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Article

Usefulness of pp65 Antigenemia for the Early Diagnosis of Cytomegalovirus Disease in Patients with AIDS

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Abstract An observational cohort study was performed to assess the effectiveness of a cytomegalovirus antigenemia (CMV-Ag) assay designed to predict clinical CMV disease in patients with AIDS. Eighty-six HIV-infected patients with CD4+ cell counts of $<100/mm^3$, positive CMV IgG, and no previous CMV disease were enrolled. Thirty-eight (44%) patients had at least one positive CMV antigenemia test, ten of whom eventually developed CMV focal disease. CMV disease was diagnosed in 13 (15%) patients. The CMV antigenemia assay was positive in ten of these 13 patients. Using a cut-off value of five positive cells in every 150 000 leukocytes sampled, the CMV antigenemia assay had a positive predictive value of 89% and a negative predictive value of 94%. The median time from the first positive CMV antigenemia test to the onset of CMV disease was 102 days. CMV disease probability at 6 months in patients with a CMV antigenemia value ≥ 5 was 77.8% versus 6% in patients with CMV antigenemia value <5 (log-rank test $=48.345$; $P<0.001$). Several independent factors were associated with the development of CMV disease: CMV antigenemia ≥ 5 cells (hazard ratio: 20.44), CD4+ count $\leq 25/mm^3$ (HR: 3.12), and sexual transmission of HIV infection (hazard ratio, 3.15). CMV antigenemia seems to be a good predictor of CMV disease in patients with AIDS.

Introduction

Cytomegalovirus disease (CMVD) in patients with AIDS occurs mostly in the form of retinitis and gastrointestinal tract involvement [1]. The main risk factor for the development of CMVD is severe immunodeficiency [2, 3], with clinical manifestations occurring almost exclusively in patients with CD4+ cell counts below $50/mm^3$. It has also been hypothesized that cytomegalovirus (CMV) could be a cofactor in the progression of HIV infection [4] and CMVD a poor prognosis factor in terms of survival [1].

Many studies have shown the relationship between the decrease in CD4+ cell counts and the frequency of CMV isolation in urine and blood [5]. Detection of CMV in urine, despite being sensitive in identifying a population that could have CMVD, is a highly nonspecific test that does not directly relate to the current or future presence of CMVD and has a limited value for determining the prognosis of patients with AIDS as well as for the monitoring and follow-up of these patients [5]. On the other hand, the detection of the virus in the blood is a more precise marker of the development of CMVD, and a number of studies have demonstrated, mainly in transplant recipients, that high levels of viremia correlate with the short-term development of CMVD [6–9]. Among the techniques available

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for detection of CMV in blood, determination of pp65 antigenemia has been shown to be useful in detecting early CMVD. This technique has been used mainly in patients who have previously undergone solid organ or bone marrow transplantation; today, it is a valuable instrument that can aid physicians in making an early decision on the start of treatment for CMV [7–11].

The primary objective of this study was to evaluate the predictive and prognostic value of the pp65 antigenemia assay in the early diagnosis of CMVD in patients with advanced HIV infection.

Materials and Methods

An observational cohort study was carried out between July 1994 and May 1996. For a period of 1 year, subjects were enrolled by sequential sampling of those meeting the inclusion criteria: adult patients with confirmed HIV infection, CD4+ cells $<100/mm^3$, IgG positive for CMV and no previous CMVD. Patients excluded were those being treated with ganciclovir or foscarnet, those with a life expectancy of less than 2 months, and those who would very likely fail to comply with the follow-up protocol. All recruited patients expressed their informed consent.

After baseline evaluation, patients were monitored every 2 months. Anamnesis, physical exam, and funduscopy were performed at every visit, and blood samples were drawn for use in the pp65 antigenemia assay, for determination of CD4+ cell count, and for haematological and biochemistry tests. Indirect ophthalmoscopy (with bilateral pharmacological mydriasis) was carried out by an ophthalmologist. Further examinations such as digestive endoscopy, fibrobronchoscopy, computed tomographic scan or magnetic resonance imaging of the brain, or lumbar puncture were performed only if patients showed specific symptoms. Patients were withdrawn from the study upon death, failure to attend follow-up, or completion of the established follow-up period.

The primary outcome variable studied was the occurrence of CMVD. Other outcome variables were death and survival time. The primary predictive variable was pp65 antigenemia; the secondary predictive variable was CD4+ cell count. Additionally, a number of covariates that could act as potential confusion factors or modifiers of the primary effect were studied (age, gender, risk group, clinical stage of HIV infection, previous opportunistic infections, treatments, and haematological and biochemical parameters).

CMVD was diagnosed according to the following criteria: (i) retinitis: characteristic findings in funduscopy; (ii) digestive involvement: consistent clinical signs/symptoms, ulcerated lesions in endoscopy, and presence of CMV inclusion bodies in the biopsies performed; (iii) lung involvement: respiratory signs/symptoms, bilateral interstitial infiltrates in chest radiograph, and presence of CMV inclusion bodies in lung biopsy, with exclusion of other organisms causing pulmonary infection; (iv) central nervous system involvement: neurological signs/symptoms, brain lesions in computed tomographic scan or magnetic resonance imaging, and CMV inclusions in brain biopsy.

The pp65 antigenemia assay directly detects the pp65 antigen of CMV in leukocytes from peripheral blood (mostly polymorphonuclear) using monoclonal antibodies in an indirect immunofluorescence technique. The commercial kit used for performing the technique was the Monofluo Kit CMV (Sanofi-Diagnostics Pasteur, France). Blood samples were processed 3–6 h after they were drawn. Leukocytes were separated by diluting the samples (3–6 ml of EDTA-anticoagulated blood) in 1.5 ml of phosphate-buffered saline and 0.5 ml of 6% dextran, followed by incubation for a period of 15 min at 37 °C. After centrifugation ($200 \times g$) at room temperature, the leukocyte layer was resuspended in 3 ml of RBC Lysing Medium (Incstar, USA) in order to lyse the residual erythrocytes. After two further centrifugations, leukocytes were quantified by the Coulter method and adjusted to a concentration of 2 000 000 cells/ml. Three aliquots of 25 µl (150 000 leukocytes) were inoculated in special slides with wells and incubated for 15–17 min at room temperature, thereby allowing sedimentation of the leukocytes. Immediately after, leukocytes were fixed by placing them in a 5% formaldehyde solution for 10 min. Then, the preparations were incubated in a moist chamber at 37 °C for 45 min with a murine anti-pp65 monoclonal antibody, washed with PBS and, again, incubated in a moist chamber at 37 °C for 44 min with a fluorescein conjugate (sheep antibodies anti-mouse IgG) diluted with Evans blue (1:10). The preparations were dried at room temperature and examined by means of fluorescence microscopy, and fluorescence leukocyte nuclei were counted. The result is expressed as the amount of positive cells per 150 000 leukocytes examined. Those who evaluated the antigenemia test were blinded to the patient status.

A statistical descriptive analysis was performed for each variable studied. The CMVD incidence rate and the CMVD cumulative incidence were estimated. The diagnostic accuracy indexes (sensitivity, specificity, and positive and negative predictive values) for the antigenemia assay as a predictor of CMVD were estimated for a number of cut-off points, along with their corresponding 95% confidence intervals (CI). Comparisons between the variables were tested by means of a bivariate analysis, depending on the positivity or negativity of antigenemia, and the development or absence of CMVD. For categorical variables, comparison was performed by means of the chi-square test and the Fisher's exact test in those cases in which the expected frequency in a cell was lower than 5. For the quantitative variables, the Student's *t* test or the Mann-Whitney U test was used, depending on whether the variables followed a normal distribution and whether the variances were homogeneous according to the Barlett test.

In order to establish the risk factors associated with the development of CMVD, a multivariate analysis was performed through the Cox proportional hazards model. The variables used were those that had been found to be significant in the bivariate analysis ($P<0.05$) and those that, not being statistically significant, could provide information to the final regression model, according to the current knowledge on the subject under study. The method of consecutive significances or the enter method was used in the selection of the optimal model (i.e. the most predictive model with the lowest number of variables). The hazard ratios (HR) and their 95% CIs were estimated from the regression coefficients of the variables. The test used to observe the global adjustment of the model was the likelihood ratio test, with a significance level of 5%. The cumulative likelihood of CMVD development was estimated by means of Kaplan-Meier curves, and the differences between subgroups were estimated by means of the log-rank test. Finally, a survival analysis was performed by means of Kaplan-Meier curves, and the Mantel-Cox method was used in order to compare the survival means.

Results

Ninety-eight patients were enrolled, 12 of whom were finally excluded due to failure to attend follow-up. The final population was made up of 86 patients, 67 males and 19 females. The mean age was 34 years (range, 22–53 years). The distribution in risk groups was as follows: 50 (58%) patients were injecting drug users, 20

(23.3%) were homosexual, 14 (16.3%) had acquired HIV infection through a heterosexual contact, and two (2.3%) were haemophilic. Thus, 60.4% of the patients had acquired the HIV infection through parenteral transmission and the remaining 39.6% through sexual transmission. The distribution in clinical stages (CDC, 1993) was as follows: 59 (68.6%) C3, 23 (26.7%) B3, and four (4.7%) A3. The mean baseline CD4+ cell count was 36/mm^3 and the median CD4+ cell count 25/mm^3. A few patients were receiving antiretroviral treatment, mainly monotherapy with zidovudine, and most of them received prophylaxis against opportunistic infections (mainly *Pneumocystis carinii* infection). The longest follow-up period was 22 months, with a mean of 8.1 months, a median of 6.5 months, and a range of 43–670 days.

CMVD was diagnosed in 13 patients (9 with retinitis, 1 with esophagitis, 1 with colitis, 1 with pneumonitis and 1 with oral aphthae), giving a cumulative incidence of 15.1% and an incidence rate of 22.81 cases per 100 individuals/year. The CMVD incidence rate in patients with CD4+ cell counts of $\leq$25/mm^3 was 37.04 cases per 100 individuals/year versus 9.68 cases per 100 individuals/year in patients with CD4+ cell counts of >25/mm^3 (HR, 3.83; 95% CI, 1.05–13.91; $P=0.028$). In patients who acquired the HIV infection through sexual transmission (homo- or heterosexual), the incidence rate was 41.18 cases per 100 individuals/year versus 15 cases per 100 individuals/year among subjects who had been parenterally infected (HR, 2.75; 95% CI, 0.92–8.17; $P=0.058$).

A total of 388 antigenemia assays were performed, with a mean of four measurements per patient (range, 1–10). Fifty-two (13%) determinations were positive. No differences were observed between the baseline biological parameters of patients with some positive results and those of patients with only negative results. No significant differences were found between the baseline biological parameters of patients who developed CMVD and those of CMVD-free patients, except for the baseline CD4+ cell count (15/mm^3 vs. 40/mm^3; $P=0.004$) and hemoglobin (10.6 g/dl vs. 12.1 g/dl; $P=0.007$).

Thirty-eight (44.2%) patients had at least one positive antigenemia test. Antigenemia was positive in 10 of 13 patients diagnosed with CMVD. The mean time between the first positive antigenemia assay and the diagnosis of CMVD was 102 days (range, 28–239 days); 26.3% of the patients with any positive antigenemia assay subsequently developed CMVD. All the measurements of antigenemia were negative in 48 (55.8%) patients, only three (6.3%) of whom developed CMVD (26.3% vs. 6.3%; $P<0.02$). In the antigenemia assay, the median number of positive cells was 5 per 150 000 leukocytes (range 0–40) among patients with CMVD compared to a median of zero positive cells per 150 000 leukocytes (range 0–8) among patients without CMVD ($P<0.0001$).

Table 1 Diagnostic accuracy rates for the pp65 antigenemia (Ag) assay as a predictor of cytomegalovirus disease

Accuracy rate	CMV-Ag (+)[a]	CMV-Ag $\geq$5[b]
Sensitivity (95% CI)	76.9% (46–95)	61.5% (32–86)
Specificity (95% CI)	61.6% (54–75)	98% (88–100)
PPV (95% CI)	26.3% (13–42)	89% (52–100)
NPV (95% CI)	94% (85–99)	94% (87–98)

[a] Qualitative CMV-Ag (positive or negative)
[b] Quantitative CMV-Ag is expressed as pp65 antigenemia (+) cells/150 000 leukocytes
PPV, positive predictive value; NPV, negative predictive value

The diagnostic accuracy indexes of the antigenemia assay as a predictor of CMVD are shown in Table 1. The most significant data include a negative predictive value of 94% for qualitative CMV antigenemia and an increase in positive predictive value and specificity from 26.3% to 89% and from 61.6% to 98%, respectively, when a cut-off point of $\geq$5 positive cells per 150 000 leukocytes was being used. In the latter case, 89% of patients with $\geq$5 positive cells (i.e., antigenemia$\geq$5) subsequently developed CMVD, while 94% of patients with negative results in the antigenemia assays were CMVD-free for the entire follow-up period.

According to the bivariate analysis, the variables associated with the development of CMVD were as follows: antigenemia $\geq$5, CD4+ cells $\leq$25/mm^3, hemoglobin <10 g/dl, history of herpes infection (chronic mucocutaneous herpes simplex or multimetameric herpes zoster), and total lymphocyte count of $\leq$500/mm^3 (Table 2). The independent risk factors related to the development of CMVD were determined by means of a

Table 2 Univariate analysis for the determination of the variables associated with the development of cytomegalovirus disease

Variable	β[a]	Hazard ratio	95% CI	P value
CMV antigenemia (+)	1.21	3.35	0.91–12.28	0.068
CMV antigenemia $\geq$5	2.90	18.34	5.93–56.74	<0.001
CD4+ cells $\leq$25/mm^3	1.36	3.88	1.06–14.16	0.04
Stage C3	0.88	2.40	0.55–10.86	0.254
Sexual transmission	0.87	2.26	0.75–6.84	0.149
Hemoglobin <10 g/dl	1.47	4.34	1.46–12.94	0.008
Herpes[b]	1.29	3.62	1.11–11.80	0.033
Lymphocytes $\leq$500/mm^3	1.15	3.17	1.05–9.55	0.040
Gender	0.34	1.41	0.31–6.36	0.658
>1 "C" event[c]	1.05	2.85	0.78–10.42	0.113

[a] β coefficients
[b] History of chronic mucocutaneous herpes or multimetameric herpes zoster
[c] More than one clinical event belonging to the C category in the baseline evaluation

Table 3 Risk factors associated with the development of cytomegalovirus disease in HIV-infected patients. Cox proportional hazards analysis. Deviance: 76.176; Likelihood ratio statistic on 1 DF: 3.372; $P=0.066$

Variable	β[a]	Hazard ratio	95% CI	P value
CMV antigenemia ≤ 5	3.02	20.44	6.09–68.61	<0.001
CD4+ cells $\leq 25/mm^3$	1.14	3.12	0.84–11.59	0.089
Sexual transmission[b]	1.15	3.15	0.91–10.95	0.071

[a] β coefficients
[b] Acquisition of HIV infection through sexual transmission (hetero- or homosexual)

multivariate analysis (Cox regression): antigenemia ≥ 5 cells/150 000 leukocytes (HR 20.44), baseline CD4+ cell count $\leq 25/mm^3$ (HR 3.12), and acquisition of HIV infection through sexual transmission (HR 3.15). The optimal regression model is shown in Table 3. The risk of CMVD at 6 months was 77.8% in patients with antigenemia ≥ 5, versus 6% in patients with antigenemia <5 (log-rank test $=48.345$; $P<0.001$). The probability of developing CMVD was also significantly higher in patients with CD4+ cell counts $\leq 25/mm^3$ than in patients with CD4+ cell counts $>25/mm^3$ (19.5% vs. 6%; log-rank test $=4.89$; $P=0.027$). No significant differences were found regarding the risk of CMVD when antigenemia was considered qualitatively: 20% in patients with positive antigenemia versus 6% in patients with negative antigenemia ($P=0.053$).

At the end of the follow-up period, 34 (39.5%) patients had died. The cumulative probability of survival after 12 months of follow-up was 37% in patients who developed CMVD versus 53% in patients without CMVD ($P=0.372$). The probability of survival at 12 months was 50% in patients with some positive antigenemia assays, and 52% in patients with persistently negative antigenemia assays ($P=0.961$). If antigenemia is quantitatively considered, patients with antigenemia ≥ 5 had a survival of 17% at 1-year follow-up versus 56% in patients with antigenemia <5 (log-rank test $=3.089$; $P=0.079$). The variable found to best predict death was the baseline CD4+ cell count, in such a way that survival at the end of the follow-up was 17% in patients with CD4+ cell counts $\leq 50/mm^3$ and 59% in patients with CD4+ cell counts $>50/mm^3$ (log-rank test $=7.639$; $P=0.022$).

Discussion

Several studies have suggested that the detection of viremia helps to predict the subsequent development of CMVD [12, 13]. CMV culture, despite being the gold standard for the diagnosis of CMV infections, is not a useful technique for the early diagnosis of CMVD: it is not sufficiently sensitive for the detection of CMV in blood, it requires 5–15 days to obtain results, and it is technically complex [14–16]. In recent years, other laboratory techniques have been developed to detect CMV in blood. These tests are more sensitive than culture and could help to predict the occurrence of CMVD. One of these techniques is the pp65 antigenemia assay. This assay has been evaluated mainly in transplant recipients, and many studies have demonstrated its high diagnostic value and usefulness in the early detection of CMVD [7–11].

Our results lead us to conclude that pp65 antigenemia is a good marker for the early diagnosis of CMVD in patients with AIDS. Qualitatively considered, the pp65 antigenemia has a low positive predictive value (26.3%); nevertheless, a negative result virtually excludes the subsequent development of CMVD in a given patient (negative predictive value, 94%). In addition, when this assay is quantitatively considered, a cut-off point can be established to select patients at high risk of CMVD. In our study, this cut-off point was five pp65 antigen-positive cells per 150 000 leukocytes examined. Using this cut-off, the positive predictive value of the assay increased greatly, from 26% to 89%, the negative predictive value was maintained (94%), and the decrease in sensitivity was minimal. The finding that the mean time between the first positive antigenemia assay and the diagnosis of CMVD was 102 days indicates that the assay is a good marker for early diagnosis in a short term.

These results are similar to those from several studies performed within the last 3 years that approached this subject from similar perspectives [17–19]. Among these, the study of Dodt et al. [18] probably clarifies this issue best. These authors identified the independent variables associated with the development of CMVD: positive PCR (OR, 10), positive pp65 antigenemia (OR, 4.4), positive CMV culture (OR, 4.3), and clinical criteria for AIDS at the baseline evaluation (OR, 3.3). In their study, PCR was more sensitive than antigenemia and viral cultures, but antigenemia was more sensitive than cultures and more specific than PCR. In our study, a multivariate analysis was also performed in order to identify the variables associated with the development of CMVD, but using the Cox regression, which includes the variable follow-up time. This way of focusing the problem is more correct than logistic regression when applied to a cohort study, and the final regression model is likely to be more predictive. The most predictive variable for the development of CMVD was pp65 antigenemia ≥ 5 at any time during follow-up. The probability to develop CMVD at 1-year follow-up with Kaplan-Meier curves was 89% (95% CI, 61–94) in patients with pp65 antigenemia ≥ 5 versus 9.6% (95% CI, 4–23.5) in patients with antigenemia <5.

Some concern may arise due to the possible effect of selection bias on the validity of the results, since those patients who would likely fail to attend follow-up

protocols were excluded. If patients excluded had a poorer prognosis, the overall risk of CMVD could be underestimated, but the evaluation of pp65 antigenemia as predictor of CMVD should not be affected.

Intravenously administered ganciclovir reduces significantly the incidence of CMVD in transplant recipients [20, 21]. Spector et al. [22] recently evaluated the efficacy of oral ganciclovir for the prevention of CMVD in HIV-infected patients with severe immunodeficiency and found a 49% reduction in the risk of developing CMVD. However, another similar study failed to demonstrate the usefulness of oral ganciclovir for prophylaxis of CMVD [23]. To define a population of HIV-infected patients at high risk of developing CMVD, the first criterion would be a CD4+ cell count of $<50/mm^3$, and then the selection of patients would be carried out through the use of early detection techniques for CMVD. The results of our study demonstrate that 89% of the patients with pp65 antigenemia ≥ 5 subsequently developed CMVD, with a probability of 77.8% at 6 months, and that 94% of patients without antigenemia remained CMVD-free at the end of follow-up.

In recent years, several authors have suggested that detection of CMV DNA by PCR could have the same or higher value as pp65 antigenemia for the prediction of CMVD [18, 24–27]. PCR is an extremely sensitive technique, but a number of inconveniences restrict its routine use in clinical laboratories. In addition, and due to its high sensitivity, the positive predictive value of qualitative PCR techniques is insufficient for the prediction of CMVD, as demonstrated for solid organ transplant recipients [28]. To improve predictivity, several strategies have been designed: detection of viral DNA in plasma or serum [18, 26] instead of in leukocytes, amplification of mRNA [29], or quantification of the CMV viral load [24, 30]. This last strategy is likely to achieve the best predictive value, but, at the moment, there have not been optimally designed prospective comparisons of CMV DNA PCR and pp65 antigenemia for identifying AIDS patients at highest risk for developing CMVD; in any case, it is not known which of these techniques has the best predictive value. Thus, we believe that the pp65 antigenemia assay is a quick and easy-to-perform technique that does not require special equipment or conditions; furthermore, it has a high positive predictive value.

A number of studies have reported that CMVD is associated with poor prognosis, regardless of the baseline CD4+ cell count and clinical category [1, 31, 32]. Although we have also found a higher mortality among patients who developed CMVD (53.8% vs. 37% for those who remained CMVD-free), this difference was not significant when the Kaplan-Meier estimation was used (log-rank = 0.795; $P = 0.372$). Our results appear to confirm that detection of CMV in blood implies a very advanced HIV infection with a short life expectancy: at the 1-year follow-up, survival was 17% in patients with antigenemia ≥ 5 versus 56% in patients with antigenemia <5. However, no differences were found when antigenemia was considered qualitatively.

It is clear from our results that the measurement of pp65 antigenemia constitutes a good marker for the early diagnosis of CMVD in patients with advanced HIV infection. Patients with pp65 antigenemia ≥ 5 are at high risk of developing CMVD; these patients would likely constitute the ideal population for the study of primary prophylaxis of CMVD.

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