Viral loads in dual infection with HIV-1 and cytomegalovirus

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Abstract

Objective—A one year study of the relation between cytomegalovirus (CMV) and human immunodeficiency virus (HIV) viral loads in a cohort of children with vertically acquired HIV-1 infection.

Design—Comparative analysis of viral load measurements for CMV and HIV-1 in peripheral blood leucocytes (PBLs) of individual children in relation to age and clinical staging.

Methods—Nested polymerase chain reaction (PCR) was used to measure HIV-1 proviral DNA and CMV genomic DNA in PBLs of 56 children.

Results—The CMV load was highest in 0–2 year old HIV positive children with stage C disease (range, 1–7143 copies/100 ng DNA; median, 125) and was significantly lower in older children. Although higher in young children, HIV-1 viral load did not show the same marked reduction with age that is seen with CMV. Over a one year period, testing of serial samples for both viruses in a subgroup of children revealed a discordant relation between viral loads for CMV and HIV-1.

Conclusions—CMV viral load falls much faster than HIV viral load in dually infected children. Screening for clinical CMV disease is most likely to be of benefit in children under 2 years of age with stage C disease. In the few children studied, levels of CMV and HIV replication appear to be independent.

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Between 40% and 80% of healthy prepubertal children are seropositive to human cytomegalovirus (CMV). High rates of coinfection have been reported in children vertically infected with human immunodeficiency virus (HIV) and CMV,^{1–5} with 30–60% excreting CMV and 18–39% of these children developing some clinical disease attributable to CMV.^{2,3}

In infected adults, HIV viral load is predictive of progression to AIDS,^{6,7} and CMV DNAemia predicts progression to disease, particularly retinitis.⁸ In younger children infected with HIV, the spectrum of CMV disease is less well established, partly because of the difficulties in performing accurate regular clinical screening for CMV retinitis. Our hypothesis was that measurement of CMV viral load might be useful in identifying those children most at risk of developing CMV

disease. Because no previous data were available, we performed this study to determine the range of CMV viral loads found in children with HIV-1 infection.

CMV disease in children can follow a primary infection or reactivation of latent infection. There is some clinical evidence that children coinfecting vertically with CMV and HIV may have a worse prognosis of their HIV disease.^{1,4} It is still not clear whether CMV acts as a cofactor in accelerating HIV pathogenesis or activates opportunistically, without clinical consequence, as a result of the general impairment in cellular immune responsiveness caused by HIV.^{1,2,4,9,10} In a subgroup of children sequential blood samples were taken over one year, allowing the changes in CMV and HIV viral loads to be studied in relation to the children's ages and stages of HIV disease.

Materials and methods

STUDY POPULATION

Fifty six vertically acquired HIV-1 seropositive children all originating from sub-Saharan Africa and aged 0.5–13 years (median, 5.6 years; 10 under 2 years) who were attending the HIV family clinics of St George's, St Mary's, and St Thomas's Hospitals in London, were enrolled from July 1996. An extra 1–2 ml of blood was taken during routine outpatient phlebotomy when the child had no clinical signs of active intercurrent infection. All children were classified using the CDC clinical and immunological staging¹¹ (fig 1), and underwent regular clinical and ophthalmological screening for CMV disease; 63% were receiving antiretroviral treatment, which consisted of various combinations of nucleoside analogues (AZT, ddI, ddC, 3TC, d4T). No child was taking protease inhibitors. All virological

Figure 1 Distribution and number of patients according to CDC immunological and clinical staging of HIV disease.¹¹

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investigations were blinded to the clinical details of the children. Monitoring for CMV IgG antibody was by in-house enzyme immunoassay.¹²

NUCLEIC ACID ISOLATION

Blood samples (1–2 ml in ethylene diamine-tetra acetic acid) for testing by the polymerase chain reaction (PCR) were processed within 24 hours for cell associated CMV or HIV-1 proviral DNA. Peripheral blood mononuclear cells were extracted for total DNA by lysis with detergents followed by treatment with proteinase K.¹³ The concentration of the extracted DNA, measured by fluorimetry, was adjusted to 100 ng/μl in the extraction buffer.

SEMIQUANTITATIVE DUPLEX PCR FOR HIV-1 AND CMV DNA

Nested PCR reactions for amplifying a 168 base pair (bp) DNA fragment from the *mtrII* immediate-early region of the CMV genome¹⁴ and a 360 bp fragment of the HIV-1 *env* V3 region¹³ were performed in the same 50 μl reaction volume. The details of the primer sequences, the composition of the PCR reaction mixture, and the cycling conditions were as described previously.^{13, 14} The HIV-1 and CMV DNA amplicons were resolved in a 6% mini polyacrylamide gel from which the appropriate specific bands were eluted for direct sequencing¹⁵ and for CMV DNA restriction analysis.¹⁶ Samples found to be negative by the HIV PCR were retested by PCR against primers specific for the “housekeeping” histidyl tRNA synthetase (*HRS*) gene¹⁴; these primers were not included routinely because the product size (360 bp) was the same as with the HIV *env* primers.

The sensitivity of the CMV *mtrII* PCR was of the order of one copy of specific plasmid DNA,¹⁶ as was that of the HIV-1 *env* PCR¹³ when determined using the MRC ADP PCR reference reagents set (No. ADP956).¹⁷

The duplex CMV-HIV PCR was performed on serial 10-fold dilutions of DNA, in duplicate, and the number of virus copies for either virus was calculated for each 100 ng of total cell DNA, on the assumption that the signal at the end point of the titration emanated from one copy of viral DNA being added at the start of the reaction. The number of CD4 positive cells was not measured on every occasion and, therefore, the number of viral copies was expressed for each 100 ng of leucocyte DNA, which is an equivalent of 15 000 cells. Preliminary testing established that duplex or multiplex PCR, when performed using the same primers, gave similar end point values for either virus on titrating blood samples that contained high levels of DNA for both viruses; this showed that there was no significant interference between the two PCR reactions when these were proceeding simultaneously. At the time of our study, the improved Amplicor HIV-1 monitor RNA PCR test using the primers SK 145/SK151 was not available, and the most comprehensive set of data for HIV viral loads throughout the study was that obtained by the nested PCR for proviral DNA. Com-

parison of the concentrations of HIV-1 *env* DNA and HIV-1 *gag* RNA in 14 children (data not shown) showed a close correlation as noted by others.^{18, 19} Therefore, all data presented were obtained by using duplex nested PCR for CMV DNA and HIV-1 proviral DNA.

Statistical analysis was performed by the Kruskal-Wallis test using the MultiStat application for the Macintosh; this compares median values for groups of non-normally distributed data.

Results

VIRAL LOAD MONITORING FOR HIV AND CMV INFECTION

The specificity of the PCR assay was tested in two groups of healthy children who served as controls for our study. The first group contained 18 HIV antibody negative children, aged 0.1–2 years (*n* = 12) and 4–9 years (*n* = 6), of HIV positive African women. All 18 children were negative by PCR for HIV-1 DNA; nine were positive for antibody to CMV and two of these were also positive for CMV DNA.

The other control group contained 44 healthy children, aged 0.1–14 years (median, 5.6) whose blood samples were obtained during routine surgical procedures; 10 were positive for antibody to CMV and one of these was positive also by CMV PCR. The levels of CMV DNA recorded in the three HIV negative, CMV PCR positive children were in all cases one copy/100 ng of total DNA.

Our study group contained 56 HIV-1 antibody positive children; 54 of these were positive for HIV proviral DNA. Of the two subjects who gave discordant results, one was a 7 year old boy who had been tested by HIV PCR for DNA three times every year since 1992, including twice during the course of our study and who, despite having CD4 positive cell counts of ~ 1000, had remained consistently negative for HIV DNA, yet weakly positive for HIV RNA. The other was an 8 year old girl (CD4 positive cell counts, 500–600 during our study) who had tested positive by HIV PCR for DNA in 1993, but had been found to be PCR negative in 1996–97, yet positive for HIV RNA. In these two patients, the discordant results between the PCR for HIV-1 DNA and HIV-1 RNA could be the result of the higher sensitivity of the HIV-1 RNA PCR, probably because the *gag* region is relatively more conserved than the highly variable *env* sequence of HIV-1, especially in viral isolates of African origin.

Forty five of the 56 HIV-1 antibody positive children were positive for CMV IgG. Among the 11 who were repeatedly CMV antibody negative, six gave negative results for CMV DNA by PCR, and the other five were weakly positive (1–2 copies of CMV DNA/100 ng of total DNA in three patients; six and 10 copies in the other two patients).

PATTERNS OF HIV DNA AND CMV DNA VIRAL LOAD
CMV and HIV viral loads were compared in relation to the age of the child at testing. All 56 children were split in to two groups: one group

Figure 2 Median HIV-1 and CMV viral loads (copies of viral DNA/100 ng of total DNA) in children of different age groups. The numbers above the bars are numbers of children in each age group.

Figure 3 Relation between HIV-1 and CMV viral load related to the CDC clinical category of HIV disease in children (A) under 2 and (B) over 2 years of age.

comprising children aged over 2 years ($n = 46$) and the other those under 2 years ($n = 10$); the latter group contained three children in clinical stage B and seven in stage C. The viral loads for CMV DNA in the 0–2 years age group (0–7143 copies/100 ng DNA; median, 125) was significantly higher ($p = 0.0028$) than in the older age group (fig 2), whereas the median viral loads for HIV proviral DNA were not significantly different between the two groups. The pattern of CMV and HIV viral loads by age of the child is shown in fig 2.

Because the levels of CMV were higher in the younger children, the two groups of children aged over and under 2 years were compared to see if there was any association between the viral loads for HIV and CMV and

the child's clinical staging. For those aged under 2 years, high viral loads for CMV were significantly associated with clinical stage C ($p = 0.014$). However, although the HIV viral loads in this group were clearly higher in children in clinical stage C, the association between HIV viral load and clinical staging was not significant (fig 3).

To examine more closely the relation between levels of viraemia for CMV and HIV in younger children, longitudinal testing of a subgroup of children under 2 years of age for both viruses was carried out over the one year period. The results for eight such patients are presented in fig 4. In four of these patients (A, C, G, and H) high levels of HIV DNA were detected initially but declined and were replaced by high levels of CMV DNA; in the other four patients (B, D, E, and F) the converse was true, with CMV DNA levels predominating early on but eventually being superseded by HIV. Only one 1 year old boy had clinical CMV disease during our study. Severe (zone 3) bilateral CMV retinitis was detected during routine screening; his peak CMV viral load was 500 CMV DNA copies/100 ng DNA and this showed no decline in response to ganciclovir treatment (fig 4H).

Discussion

Several studies have confirmed the relation between HIV-1 viral load and disease progression in both adults^{6,7} and children,^{20–23} and have indicated that CMV DNAemia levels are predictive for the development of CMV end organ disease in cases of adult HIV infection.^{3,8,24} Quantitative CMV PCR has shown a strong association between higher baseline copy number and the earlier development of CMV disease in a recent anti-CMV drug study.²⁵

In our study, most of the HIV infected children appeared to be infected with CMV, as indicated by the detection of CMV IgG antibody and viral DNA. Five children were CMV antibody negative or equivocal, while being weakly positive for CMV DNA. A similar discrepancy between CMV serology and PCR has been noted previously in a group of heart transplant patients.¹⁶ Interestingly, this suggests that there might be a qualitative or quantitative effect of immunosuppression on CMV antibody production in some patients. For example, a response comprising mainly low avidity antibody could give a false negative result by standard antibody assay.

The cross sectional component of this study detected no clear association between a high CMV viral load and the development of CMV end organ disease because of the low frequency of CMV retinitis in the study group (one of eight patients with high CMV load; none of four patients who died). The very high levels of CMV viral load in the first 2 years of life, and the rapid apparent subsequent decline suggest that sequential long term testing for CMV viral load will not be useful in predicting clinical CMV disease in HIV infected children. The introduction of "highly active antiretroviral therapy" (HAART) in adults has recently been

Figure 4 Discordant HIV-1/CMV load patterns (copies of viral DNA/100 ng total DNA) in eight individual patients under 2 years of age, determined by duplex PCR (similar results were obtained by monoplex PCR for each virus alone). Closed symbols, HIV-1 load; open symbols, CMV load. Patients mean CD4 positive lymphocyte count over study period ($\times 10^6$): (A) 0.5; (B) 1.48; (C) 0.6; (D) 1.77; (E) 1.43; (F) 0.8; (G) 0.95; (H) 0.3.

associated with a dramatic reduction in the frequency of CMV disease.

In agreement with earlier studies,²²⁻²³ the highest HIV-1 viral loads were found in younger children in clinical stage C. High CMV viral loads were found only in the 0–2 year old age group, remaining low in the older age groups. All the infants in our study had presented with rapidly progressive disease and high HIV viral loads. In the absence of any infants followed prospectively from birth it is not possible to identify whether the age or stage of HIV disease is the principal explanatory variable for determining the high CMV load.

An unexpected finding was that the levels of HIV-1 and CMV DNA showed no consistent association in individual patients. In the blood, latent CMV and HIV-1 occupy different cell compartments. For CMV these are usually monocytes and polymorphs,²⁶⁻²⁸ and other white blood cell types in cases of active infection.²⁹ For HIV-1, proviral DNA is restricted predominantly to CD4 positive lymphocytes.³⁰ For these reasons, HIV and

CMV coinfection of white blood cells is considered to be an unlikely event,³¹ and this is borne out in our small study by the finding that high viral loads for these two viruses appeared to be mutually exclusive. However, HIV and CMV might interact in tissues such as lymphoid organs, either by coinfecting the same cell type (for example, macrophages), or by a CMV encoded superantigen that enhances HIV-1 replication in V β 12 positive, CD4 positive cells.³² Why levels of cell associated HIV-1 and CMV in the peripheral blood appeared to be mutually exclusive is not clear, but might reflect the differential effects of cytokines on virus replication.³³

Several conclusions can be drawn from our results. First, because high level CMV DNAemia is most common in the 0–2 year old age group of HIV-1 vertically infected children, it implies that the CMV infection in this group of patients is the result of intrauterine or perinatal primary infection, rather than reactivation of latent CMV. Because most CMV infected children with a high CMV load were in clinical stage C of their HIV disease, this suggests that early, possibly intrauterine, onset of infection with HIV, when followed by rapid development of immunosuppression, predisposes these patients to systemic CMV infection. In the absence of widespread availability of CMV viral load measurements, it is this group of patients who would probably benefit most from regular ophthalmological surveillance.

In children over 2 years there was no clear association between CMV load and stage of the HIV disease. High levels of CMV in young children with immature immune systems might be associated with impaired cellular immunity to CMV.³⁴ In older children, whose immune system is able to bring CMV under control,³⁴ the most important factor in progression to CMV disease is probably the rate at which HIV infection is able to overwhelm the overall immune response. The temporal relation between the levels of HIV-1 infection and CMV and the discordant HIV/CMV load pattern in individual patients suggests that both viruses replicate independently of one another, and that CMV is not acting as a major promoter of HIV disease progression.

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