

PP65 antigenemia in the diagnosis of cytomegalovirus infection in AIDS patients

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Abstract: Cytomegalovirus causes significant morbidity and mortality in AIDS patients and those having undergone bone marrow or another transplant. PP65 antigenemia is based on detecting viral antigen in peripheral blood leukocytes through immunochemistry and by monitoring the infection in immunocompromised individuals. The present study aimed to set up this diagnostic technique in AIDS patients with active cytomegalovirus infection and verify its occurrence in the Botucatu region of São Paulo state, Brazil. Fifty patients, 35 men and 15 women aged from 24 to 69 years, were recruited from those attended at the Department of Tropical Diseases of Botucatu Medical School, UNESP, and divided into three groups according to CD₄⁺ T lymphocyte counts and antiretroviral treatment. The control group comprised bone marrow transplant patients. Fourteen AIDS patients with low CD₄⁺ cell counts tested positive for PP65 antigenemia, which could predict cytomegalovirus infection and indicate prophylactic treatment.

Key words: acquired immunodeficiency syndrome, cytomegalovirus, pp65 antigen, laboratory diagnosis.

INTRODUCTION

Cytomegalovirus (CMV), the commonest opportunistic viral pathogen found in AIDS and bone marrow and other organ transplant patients, causes significant morbidity and mortality. Studies conducted before and after the introduction of highly active antiretroviral therapy (HAART) have consistently identified CMV viremia as a predictor of mortality, independent of absolute CD4 T-cell count and HIV viral load. The clinical manifestations of CMV disease are related to lesion location and include retinitis, gastrointestinal tract problems, hepatitis, pneumonia, myocarditis, and changes in the central and peripheral nervous systems (1-4). Diagnosis of active CMV infection in immunodepressed patients is hampered by the fact that they are often infected without any

clinical symptoms. Fast detection methods for CMV viremia include: the antigenemia assay for detecting PP65 antigen in polymorphonuclear leukocytes from peripheral blood, polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), and molecular methods such as branched-DNA signal amplification assay (b-DNA) and hybrid capture.

Blank *et al.* (5) demonstrated that PP65 antigenemia assay is sensitive, specific, easy to perform, and does not require special equipment, thereby allowing indirect quantification of CMV in leukocytes from AIDS and bone marrow transplant patients. Early diagnosis of active CMV infection is related to the increased number of PP65 antigenemia-positive cells in leukocytes, which can occur three months before disease development (6-10). Antigenemia is used as a tool to monitor disease caused by CMV in bone

marrow and solid organ transplant patients (11, 12). The objective of this work was to adopt the PP65 antigenemia technique for laboratory diagnosis of active CMV infection in AIDS patients at the Tropical Disease Experimental Laboratory, Botucatu Medical School, São Paulo State of University (UNESP), and evaluate the occurrence of this infection in the Botucatu region of São Paulo state, Brazil.

PATIENTS AND METHODS

Fifty confirmed AIDS patients were recruited, having been attended at the Department of Tropical Diseases, Botucatu Medical School, UNESP, between December 2004 and December 2005. Thirty-five (70%) were male and 15 (30%) female, with a mean age of 41.1 years (varying from 24 to 69 years). A control group was also studied, consisting of 34 bone marrow transplant patients from the Amaral Carvalho Hospital in Jaú, Brazil, of which 21 (61.8%) were male and 13 (38.2%) female. The present work was approved by the Research Ethics Committee of Botucatu Medical School of UNESP.

Inclusion criteria for AIDS patients were adults from either sex, with confirmed AIDS diagnosis, aged 18 years or more, who at the time of selection had undergone CD₄⁺ T lymphocyte determination, and gave their written informed consent to participate in this study. They were divided into the following groups: Group G1: 18 individuals with AIDS, CD₄⁺ T lymphocyte cell count < 100 cells/mm³, without antiretroviral treatment. Group G2: 17 individuals with AIDS, CD₄⁺ T lymphocyte cell count < 100 cells/mm³, having received antiretroviral treatment. Group G3: 15 individuals with AIDS, CD₄⁺ T lymphocyte cell count > 100 cells/mm³, and with and without having received antiretroviral treatment. Group G4: 34 bone marrow transplant individuals independent of the CD4⁺ T lymphocyte cell count.

Suspected CMV infection was defined in AIDS patients as the presence of clinical signs suggestive of adrenal compromise or the presentation of retinitis with the characteristic lesions. CMV infection was considered confirmed in those presenting positive culture or cytomegalic inclusion found in tissue submitted to histopathology.

Laboratory diagnosis of human

immunodeficiency virus (HIV) infection was by enzyme immunoassay (ELISA) in two serum samples from each patient to detect TYPE 1 and 2 HIV antibodies. Flow cytometry was used to count CD₄⁺ T lymphocytes, and results were expressed as the absolute number of cells/mm³. These exams were performed at the UNESP Blood Center in Botucatu.

Antiretroviral treatment was indicated as per the Brazilian Ministry of Health National DST/AIDS program (13).

Antigenemia Assay

This technique was performed as described by van der Bij *et al.* (2), in 1988, with a few modifications. Detecting PP65 antigen requires 4 mL of blood by venopuncture collected with EDTA anticoagulant, with the addition of 2 mL of dextran in a conical plastic tube to separate the supernatant containing polymorphonuclear leukocytes. The pellet obtained was resuspended in phosphate buffer at a final concentration of 3 x 10⁶ per mL. Monoclonal (Clonab CMV[®] pp 65, Bio-Rad, Germany) and conjugate peroxidase were used.

A slide was stained with Mayer's hematoxylin and read under light microscope; the sample was considered positive when it showed at least one cell with the nucleus or perinuclear membrane stained red or brown. The presence of cells with the nucleus stained blue and cytoplasm stained brown was defined as a negative slide. The reaction was considered false positive when any cell presented cytoplasm stained only light brown (endogenous peroxidase). Slides were read by two different observers under a double blinded scheme without either knowing the result of the other's evaluation. Observer one was an experienced biologist who has routinely used this method in the Antigenemia Laboratory at the Amaral Carvalho Hospital. Observer two was a biologist who had been trained by observer one before the start of the study, and who installed the method at the Tropical Diseases Experimental Laboratory.

Statistical Analysis

Frequency comparisons in the same sample were made by the binomial test and comparison of medians in dependent samples by the Wilcoxon test. Frequency comparisons in independent samples were by the χ^2 and Fisher's exact tests.

The statistical analyses permitted a probability of a type I error less than or equal to 0.05 to reject a null hypothesis.

RESULTS

The study groups presented a homogenous gender distribution (Table 1). There was a predominance of males in the study groups without a significant inter-group difference. Among the 50 HIV or AIDS patients studied, only 14 presented PP65 antigenemia, while in the transplanted group none tested positive.

There was significant difference between the patients with HIV or AIDS and those with bone marrow transplants who tested positive for PP65 antigenemia.

Patients in Groups G1 and G2 who presented CD4⁺ T lymphocyte counts below 100 cells/mm³ had a higher number of PP65 antigenemia-negative cases than Group G3. The antigenemia-positive frequency in HIV/AIDS patients did not differ among the three study groups (Table 2).

There was no difference between independent observers for PP65 antigen neutrophil quantification and CD4⁺ T lymphocyte counts below or above 100 cells/mm³, in AIDS patients with or without antiretroviral treatment and in bone marrow transplant patients.

Evaluation of the 14 HIV/AIDS patients with positive PP65 antigenemia revealed that only two presented symptoms of cytomegalovirus (Table 3). The number of PP65 positive neutrophils was less than ten in 13 of these 14 cases. The only patient with confirmed cytomegalovirus

diagnosis by cutaneous lesion histopathology presented a negative PP65 evaluation in an exam performed after treatment with ganciclovir, which is specific for this virus. Six patients with CD4⁺ T cell counts above 100 were found with neutrophils positive for PP65 and the highest number of cells (21 cells) was found in one of these patients. All patients with lymphocyte counts below 100 cells/mm³ presented only 1 to 5 cells positive for PP65.

DISCUSSION

This study analyzed 50 HIV/AIDS patients with and without antiretroviral treatment and with CD4⁺ T lymphocyte counts above and below 100 cells/mm³, the latter situation having a predisposition to many opportunistic infections including more serious ones like cytomegalovirus. Thirty-four bone marrow transplant patients were also analyzed as this also presents a risk of infection by CMV.

AIDS patients with less than 100 CD4⁺ cells per cubic millimeter were included in the study before antiretroviral treatment. G2 patients, who also presented AIDS and less than 100 CD4⁺ cells per cubic millimeter, were on antiretroviral treatment but had revealed treatment failure. The group with and without antiretroviral treatment and more than 100 CD4⁺ cells per cubic millimeter (G3), included patients who were not indicated for treatment as their CD4⁺ cell counts were found to be over 200 per cubic millimeter. Therefore, most patients were in the advanced stage of the disease, without having received antiretrovirals or with treatment failure, with a high possibility

Table 1. Group and gender distributions of 50 HIV infected patients and 34 bone marrow transplant patients

Group	Gender		Total (n %)
	Male (n %)	Female (n %)	
G1	14 77.8	4 22.2	18 100.0
G2	11 64.7	6 35.3	17 100.0
G3	10 66.7	5 33.3	15 100.0
G4	21 61.8	13 38.2	34 100.0
Total	56 66.7	28 33.3	84 100.0

G1: CD4⁺ T lymphocyte cell count < 100 cells/mm³, without antiretroviral treatment; G2: CD4⁺ T lymphocyte cell count < 100 cells/mm³, and with antiretroviral treatment; G3: CD4⁺ T lymphocyte cell count > 100 cells/mm³, and with and without antiretroviral treatment; G4: bone marrow transplant patients.

No statistically significant difference among groups (p > 0.05).

Table 2. Distribution of HIV/AIDS patients according to presence of PP65 antigenemia (n = 50)

Groups	Positive n (27.7%)	Negative n (72.3%)	Total n (100%)
G1	5	13	18
G2	3	14	17
G3	6	9	15
Total	14	36	50

G1: CD4⁺ T lymphocyte cell count < 100 cells/mm³, without antiretroviral treatment; G2: CD4⁺ T lymphocyte cell count < 100 cells/mm³, and with antiretroviral treatment; G3: CD4⁺ T lymphocyte cell count > 100 cells/mm³, and with and without antiretroviral treatment.

Contrasts: Exact Fisher Test G1 = G2 (p > 0.05); (G1 + G2) = G3 (p > 0.05).

Table 3. Distribution of HIV/AIDS patients according to clinical and laboratory characteristics (n = 14)

Patient	Group	CD ₄ ⁺ T lymphocytes (n/mm ³)	Clinical symptoms suggesting cytomegalovirus	PP65 positive cells (n)
1	1	6	No	1
2	1	97	No	3
3	1	10	Yes	3
4	1	58	No	3
5	1	2	Yes	2
6	2	46	No	3
7	2	66	No	3
8	2	11	No	5
9	3	221	No	1
10	3	391	No	4
11	3	146	No	3
12	3	290	No	21
13	3	425	No	3
14	3	107	No	1

of presenting opportunistic infections including cytomegalovirus (14-16).

The number of evaluated leukocytes varies according to the author consulted and must be between 5 x 10⁴ and 2 x 10⁵. We considered 3 x 10⁵ leukocytes to be adequate for this study (8, 17).

Another aspect which varied according to the consulted work is the positivity criterion. In our study, finding just one stained cell was a sufficient definition of positivity, but Landry and Ferguson (18) defined a low antigenemia level as a number of positive cells between one and ten, intermediate

between 11 and 50, and elevated when over 50. They observed that most individuals with a low antigenemia level were asymptomatic or were on CMV-specific treatment. In our study, 14 AIDS patients presented study-positive PP65 antigenemia, however 13 presented with less than 10 positive cells and only one patient exceeded ten. This latter patient revealed a CD₄⁺ T lymphocyte count greater than 200 cells/mm³ and did not present clinical signs suggesting CMV infection.

The importance of testing for pp65 antigenemia in AIDS patients with low CD₄⁺ cell counts is,

according to Dodt *et al.* (19), that a positive exam result can predict infection by CMV well before disease development, which could indicate its prophylaxis. Torrús *et al.* (16) observed that 26.3% of patients with positive antigenemia tests developed the disease and that the time between first positive test and cytomegalovirus diagnosis was 102 days, suggesting that the test is a good early marker for the disease. A similar result was found by Blank *et al.* (20) where positive antigenemia was seen in AIDS patients three months before they developed the disease caused by CMV.

Antigenemia pp65 was performed only once in our study, and not repeated to monitor patients at high risk of developing the disease caused by CMV, as suggested by some cited authors (2, 16).

The *et al.* (21) argued that repeated PP65 antigenemia testing at short intervals, at least weekly, was important in bone marrow transplant patients for diagnosing CMV activity and indicating treatment as early as possible. There is no precise indication of the ideal interval to repeat the test in AIDS patients; however, Francisci *et al.* (22) suggested an interval of 4 to 6 months when negative and three months when positive. They also suggested performing a course of anti-CMV specific treatment, following prophylaxis. Perhaps the antigenemia assay should be performed repeatedly on AIDS patients whose CD₄⁺ T lymphocyte counts remain below 100 cells per mm³, when the risk of CMV infection is higher (22). There is evidence, however, that even in patients whose count remains below this level, but are on a potent antiretroviral treatment program, the incidence of opportunistic infections is low due to the presence of a virological response to treatment, or that control of plasmatic viremia from HIV is kept below the detection limit, or at least below 10,000 copies per mL or log₄0.

The present study found a small number of cells positive for CMV pp65 antigen in all 14 patients, and only two of them presented clinical signs suggesting the infection. According to Landry and Ferguson (18) and Mazzulli *et al.* (23), finding less than ten positive cells generally occurs in asymptomatic patients, which was also seen in our study. The latter authors, who found positive antigenemia in all patients clinically presenting infection by CMV and in those with suspected infection, suggested repeating the exam after 10 days.

Our intention is to use the pp65 antigenemia technique from this study in routine consultations with AIDS patients seen at the AIDS Day Hospital at the Botucatu Medical School, UNESP, to perform early diagnosis and monitoring, with periodically repeated tests for active infection caused by CMV in AIDS patients, the aim being to provide disease prophylaxis or early treatment.

The present work has a few limitations, since antigenemia testing was performed only once for each patient and was not compared with other methods like PCR.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Research Ethics Committee (CEP) of the Botucatu Medical School, UNESP, under the protocol number 234/2002.

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