Cytomegalovirus co-infection in patients with HIV/AIDS in north India

S. Mujtaba, S. Varma & S. Sehgal

Department of Immunopathology & Internal Medicine, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Received April 16, 2001

Background & objectives: Cytomegalovirus (CMV) is a frequent opportunistic infection in immunocompromised individuals particularly those receiving organ transplants and harbouring HIV infection. The classical CMV syndrome may be seen in only a small percentage of patients and tissue diagnosis is cumbersome, costly and requires hospitalization. Hence there is an urgent need to establish accurate and early diagnosis for proper institution of therapy. An attempt was made to detect active CMV co-infection in patients with HIV/AIDS using three assays and the positivity rates in the 2 groups compared.

Methods: In the present study, we used a highly sensitive polymerase chain reaction (PCR) for immediate early gene of CMV, pp65 antigenaemia assay and IgM ELISA assay to detect the presence of CMV co-infection in 37 patients with AIDS and 32 healthy HIV seropositives. Thirty healthy laboratory workers served as normal controls.

Results: Of the 37 patients with AIDS, 12 (32.4%) showed a positive reaction by PCR and only 4 patients were positive by the antigenaemia assay. Of the 32 HIV seropositives, only one was positive by PCR (3%), and all were negative for antigen assay. None of the controls showed positivity by any of the tests. The difference in PCR positivity rates between HIV seropositives and patients with AIDS was significant (P<01). IgM antibodies were positive in four (10.3%) AIDS patients and only one (3%) HIV seropositive, the difference was insignificant. The difference in antigen positivity between HIV seropositives and AIDS patients was also insignificant.

Interpretation & conclusion: CMV appears to be an important co-infection in patients with AIDS in India and PCR is a powerful tool for detection of CMV in blood and is superior to the antigenaemia assay. PCR can be performed with a small volume of blood avoiding any invasive procedure, and can provide quick information for timely institution of therapy.

Key words Cytomegalovirus (CMV) - HIV/AIDS - PCR - pp65 antigen

Infection by cytomegalovirus (CMV) is the major cause of morbidity and mortality in individuals with depressed cell mediated immunity of congenital origin, iatrogenic origin and that associated with acquired immunodeficiency syndrome (AIDS)1-3. Although one of the important routes of infection is intraterine4, patients with organ transplants and AIDS are at serious risk of developing CMV diseases5-6. The clinical diagnosis of AIDS with CMV infection can be difficult in the absence of CMV retinitis, polyradiculopathy and the classical CMV syndrome7-9. It has been documented6 that at
least 25 per cent of AIDS patients develop serious CMV disease. Retinitis occurs in 6-15 per cent of patients, gastroduodenal disease develops in 5-10 per cent and at autopsy 30 per cent reveal active CMV infection. The diagnosis poses difficulties because a 2-3 wk period is mandatory for virus isolation while IgM antibodies as detected by ELISA correlate poorly with the clinical status of CMV infection and facilities for culture are usually not available in most centers. PCR and antigenaemia assays have been introduced for a rapid diagnosis of CMV and a more recent report indicates that PCR and antigenaemia assays are sensitive methods, which predict the development of CMV disease up to several months prior to clinical manifestations.

The emergence of AIDS in India has necessitated the establishment of reliable tests for the diagnosis of co-infection with CMV. There are a few reports available of CMV infection in Indian patients with HIV/AIDS, which are based primarily on clinical or autopsy evaluation. In the present study an attempt was made to detect the presence of active CMV co-infection in HIV/AIDS patients, referred to the surveillance center attached to the Nehru hospital at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. Further, the positivity rates of the three assays viz., PCR, antigenaemia assay and IgM ELISA assay were compared in the two groups.

Material & Methods

Patients reporting to the AIDS Surveillance Center, Department of Immunopathology, PGIMER, Chandigarh between 1997-1999 were included in the study. HIV infection was established by two different ELISA tests i.e., Recombigen (Cambridge Biotech, Ireland), Detect (Biochem Immunosystem Inc., Canada) and a rapid test, Capillus (Cambridge Biotech, Ireland) or Immunocomb (Ogenics, Israel). If all the three tests were positive, case was designated as HIV positive. In case of even a minor discord, Western blot was performed (New Lav Blot I/II Sanofi Diagnostics, Pasteur, France) Clinical diagnosis of AIDS was established by the Centers for Disease Control (CDC) clinical criteria. Seropositive patients without symptoms and with a CD4 count of >200/mm³ were designated as healthy seropositives. Normal laboratory workers were used as controls after consent and necessary ethical clearance. Five ml of blood was collected for the following assays.

**CMV antigenaemia assay:** The antigenaemia assay directly detects the CMV antigen in the nucleus of polymorphonuclear leucocytes expressing viral phosphoprotein-65 (pp65). The assay was performed according to the method of Gerna et al. Anti-pp65 monoclonal antibody was a gift from Dr Giuseppe Gerna, Italy which was used as a positive control for nuclear positivity in the polymorphonuclear cells.

**CMV PCR:** DNA was prepared from peripheral blood leucocytes by the method of Higuchi. To avoid contamination by PCR product carry over and false positive results, the samples were prepared in a different laboratory from that in which the amplification reaction was performed. In addition, all other recommended precautions were taken. Primers specific for CMV were obtained from Gibco BRL, USA. They were selected from exon 4 of major immediate early gene as advocated by Stenberg et al. Strain AD 169 of human CMV, used as a positive control, was a gift from Dr Philip E. Pellet (CDC, Atlanta). The sequences of the primers IE1, IE2 and probe IE3 used were as follows:

IE1; CCACCCGTGGTGCCAGCTCC,
IE2; CCGGCTCTCTGAGCACCC,
IE3; CTGGTGTCACCCCCAAGATCCCTGATGATCC.

The PCR reaction mixture of (master mix) 25 µl contained 2.5 µl of 10X Taq buffer (Boehinger Mannheim, Germany), 2.5mM MgCl₂, 70 pM of each primer IE1 and IE2, 200µM dNTPs, 2.5 U of Taq polymerase (Boehinger Mannheim, Germany) and autoclaved water. Each reaction tube received 25µl of PCR mixture plus 25 µl of DNA and 50 µl mineral oil (Sigma, USA). The reaction was carried out in a DNA thermal cycler (Perkin Elmer, USA) using the following programme, 1 min each for denaturation at 94°C, annealing at 42°C and extension at 72°C for 48 cycles except that the first
cycle had denaturation of 5 min and the last cycle had an extra extension for 10 min.

**Dot hybridization:** Dot hybridization was performed according to Shibata et al. Briefly, heating at 95°C denatured the PCR products, 10 µl was spotted on nylon membrane (Boehringer Mannheim, Germany). After baking, the membrane was prehybridized at 42°C for 4-5 h and finally hybridized with γ³²PATP radiolabelled probe. The isotope was procured from the Board of Radioisotope Technology (BRIT), Trombay. After washing, the membrane was subjected to autoradiography for 48 h at -70°C.

**CMV IgM ELISA:** CMV IgM ELISA was performed according to the manufacturer’s protocol (Vironostika, Organon Teknika, Belgium). The cut-off value (control+3SD) was calculated from the mean of control value. The data were analysed using Chi square analysis.

**Results**

Of the 37 patients with AIDS, 32 were males and 5 females with a mean age of 30.79±11.6 yr. The majority presented with fever, severe weight loss, diarrhoea, oral candidiasis etc. CMV IgM antibodies were positive in 4 (10.8%) patients. CMV antigen was also positive in 4 (10.8%) patients. Of these 4 patients, 3 (8%) had both antigenaemia as well as IgM positivity. Only IgM or only antigen was positive in one patient each. However, by PCR, 12 of 37 (32.4%) patients with AIDS were positive. Of these 12 patients, PCR alone was positive in 7 (18.9%), three patients were positive by all the three tests and one patient each was positive by PCR+IgM or PCR and antigenaemia assay.

Of the 32 healthy seropositives, 21 were males and 11 were females with the mean age of 29.7±5.7 yr. CMV IgM antibodies were positive in one case (3.0%). Antigenaemia assay was negative in all. PCR was positive in one case (3.0%). The patient showing positive CMV IgM antibodies was however negative by PCR.

Of the 30 normal controls, 20 were males and 10 females with a mean age of 30.4±7.2 yr. None showed ELISA, antigenaemia or PCR positivity. For a definite diagnosis of CMV infection, a positive PCR assay was considered, mandatory. The difference in IgM positivity and CMV antigen positivity between HIV seropositives and AIDS patients was not significant. However, the difference in PCR positivity rates between HIV seropositives and patients with AIDS was significant (P<0.01).

**Discussion**

Although tuberculosis is the single most important opportunistic infection in Indian patients with AIDS, it appears that CMV infection is one of the important causes of morbidity in our patients, as many presented with diarrhoea, fever, pneumonia, where histopathological diagnosis was not possible. The PCR assay used in the present study appeared quite specific, as the controls were all negative *i.e.*, a specificity of 100 per cent and the healthy seropositive subjects showed a significantly lower positivity of 3.0 per cent (*i.e.*, 1/32) by the PCR test. This patient could have been in the sub clinical stage of infection. In fact Dodd et al. have indicated that PCR can reliably predict the emergence of clinical disease. A large number of histologically proven cases were not available for ascertaining the sensitivity of the assay but a number of studies are available from transplant recipients which indicate a sensitivity of upto 92 per cent. Similarly Gerna et al. documented 46 per cent of patients to be antigen positive out of total positives by PCR. Hoshino et al. also reported low sensitivity of the antigenaemia assay. In this study IgM ELISA was positive in only 33 per cent of the AIDS patients positive by PCR. Lack of correlation could be attributed to low sensitivity of the test, severe immunosuppression or reactivation of CMV where the patient may not exhibit IgM antibodies. The PCR assay in this study appeared superior to the antigenaemia assay. PCR can detect one positive cell in a million cells while in the antigenaemia assay at best 50-100 thousand cells can be screened. Boland et al. monitored CMV infection in 201 samples from 19 heart and 2 lung transplant patients and reported 27 per cent positivity by antigen assay and 50 per cent by the PCR test. Frymuth et al. reported PCR
and culture positivity in 53 and 43 per cent respectively in transplant recipients while in patients with AIDS the figures were 40 and 24 per cent. Thus, PCR appears to be more sensitive than either antigenaemia assay or shell vial culture. Further, with the PCR and hybridization protocol used in this study none of the healthy controls was found to be positive and only 3 per cent of HIV seropositives showed a positive signal. This makes PCR the most reliable test for monitoring antiviral therapy. According to Dodt et al.\textsuperscript{7} PCR can detect CMV DNA at a median of 46 days before the onset of clinical disease.

Our data show that 32.4 per cent of patients with AIDS had CMV co-infection and part of the symptoms may be attributed to CMV. Seven out of 12 patients had fever and wasting, 6 had diarrhoea and 3 had CNS manifestations. These are typical features of CMV syndromes involving different organs as proved histologically\textsuperscript{7-9}. However, in the developing countries such procedures are not routinely available. Thus, PCR assay could guide the timely institution of therapy in patients who are symptomatic yet do not present with a classical CMV syndrome or where a biopsy diagnosis is not feasible. Further, patients with a positive PCR need to be vigilantly followed up for development of symptoms and early initiation of therapy.

**Acknowledgment**

The authors thank the Department of Biotechnology, New Delhi for financial support. Junior research fellowship was granted to SM by the University Grants Commission, New Delhi. Technical assistance provided by Ms N. Pasricha and Shri I. James is gratefully acknowledged.

**References**


Reprint requests : Dr (Mrs) Shobha Sehgal, Emeritus Professor, Department of Immunopathology, Postgraduate Institute of Medical Education & Research, Chandigarh 160012