates the predictability of further data.

In two of three seronegative healthy adults, neither cell fraction was HCMV PCR-positive yet the control PCR showed the DNA was amplifiable. In the one additional seronegative subject, HCMV was reproducibly detected in the PBMC, whereas the PMNL DNAs were HCMV PCR-negative for samples obtained on two separate occasions. These results were confirmed using another HCMV PCR targeting the glycoprotein B gene to rule out the possibility of contamination from PCR product carry-over. As before, the PMNL DNA was shown to be amplifiable using a control PCR. It therefore appears that this individual represents a seronegative HCMV carrier, as reported previously (Taylor-Wiedeman et al., 1991; Stanier et al., 1989). Interestingly, clinical evidence suggests that some seronegative subjects may harbour transmissible virus, because seronegative transplant recipients receiving organs and blood products from seronegative donors demonstrate a low incidence of HCMV infection (Bowden, 1991).

Our work has suggested that monocytes are a major site of HCMV persistence in peripheral blood (Taylor-Wiedeman et al., 1991). However, as it is difficult to infect freshly isolated monocytes with HCMV and monocytes appear not to replicate virus (Rice et al., 1984), the issue arises as to how these cells may acquire and maintain persistent HCMV. It is known that bone marrow progenitors can be infected in vitro (Sing & Ruscetti, 1990); therefore, one explanation of monocytic cell persistence may be that the cells acquire HCMV at an early stage of their differentiation in the bone marrow. However, the same myeloid precursors give rise to both monocytes and granulocytes, but PMNL do not appear to harbour HCMV in healthy carriers whereas monocytes do. Therefore, if early myeloid precursors are targets for HCMV infection it would appear that differentiation results in a partitioning of HCMV to the monocyte fraction. One explanation for this could be that HCMV-infected PMNL precursors may be unresponsive to growth factors, resulting in a loss of these cells (Sing & Ruscetti, 1990).

Finally, it is well documented that HCMV can be detected in PMNL of infected immunocompromised patients and, in rare instances, in immunocompetent subjects (Saltzman et al., 1988; Dankner et al., 1990; Fiala et al., 1975; Rinaldo et al., 1977; Revello et al., 1992; Gerna et al., 1991). The common factor in all these cases, however, is viraemia usually with serious active disease. However, in one report (Gerna et al., 1990), 30 HCMV seropositive/HIV-infected patients with > 400 x 10^9/1 CD4 cells and no HIV disease showed no evidence of HCMV DNA in PMNL-enriched leukocyte populations by PCR. Although these latter patients may be similar to the normal subjects presented here, the PCR sensitivity in the latter report was adjusted to detect only high copy number DNA, not low copy number genomic DNA as might be present in normal subjects with latent virus. The lack of sensitivity to detect low copy number genomic DNA precludes drawing definitive conclusions concerning normal subjects.

Some reports have shown evidence for active expression of the viral genome in PMNL of viraemic patients either by in situ hybridization demonstrating the presence of RNA transcripts for immediate early, early and late genes (Dankner et al., 1990), or by immunofluorescent detection of nuclear localized immediate
early proteins and late HCMV antigens (Revello et al., 1992). It is, however, not clear whether this detection of HCMV gene expression results from phagocytosis of intact viral particles with subsequent gene expression or from direct infection of PMNL cells (Dankner et al., 1990; Revello et al., 1992). Nonetheless, our results would suggest that HCMV is not normally present in PMNL of non-viraemic healthy HCMV carriers.

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References


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