Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells

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We have used the nested polymerase chain reaction (PCR) combined with fluorescence-activated cell sorting to define sites of latency of human cytomegalovirus (HCMV) in the peripheral blood of healthy subjects. Peripheral blood mononuclear (PBM) cells were separated into T cell or non-T cell populations and monocytes, and were then analysed by PCR for the presence of HCMV DNA. In five of six seropositive subjects, HCMV was found predominantly in the non-T cell population. Further analysis suggested that the virus was present in adherent cells and CD14+ cells. In three of nine seronegative subjects we could demonstrate HCMV DNA, which we do not believe was due to contamination, reproducibly by PCR. In one of these seronegative subjects, HCMV DNA was present predominantly in the non-T cell fraction of PBM cells. No HCMV DNA was detectable in the remaining six seronegative subjects. We conclude that, within the PBM cells of normal asymptomatic seropositive and some seronegative subjects, HCMV is present predominantly in the monocyte fraction. In addition, the detection of HCMV sequences in seronegative subjects may indicate that infection with HCMV is more widespread than conventional seroepidemiology suggests.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that infects 50 to 90% of adults, with seroprevalence depending on the socio-economic background of the individual, as well as other risk factors (Alford & Britt, 1990). Despite its prevalence, severe morbidity in the adult due to HCMV infection is limited mainly to patients with AIDS and immunosuppressed recipients of organ transplants. The source of infection in these patients is most commonly reactivation of the individual's own persistent HCMV and/or reactivation of persistent HCMV acquired through donor organs or blood transfusions. However, many aspects of the biology and pathogenesis of HCMV are poorly understood. One area of uncertainty involves the site of virus persistence in the healthy seropositive individual.

HCMV infection clearly can be transmitted by blood products from healthy donors to susceptible recipients and the incidence of transfusion-associated HCMV infection can be markedly reduced by using leukocyte-depleted blood products (Adler, 1983). However, it has been isolated from healthy donors only rarely (Diosi et al., 1969). Immunohistochemical detection methods might be expected to lack the sensitivity necessary to detect latent viral gene expression directly, although one recent report using immunohistochemical methods suggests that immediate early viral proteins are present in many cell types (Toorkey & Carrigan, 1989). In addition, it is not possible to detect HCMV sequences by dot blot hybridization analysis of leukocyte DNA from healthy seropositive subjects (Jordan, 1983). Consequently, more sensitive techniques are required to detect sites of HCMV persistence.

Using in situ hybridization, it has been reported that HCMV immediate early 1 (IE1) gene transcripts are present in 0.03 to 2% of peripheral blood mononuclear (PBM) cells from healthy seropositive individuals and in 0.1% of PBM cells from one of 12 healthy seronegative subjects; most positive cells in this study were thought to be T cells (Schrier et al., 1985). However, other reports have been unable to detect HCMV and, consequently, it appears that the sensitivity of in situ hybridization may limit its use in detecting sites of persistence in healthy carriers (Dankner et al., 1990).

We have therefore used the polymerase chain reaction (PCR) to detect HCMV DNA in highly purified by fluorescence-activated cell sorting (FACS) populations of PBM cells from healthy seropositive and seronegative subjects.
Methods

Subjects. All individuals were healthy adult volunteers.

Serology. Serological status was determined using a competitive ELISA system (CompEnz-CMV; Northumbria Biologicals) or an indirect ELISA (Virenz G CMV; Northumbria Biologicals); sera which gave negative results in these assays but which were PCR-positive were also tested using an HCMV latex agglutination assay (Becton Dickinson).

Cell separation. Heparinized blood samples were obtained, and PBM cells were isolated by centrifugation on Ficoll-Hypaque gradients and sorting using a Coulter EPICS C flow cytometer. Cells were labelled with mouse monoclonal anti-human CD3 IgG (derived from the mouse hybridoma cell line OK T3 CRL 8001, purchased from the European Collection of Animal Cell Cultures) and a fluorescein-conjugated F(ab')2 fragment of rabbit anti-mouse IgG (Dakopatts). Cells from one subject were also sorted using a fluorescein-conjugated mouse anti-human CD14 antibody (Becton Dickinson), an antigen present on mature monocytes in PBM cells.

In addition, adherent cells were isolated from PBM cells of seven seropositive individuals by plating on plastic Petri dishes in PBS and incubating for 1 h at 37 °C. Non-adherent cells were collected and, after vigorous washing, the adherent cells were collected by dislodging with a rubber policeman (Treves et al., 1980).

Purity. After FACS, purity analyses were performed by either sorting a small sample of cells with the EPICS C flow cytometer or by paraformaldehyde fixation and analysis on the Coulter EPICS Profile machine.

Adherent and non-adherent cells were stained with a fluorescein-conjugated mouse anti-human CD14 or CD3 antibody to determine the percentage of mature monocytes present in cells adhering to plastic.

DNA. DNA was extracted, after RNase and proteinase K treatment, using phenol followed by chloroform/isoamyl alcohol. DNA was precipitated, and washed with 100% and then 70% ethanol. The DNA concentration of each sample was equilibrated by visualization on ethidium bromide-stained gels and an equal amount of DNA from the CD3− and CD3+, or CD14− and CD14+, cells of each subject was analysed by PCR.

For seronegative subjects, heparinized blood and similar volumes of control solutions (PBS containing the same batch and concentration of heparin) were processed side by side on Ficoll-Hypaque gradients to yield PBM cells and controls. This additional control was used as a further precaution to detect any possible false positive results due to amplification of contaminating DNA. PBM cells and controls were divided into three aliquots and frozen at -70 °C to be processed into DNA on separate occasions, as described above.

HCMV PCR. The initial PCR was performed as an asymmetric nested amplification. Initially, a forward and reverse primer from exon 4 of the HCMV major IE gene were used to yield an amplification product of 564 bp. This was followed by a second, 30 cycle amplification using a more internal forward primer but the same reverse primer as in the first 30 cycles, yielding an amplification product of 403 bp (Fig. 1).

In later experiments, a fully nested PCR was used which consisted of two 30 cycle sets, and initially yielded a 373 bp and subsequently a 293 bp amplification product (Bell, 1989; Mullis & Faloona, 1987). All primers used for the HCMV amplification were located in exon 4 of the IE1 gene of HCMV (Fig. 1) (Akrigg et al., 1985). This area of the genome has no complementarity to that of other herpesviruses or humans. These primers were used successfully to amplify HCMV DNA from six clinical HCMV isolates, the AD169 strain, and six seropositive healthy subjects in addition to the six for which detailed results are presented.

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\begin{align*}
\text{Forward primer:} & \quad 5' > \text{AAGTGAGTTCTGTCGGGTGCT} <3' \\
\text{Reverse primer:} & \quad 5' > \text{GTGACACCAGAGAATCAGAGGA} <3'
\end{align*}
\]

\[
\begin{align*}
\text{Nested forward primer:} & \quad 5' > \text{GTGCACCATGGAACCTCGATAGAGG} <3' \\
\text{Nested reverse primer:} & \quad 5' > \text{GTAGACGAGAGATCGAGAGGG} <3'
\end{align*}
\]

Fig. 1. Oligonucleotide primers used in the asymmetric HCMV PCR (a), the fully nested HCMV PCR (b) and the histidyl-tRNA PCR (d). The oligonucleotide probe (c) was used to detect HCMV-specific signals in the Southern blot analyses of the PCR amplifications.

The standard 1 × reaction mix contained 50 mM-KCl, 10 mM-Tris-HCl pH 8-5 at room temperature, 2 mM-MgCl2, 0-01% gelatin, 0-5% Tween 20, 1 μM of each primer, 200 μM of each dNTP and 1-25 units of Taq polymerase. The cycle parameters, performed on a Perkin Elmer Cetus Thermocycler, were 5 min initial denaturation at 94 °C followed by 30 cycles of 94°C for 30 s, 55 °C for 30 s and 72 °C for 90 s.

Histidyl-tRNA synthetase PCR. PCR amplification of the human histidyl-tRNA synthetase gene was used to confirm that negative PCR results for HCMV PCR were not due to any general inability to amplify DNA and that genomic DNAs were equally able to be amplified (Tsui & Siminovitch, 1987; Corrochano, 1991). This PCR had already been well defined and was considered to be a good positive control for the ability to amplify DNA, especially for extremely small amounts of DNA (yields from FACS are very pure, but often very small), as it is a monocopy gene. Thirty cycles of amplification were used to produce a 360 bp product (Fig. 1). The standard 1 × reaction mix was the same as above with the exception of the presence of 1-5 mM-MgCl2. Cycling parameters were identical to the HCMV PCR.

Analysis of PCR-amplified DNA. PCR samples were denatured with 0-4 M-NaOH and boiled for 5 min immediately before application to a Duralon-UV (Stratagene) nylon membrane for slot blot hybridization or were transferred directly from an agarose gel to a nylon membrane using standard Southern blot methodology (Schatz, 1989). Membranes were u.v.-fixed for 5 min prior to overnight incubation at 42 °C in standard prehybridization solution. For slot blot hybridization, samples were incubated overnight at 42 °C with a 32P-end-labelled 480 bp DNA probe that spanned the entire HCMV PCR amplification product. This probe was a BamHI–BglII fragment of the HCMV IE1 cDNA plasmid pJD083 (Akrigg et al., 1985). The probe was labelled using a random hexanucleotide priming kit (Prime-a-Gene; Promega). After hybridization, membranes were washed twice in 2 × SSC and 0-1% SDS for 15 min at room temperature, twice in 1 × SSC and 0-1% SDS for 15 min at room temperature, and finally once in 0-1% SSC for 1 h at 65 °C. For Southern blotting, hybridization was carried out for 4 h at 42 °C with a 32P-end-labelled, 36 bp oligonucleotide probe internal to the final HCMV PCR amplification product (Fig. 1). Following oligonucleotide hybridization, membranes were washed twice in 6 × SSC for 10 min at room temperature and once in 6 × SSC for 5 to 10 min at 55 °C. Autoradiographs were kept at -70 °C overnight prior to developing.
Results

Serology
All samples were unequivocally seropositive or seronegative. Indirect ELISA results from four seropositive individuals were two- to threefold the negative cutoff value; these were also confirmed by latex agglutination. The other two seropositive subjects and the nine seronegative subjects were analysed by competitive ELISA and yielded values of 4% and 9% (positive values <50%), and 82 to 104% (negative values >66%), respectively. For the three individuals who were PCR-positive but seronegative, additional samples were obtained 2, 5 and 10 months after the first sample. These were all confirmed as being seronegative by both competitive ELISA and latex agglutination.

Purity
Analyses of sorted populations of PBM cells were performed on cells derived from eight sorts. Purity ranged from 93-8 to 99.77% CD3+ cells in the positively sorted populations and from 94 to 99.6% CD3- cells in the negatively sorted populations.

In six subjects, anti-CD14- and anti-CD3-specific antibodies were used to examine adherent and non-adherent cells by indirect immunofluorescence. In all cases the adherent cells showed specific fluorescence for the CD14 marker, with less than 5% of cells positive for the CD3 marker, indicating a highly pure population of mature monocytes. Similarly, non-adherent cells showed specific fluorescence for the CD3 marker with less than 5% of cells positive for CD14.

PCR sensitivity
After the initial 30 cycles using a known copy number of a pGEM-2 vector containing a full-length cDNA clone of IE1 as a template, as few as 103 copies could be directly visualized on an ethidium bromide-stained agarose gel (Fig. 2a). After the additional 30 cycles using the nested primers and 1 μl of the initial PCR reaction mix as template, as few as 10 copies of the IE1-containing plasmid could be visualized easily on an ethidium bromide-stained agarose gel. The identity of these bands was confirmed by Southern blotting and probing with an HCMVIE1-specific oligonucleotide probe, which increased the sensitivity to the level of one copy of an IE1-containing plasmid (Fig. 2b).

HCMV DNA is present primarily in monocytes of asymptomatic seropositive individuals
PBM cells from six seropositive individuals were sorted by FACS using the CD3 marker for T cells. DNA from the CD3+ and CD3− populations was amplified by asymmetric nested PCR for HCMV. For five of the six subjects, autoradiography of slot blot hybridization analyses reproducibly showed more signal in the CD3- than in the CD3+ amplification products (Fig. 3). In the remaining seropositive subject, the positive signal was equal in the CD3+ and CD3- populations. It is interesting to note that in four of the five subjects little or no HCMV signal was detectable in the CD3+ populations. These cells, however, were equally as positive for the endogenous histidyl-tRNA synthetase gene by PCR as was the CD3- population (Fig. 3b).

To determine whether monocytes in the CD3- population accounted for this positive signal, DNA from adherent cells from seven seropositive individuals was examined by PCR and Southern blotting. In all cases, after nested PCR an HCMV-specific band was detected on ethidium bromide-stained gels and by Southern blot analysis, whereas total PBM cells from one seronegative subject prepared at the same time and the non-DNA-containing control showed no amplification product (Fig. 4 and data not shown). As with the CD3 sorts, non-adherent cells showed significantly less HCMV-specific PCR signal than adherent cells (data not shown).

As cells which adhere to plastic may not consist entirely of monocytes, PBM cells from one individual were sorted by FACS into CD14+ and CD14- cells.
Fig. 3. (a) Autoradiograph of slot blot hybridization analysis of six seropositive subjects (S1 to S6), two seronegative subjects (S7* and S8*), a positive control of 100 pg pES (Boom et al., 1986) blotted directly without PCR amplification (+) and a non-template, negative control (−). Cell populations are identified as CD14+ or CD14−, and CD3+ or CD3−. (b) DNA from subjects in (a) was analysed by histidyl-tRNA synthetase PCR. Amplified products were separated on agarose gels and stained with ethidium bromide. Subjects S1 CD14+ and CD14− (lanes 2 and 3), S2 CD3+ and CD3− (lanes 4 and 5), S6 CD3+ and CD3− (lanes 6 and 7), S3 CD3+ and CD3− (lanes 8 and 9), S7 CD3+ and CD3− (lanes 10 and 11), and S8 CD3+ and CD3− (lanes 12 and 13). Lane 1 is a reaction mix negative control and lane 14 is a DNA-positive control. Lane m, a 100 bp marker ladder.

(Hunt, 1987); CD14 is a marker for mature monocytes. DNA was then amplified by asymmetric PCR and analysed by slot blot hybridization and probing (Fig. 3). CD14+ cells gave a clear positive signal whereas CD14− cells did not, and histidyl-tRNA synthetase PCR amplified both these samples equally.

Some seronegative subjects are PCR-positive

In six of nine seronegative subjects, HCMV PCR results were negative by gel electrophoresis, and Southern blot analysis and specific oligonucleotide probing of at least two DNA and control samples prepared separately (Fig. 5 and data not shown). However, in three subjects, on at least two DNA samples prepared separately HCMV PCR results were positive whereas the comparable control samples were negative (Fig. 3 and 5). For these...
three individuals new specimens of PBM cells were obtained and prepared as in Methods. On re-analysis of at least two DNA samples from these three individuals prepared separately, HCMV PCR remained positive. One of these individuals was analysed further by cell sorting to yield T cell (CD3+) and non-T cell (CD3−) PBM populations. The HCMV PCR results showed that the non-T cell population harboured the HCMV signal, as determined by slot blot hybridization analysis and probing (Fig. 3). These results were comparable to the findings in five of the seropositive subjects, in whom HCMV DNA was present reproducibly, predominantly in CD3− cells.

Discussion

We were able to detect as little as one copy of an IE1-containing plasmid using nested PCR to amplify exon 4 of the HCMV IE1 gene. This target was amplified from multiple clinical isolates and from PBM cells of all healthy seropositive individuals tested, and hybridized specifically to corresponding HCMV probes. Consequently, this technique was sensitive and specific, and allowed a detailed analysis of the sites of low copy persistence of HCMV.

Employing the above technique, we have shown the presence of HCMV probe-positive signals primarily in the CD3− or non-T cell population of PBM cells from healthy seropositive carriers. This is in contrast to the results of Schrier et al. (1985) obtained using in situ hybridization. However, from our data, it remains possible that T cells or a subset of T cells are a minor site of persistence in some individuals. Further analysis of both adherent cells and CD14 positive sort cells (representing mature monocytes in both cases) suggests that the source of the HCMV probe-positive signal is primarily monocytes. In every case in which HCMV signal was not observed (for example in DNA from some CD3+ and CD14− cells, as well as most seronegative subjects), PCR amplification of the histidyl-tRNA synthetase gene confirmed that negative PCR results were not due to any general inability to amplify DNA.

In six of nine seronegative subjects, HCMV sequences were not detectable by blotting and probing. However, for the other three seronegative subjects tested, on at least two separately prepared DNA samples from two separate blood samples, HCMV PCR results were positive. In addition, in one of these seronegative subjects the predominant HCMV-specific signal was in the CD3− population, as in the seropositive subjects. Our frequency of seronegative but PCR-positive samples is similar to that reported by Stanier et al. (1989), who found that three of 10 healthy HCMV seronegative adults were HCMV PCR-positive. This implies that HCMV infection may be more widespread than conventional seroepidemiology suggests. Although this is an intriguing finding, at present it is not known whether there is any clinical correlation between the existence of seronegative but PCR-positive individuals and transmission of HCMV infection or disease. We have no evidence that these subjects are infected with functional virus. It should be noted that, in general, seronegative adult transplant recipients who receive organs from seropositive donors, and seronegative neonates receiving seronegative blood products show little evidence of HCMV transmission (Paya et al., 1989; Bowden et al., 1986; Chou, 1986; Adler et al., 1983; Yeager et al., 1981). Therefore, further studies are needed to analyse the level of transcription, if any, in seronegative/PCR-positive healthy carriers to determine whether these viral gene sequences are expressed.

Owing to the extreme sensitivity of the PCR, it is well known that contamination is a major problem. For this reason contamination was rigorously excluded by appropriate controls, in addition to instituting general procedures to minimize PCR contamination. The latter measures consisted of (i) physical separation of pre- and post-PCR work areas, including use of separate supplies and pipettors, (ii) use of disposable plastics or new glass bottles when preparing reagents, (iii) preparing stocks of reagents in a DNA-free work area and (iv) practice of meticulous laboratory technique, such as frequent glove changes and minimizing aerosolization of samples when uncapping tubes. Clearly, others have been able to amplify HCMV DNA successfully by PCR (Hsia et al., 1989; Demmler et al., 1988; Jiwa et al., 1989; Shibata et al., 1988). However, given the ease of contamination it is clear that PCR, especially diagnostic PCR, has to be applied carefully if it is to be discriminatory.

Evidence presented in this report suggests that HCMV can be detected in monocytes of healthy subjects. This is consistent with previous evidence that clinical isolates can preferentially infect monocytes, albeit at low levels (Rice et al., 1984), and with in situ evidence suggesting monocyte carriage of HCMV in post-transplant kidneys (Gnann et al., 1988). However, our data do not address the question of how HCMV is maintained in an asymptomatic individual. There are at least two possible methods of maintenance. First, low level productive infection is feasible, which would necessitate extremely low levels of virus release because it is impossible to coculture HCMV from healthy carriers. Second, limited virus gene expression could occur during persistence without release of infectious virus, more analogous to the persistence of Epstein–Barr virus, which is maintained in an extrachromosomal state; in this case, limited expression of the HCMV gene products necessary for
episomal maintenance might be expected. Work is in progress to determine the extent of HCMV gene expression in asymptomatically carriers by reverse transcription PCR.

References


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