EVALUATION OF GLYCOPROTEIN B GENOTYPES AND LOAD OF CMV INFECTING BLOOD LEUKOCYTES ON PROGNOSIS OF AIDS PATIENTS

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SUMMARY

Background: Cytomegalovirus (CMV) remains an important pathogen to immunocompromised patients even in the era of HAART. The present study aimed at evaluating the influence of CMV viral load and its gB genotypes on AIDS patients' outcome. **Methods:** Blood samples of 101 AIDS patients were collected and tested for HIV load, CD4 - cell count and opportunistic pathogens, including CMV. Seminested PCRs were run to detect CMV genome and in the positive samples, gB genotyping and CMV load were established using enzymatic restriction and real time PCR, respectively. All patients were clinically followed for four years. **Results:** In thirty patients (31%) CMV was detected and all fatal cases (n = 5) occurred in this group of patients (p = 0.007), but only two patients had CMV disease (1.9%). However, viral load was not statistically associated with any analyzed parameter. The most frequently observed CMV genotype was gB2 (45.16%) followed by gB3 (35.48%). gB2 genotype was more frequently found in patients with CD4-cell counts under 200 cells/mm^3 (p = 0.0017), and almost all fatal cases (80%) had gB2 genotype. **Conclusions:** Our study suggests that CMV and its polymorphisms in biologically relevant genes, such as the gB encoding ORF, may still influence the prognosis and outcome of AIDS patients. The gB2 genotype was associated to patient's bad outcome.

KEY WORDS: CMV, CMV load, HIV, AIDS, CMV genotypes.

INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous herpesvirus that is the causative of asymptomatic or mild symptomatic infections in immunocompetent hosts[25]. Conversely, in immunocompromised patients, such as those with AIDS or in transplant recipients, CMV might produce significant morbidity and mortality[2,17].

CMV glycoprotein B (gB) is a highly immunogenic protein incorporated into the viral envelope, which is believed to exert an essential biological role in virus host interaction, since it participates in the entry, propagation and replication of the virus in different host cells[3,18]. Wild type CMV strains can be classified into four major gB genotypic variants (gB 1-4) based on gB sequence. Each of them have tropism for distinct cell lines, leading to different pathogenesis and severity of disease[6,20,23,27]. Thus, the genotypic characterization of CMV strains infecting immunocompromised individuals can contribute to epidemiological molecular studies and to the definition of the role of viral genetic variability in clinical expression and prognosis. Besides this, CMV infection might reduce patient's immune response, favoring the onset of opportunistic diseases and in some cases, death[24].

Several studies have tried to correlate the infection by different gB genotypes of CMV with clinical prognosis of individuals[4,5,27,28]. Therefore, the aims of the present study were to determine the incidence of CMV infecting blood leukocytes of HIV-infected individuals in a Brazilian care unit, the incidence of gB genotypes and loads of their infective CMV strains, and correlate this information with laboratorial and clinical data obtained during a 4-year follow-up.

PATIENTS AND METHODS

One hundred one AIDS patients at different phases of the disease and followed at the General Hospital of the School of Medicine of Ribeirão Preto/University of São Paulo (HCFMRP/USP) and at the county ambulatories of Jaboticabal and Monte Alto (São Paulo State, Brazil), were invited to participate in this study. This protocol was approved by the Ethics Review Board of HCFMRP-USP (process # 1976/2002).

Blood samples were collected from 2001 to 2002 and all participants attended follow-up visits every three months during four years. The following clinical data were registered during their visits: gender, age, stage of HIV infection, occurrence of opportunistic infections including those caused by CMV, and information on the intake of highly active antiretroviral therapy (HAART). T-CD$_4$ cell counts and HIV loads by

the time of first sampling and in the last clinical visit were included in the study as laboratorial parameters of patient development.

DNA extraction and nested-PCR for detection of CMV genome: For PCR test, the blood samples (5 mL), collected in EDTA-containing tubes, leukocytes were separated with the addition of 1% dextran solution and the DNA of the cells from the buffy coat were extracted by the phenol-chlorphorm method[16]. To amplify CMV DNA we used a nested-PCR technique previously reported by CUNHA et al.[7]. The PCR was performed using CMV primers as follows: gB 1319 (5'-TGGAACTGGAACGTTTGGC3') and gB 1604 (5'GAAACGCGCGGCAATCGG-3') as external primers. In the second round a nested PCR was performed using gB 1319 (5'-TGGAACTGGAACGTTTGGC3') and gB 1676 (5'TGACGCTGGTTTGGTTGAATG3') as external primer. The reaction mixture of the PCR contained in a total volume of 50 μL, 75 mM of Tris HCl (pH 9), 2 mM of $MgCl_2$, 50 mM of KCl, 20 mM of $(NH_4)_2SO_4$, 50 μM of each one of the deoxynucleoside triphosphates, 0.3 μM of primers gB1319 and gB1604, and one μg of DNA obtained from primary blood lymphocytes. The reaction mixture was first incubated at 94 °C for three min. Next, the temperature was reduced to 80 °C, when 2 U of Taq DNA polymerase were added and the mixture was submitted to 15 cycles of 60 sec at 94 °C, 120 sec at 65 °C, and 120 sec at 72 °C, and to 30 cycles of 60 sec at 94 °C, 90 sec at 55 °C, 120 sec at 72 °C, and finally to three min at 72 °C. Two microliters of these reaction products were used in the nested-PCR, included in a reaction mixture similar to that mentioned above, except for the primers gB1319 and gB1604. PCR and nested-PCR products were subjected to electrophoresis in a 2% agarose gel and the amplicon bands were visualized under UV light after ethidium bromide staining. A 100 base pair (bp) marker (Amersham Pharmacia, USA) was used to estimate the size of bands. Amplicons from nested-PCR were further used for genotyping CMV.

CMV load by real time PCR: In DNA extracts of leukocytes in which CMV was detected by nested-PCR, the CMV load was also determined by real time PCR. The reaction was carried out using a 40-cycle TaqMan PCR assay. The reaction mixture, containing primers and probe designed in our laboratory from the region of gB-CMV, included 10 picomols/10 ηl of the forward primer (5'-TGGAATCGGTGCACAATCTG-3') and 10 picomols/10 ηl of the reverse primer (5'-CGCGCAACGTGTCATAGG-3') in a volume of 1 μL, five picomols/10 ηl of a probe (6FAMACGCCCAGCTGCAGTT MGBNFQ) in a volume of one μL, master-mix (2x concentrated) buffer containing TaqMan in a volume of five μL, plasmid or clinical sample DNAs in a volume of one μL of DEPC treated water, in a final volume of 10 μL. The reaction was carried out in a RT-PCR 7500 device (Applied Biosystems, USA), and was monitored from 700 to 770 nm. To avoid false positive results, specimens were processed in parallel with negative-control aliquots of phosphate buffered saline. PCR were run in triplicate and the final CMV load was considered as the mean of the three values.

CMV genotyping: The amplicons of CMV obtained by nested-PCR were submitted to an enzymatic cleavage for gB genotyping. In this case, 10 μL of a mixture containing the amplicons were added to a microtube containing two uL of the proper buffer solution. The enzymes H*inf*I or *R*saI (New England Biolabs, USA) (10 U, from each enzyme) were added to the solution, the volume being 20 μL. After one hour incubation at 37 °C, the digested products were visualized in a 1% agar

gel, after electrophoresis at 100V, for 45 min. This procedure allowed discrimination of fragments with similar weights (up to 10 bp), and thus the identification of the four described gB genotypes[6].

Statistical analysis: The model of logistic regression was used to verify the influence of CMV viral load over T-CD4 counts, HIV viral load, medication intake and incidence of death and opportunistic infections. The unpaired Student´s t-test was used for analysis of parametric data, such as time of diagnosis and age from CMVpositive and negative individuals.

To analyze differences in CMV viral loads and T-CD4 counts among groups of samples with different CMV genotypes, Kruskal-Wallis non-parametric test was used and in case of significance, the post-test of multiple comparisons[10] was performed.

RESULTS

CMV detection by semi-nested-PCR: Analyzing leukocyte samples from 101 AIDS patients CMV genome was detected in 31 (31%). In these patients, CMV was detected at different times during follow-up. Baseline information on patients with positive and negative samples for CMV is shown in Table 1. In the group of patients with CMV positive samples, 30 to 35 year-old females predominated, while in the CMV negative group 35 to 40 year-old males predominated (p 0.05353, for gender and p 0.6, for age). The time since AIDS was diagnosed did not differ for both groups (p 0.141).

Table 1
Characteristics of patients with detected CMV genome and patients with negative blood samples

	CMV negative n = 70	CMV positive n = 31
Gender		
Male	60%	33%
Female	40%	67%
Age (Mean ± SE)	37.64 ± 1.190	38.77 ± 1.797; p = 0.60
Current HIV treatment		
None[1]	20%	23%
3 ARVs	60%	71%
Booster	20%	6%
Use of ARVs[2]		
Regular use	44%	46%
Irregular use	56%	54%
T-CD4 counts, cells/mL (Mean ± SE)	426.1 ± 32.49	290.6 ± 47.15*; p = 0.02
Time (yrs) since HIV diagnosis (Mean ± SE)	5.66 ± 1.93	5.00 ± 1.77; p = 0.141

[1]Patients in this group either abandoned medication, had it suspended for any reason or did not start the medication. [2]Data from this item correspond to what is declared by patients. *Data compared using non-paired t-test, 95% confidence interval.

At the end of the follow-up, most of the patients with detected CMV were also symptomatic for HIV-related infections (53%). Among then, toxoplasmosis was the opportunistic infection most frequently detected (12%). The group of participants without CMV infection of leukocytes was mostly asymptomatic (70%) and tuberculosis was the most frequent opportunistic infection (14%). Moreover, in the majority of patients at C3 AIDS stage CMV was detected in leukocytes (p 0.0064) (Table 2). Also, five of the patients died during this period (Table 3).

During follow-up the CD4-cell counts increased in values for the majority of the enrolled patients, and the HIV loads remained stable throughout the study period; in spite of most participants making irregular use of HAART.

Regarding CMV disease, only two patients in the CMV positive group had diagnosis of encephalitis and esophagitis (6%, p 0.1005, OR 0.08936) by this virus. However, 16% of the patients with CMV detected in the leukocytes died during the follow-up while no deaths occurred among those uninfected by CMV (p 0.0021).

CMV viral load: The real time PCR was able to detect one copy of the plasmid containing part of the gB gene of CMV. Therefore, CMV load of the participants ranged from 100 to 35,000 viral copies/1.5×10^5 leukocytes. Considering as high CMV load values above 200 viral copies/1.5×10^5 leukocytes, two groups of CMV-AIDS patients were created, including those with < 200 and > 200 CMV copies/1.5×10^5 leukocytes. No association was observed for the values of CMV and HIV viral loads (p = 0.6144) and neither for CMV loads and T-CD4 cell counts (p 0.3947) (Table 4). However, three out five fatal cases had > 200 viral copies of CMV/1.5×10^5 leukocytes and also T-CD4 cell counts under 55 cells/mL.

A number higher than 200 viral copies of CMV/1.5×10^5 leukocytes had 60% sensitivity, 34% specificity, 15% predictive positive value and 82% predictive negative value for prediction of bad prognosis of AIDS.

gB genotypes of CMV: All 4 gB genotypes of CMV were observed in the leukocytes of the AIDS patients and frequency distribution of genotypes was 45.16%, in 35.48%, 12.9% and 6% for gB2, gB3, gB4 and gB1, respectively.

Table 5 shows median values of CD4-cell counts and CMV viral loads among samples having different gB genotypes. Although most individuals infected with gB2 genotype had CMV loads higher than 200 viral copies/1.5×10^5 leukocytes, no statistical difference was observed for mean CMV loads (p 0.11). However, most patients infected with gB2 strain had T-CD4 counts below 200 cells/mL (p 0.0017). Also, gB2 genotype was present in leukocytes of 80% (4/5) of the fatal cases. Conversely, the frequency of gB3 genotype was higher among individuals with CMV loads < 200 viral copies/1.5×10^5 leukocytes.

The presence of gB2 genotype of CMV infecting leukocytes as a predictor of bad prognosis for AIDS showed 80% sensitivity, 61.5% specificity, 28% positive predictive value and 94% negative predictive value.

DISCUSSION

This 4-year follow-up work focused on the effects of CMV infection in AIDS patients, analyzing the association of CMV load and gB

Table 2
Characteristics of enrolled patients at the end of the study

	CMV negative n = 70	CMV positive n = 31
Asymptomatic	70%	47%
Opportunistic infections		
Toxoplasmosis	10%	12%
Tuberculosis	14%	9%
Cryptococcus	0%	6%
Pneumocystosis	0%	6%
CMV Encephalitis	0%	3%
Other opportunist infection	10%	9%
Deaths	0%	12%

Table 3
Key findings from patients that came to death during the follow-up

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
CD4-cell count	38	35	53	41	6
CMV viral load	117	16900	758	120	707
HIV viral load	67170	230000	256247	71556	16761
CMV genotype	gB3	gB2	gB2	gB2	gB2
Opportunistic infection		Neurocripto	Pneumocystosis	Cryptococcosis	Neurotoxoplasmosis
Years from AIDS diagnosis	3	5	2	>5	3
Medication intake	NA	Irregular	Irregular	Irregular	Regular
HAART regimen	NA	Biovir+EFV	Biovir+EFV	Biovir+EFV	Biovir+EFV

NA – Not applicable.

Table 4
Frequencies of individuals in two ranges of values for CMV viral loads and different T-CD4 cell counts

	CMV load		p-value
	<200	>200	Odds Ratio
T-CD4 cell counts			0.7284[a]
0-200	4	11	1.173(0.477;2.880)
200-500	3	6	
>500	1	6	0.3947[b]
			1.732(0.489;6.137)
HIV viral load			
<10000 copies	3	5	0.6144
>10000 copies	11	12	1.528(0.294;7.944)

Data in this table is represented by number of patients. Statistical analysis used logistic regression model with 95% confidence interval. (a) p-values and odds ratio for the influence of CMV loads on T-CD4 counts from 0-200 and 200-500. (b) p-values and odds ratio for the influence of CMV loads on T-CD4 counts from 200-500 and >500.

Table 5
Values of CD4-cell counts and CMV viral loads in samples of patients with different gB genotypes

Genotype	Variable	Median	Min value	Max value
gB1	CD4	487	449	525
	CMV	1615	660	2570
gB2	CD4	99.5	6	334
	CMV	1560**	120	32300
gB3	CD4	341	38	1149
	CMV	125	100	3380
gB4	CD4	510	492	548
	CMV	289	100	2400

Data from 31 patients with CMV detected genome by PCR. Analyses were performed using Kruskal-Wallis test for CD4-cell counts (cell/mm^3) and viral loads (viral copies/1.5 x 10^5 leukocytes). Dunn's test was used as post test. ** $p < 0.001$.

genotypes as parameters of disease development and prognosis. This information was obtained by molecular biology techniques such as PCR, which are widely considered as suitable tools. The drawback of this technique is the high sensitivity, which raised concern regarding the diagnosis of the pathogen effectively causative of patient's clinical symptoms. Previously, we have used PCR for CMV genome detection in samples of children and patients with kidney transplant, obtaining specific and reproducible results[1,7,29], although with a highly sensitive method, we could be detecting latent CMV incorporated into leukocytes DNA[19]. Thus, to improve the diagnosis of CMV disease, a real time PCR virus quantification method was used trying to establish a clinically relevant cut-off value for CMV viral load in leukocytes.

Our data point to a high prevalence of CMV (31%) among AIDS patients when compared to that observed by DEYTON et al.[8]. These authors looked for CMV genome by PCR in blood samples of a group of 374 patients during a follow-up study of 37 months. According to these authors, in the initial test, CMV genome was detected in 15.8% of samples (59 patients). Although the rate of positive samples was not stable throughout the above follow-up study, the difference in values of initially positive samples with the present study could be explained by the high prevalence of CMV infection in Brazil, about 95%[7,29].

Considering the baseline population characteristics, our results are similar to those reported by a multicentre study conducted by JABS et al.[15], except for the high prevalence of females in the CMV detected group. Moreover, similar to our data, most patients in this previous report were using HAART. Interestingly, our results demonstrate that a large proportion of patients had referred to an irregular use of HAART during the follow-up period, although there was no increase of their HIV loads, indicating that even used irregularly, HAART may decrease HIV replication and thus allow the rescue of immune function.

The issue of whether CMV remains as a risk factor for patients receiving HAART has been reviewed elsewhere[14,26]. Both works came to the conclusion that HAART reduced the incidence of CMV disease among patients, although CMV remains a risk factor for prognosis of AIDS patients with CD4-cell counts below 100 cells/mm^3. Our results showed that the presence of CMV in blood leukocytes was associated with case fatality and in all death cases patients had CD4 below 55 cell/mm^3, indicating that they were severely immune depressed. Probably, as a consequence, these patients had high CMV loads, which could also contribute to disease progression. None of the fatal cases had symptoms of CMV disease, suggesting that CMV itself was not the direct cause of death of these patients. In this regard, SPENCER et al.[24] suggested that CMV indirectly contributes to immune depression of AIDS patients. These authors related the presence of an interleukine-10-like protein that is expressed during CMV infection. This protein suppresses Th1 immune response, acting synergistically with HIV aggravating immune system depression.

Considering CMV loads, our real time PCR was highly sensitive being able to detect one copy of plasmid per μL, similar to that reported by SANCHEZ & STORCH[22] using the same technique. Therefore, the values for CMV viral loads ranged from 100 to 35,000 viral copies/1.5x10^5 leukocytes with the majority of patients presenting > 200 viral copies/1.5 x 10^5 leukocytes. GOURLAIN et al.[13] reported that six out of 16 patients with CMV loads higher than 100 viral copies/1.5 x 10^5 leukocytes (detected by RT-PCR) developed CMV disease. Plasma samples from these patients had CD4+- cell counts below 75 cells/mm^3 and HIV loads higher than 100,000 viral copies/mL which was also predictive of CMV disease. In our work, CMV viral loads were not correlated with CD4 counts, HIV loads or incidence of death, although high CMV viral loads were observed in one fatal case. Also, our data points to a low incidence of CMV disease, two out of 31 patients with CMV genome detected had CMV disease based on suggestive clinical presentation of CMV encephalitis and esophagitis. In both cases, viral loads were higher than 200 viral copies/1.5 x 10^5 leukocytes.

Considerable attention has been paid to the glycoprotein B from CMV viral envelope and many studies have tried to establish the influence of different gB genotypes (gB1-4) on the patient's outcome [for review see

26]. However, the correlation of gB genotypes and disease progression is still unclear. The distribution frequencies of gB genotypes may vary according to groups of immunocompromised patients. In bone marrow transplant recipients and AIDS patients, gB2 and 3 are more often found, while in congenitally infected babies gB1 is more frequent[3]. Our findings indicate that gB2 genotype was most encountered (45.16%) in samples of AIDS patients with CD4-cell counts below 200 cells/mm^3, although CMV disease was rarely observed. Also, patients with gB2 strains had CMV loads higher than 200 copies/1.5x10^5 leukocytes, but again no association between genotype and viral load was observed. Besides, it was observed in another study that neutralizing an antibody to gB2 human cytomegalovirus does not prevent reactivation in patients with human immunodeficiency virus infection[21].

Interestingly, in our work, 80% (4/5) of patients that died were infected with gB2 CMV genotype, which could be due either to the high prevalence of this genotype or to a real association of gB2 and immune suppression. In a previous study, GILBERT *et al.*[11] observed a similar predominance of gB2 genotype patients with low CD4-cell counts in AIDS patients with and without CMV retinitis, indicating no association between genotype and disease. DREW *et al.*, in 2002, found that half of AIDS patients had gB2 genotype and that this genotype is not a major determinant of retinitis pathogenicity but appears to be highly prevalent among HIV-infected patients[9]. In the Netherlands, differently, however, it was found that CMV gB3 was the most prevalent gB genotype in AIDS patients, but also double or triple infection with other CMV gB strains were common[12]. Nevertheless, the confirmation of the virulence of gB2 genotype observed in the present study must be assessed in a larger casuistic study.

CONCLUSIONS

Cytomegalovirus infection remains as a risk factor for immunocompromised individuals, even in the HAART era. Although the presence of CMV genome was not associated with the incidence of CMV disease, a relevant proportion of patients coinfected with HIV and CMV died (12%), while CMV genome was detected mostly in individuals with CD4-cell counts lower than 100 cells/mm^3. Moreover, our results may raise the issue of whether the presence of CMV gB2 genotype could be a predictor of bad prognosis and decrease of immune response, even in patients using HAART. In the light of these facts, an integration of clinical and virological data may favor the evaluation of prognosis of AIDS patients.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest whatsoever.

RESUMO

Avaliação de genótipos de glicoproteína B e carga de CMV de leucócitos no prognóstico de pacientes de Aids

Antecedentes: O citomegalovírus (CMV) permanece um importante patógeno para pacientes imunocomprometidos, mesmo na era da HAART. O presente estudo teve como objetivo avaliar a influência da carga viral do CMV e seu genótipo gB sobre a evolução de pacientes com AIDS. **Métodos:** Amostras de sangue de 101 pacientes com AIDS foram coletadas e testadas para carga viral de HIV, a contagem de células CD4 e patógenos oportunistas, incluindo o CMV. Um sistema de PCRs seminested foi utilizado para detectar o genoma do CMV e em amostras positivas a carga viral de CMV e genotipagem foram estabelecidos por restrição enzimática e PCR em tempo real, respectivamente. Todos os pacientes foram acompanhados clinicamente durante quatro anos. **Resultados:** Trinta pacientes (31%) tiveram CMV detectado e todos os casos fatais (n = 5) ocorreram em pacientes deste grupo (p = 0,007), porém apenas dois pacientes tinham doença por CMV (1,9%). No entanto, a carga viral não foi associada estatisticamente a nenhum dos parâmetros analisados. O genótipo de CMV mais freqüentemente observado foi gB2 (45,16%), seguido por gB3 (35,48%). O genótipo gB2 foi mais freqüente em pacientes com contagens abaixo de 200 células/mm^3 CD4cell (p = 0,0017), e quase todos os casos fatais (80%) tinham o genótipo gB2. **Conclusão:** Nosso estudo sugere que CMV e seu polimorfismo em genes relevantes biologicamente, como a gB, pode ainda influenciar no prognóstico e evolução de pacientes com AIDS. O genótipo gB2 foi associado ao mau prognóstico do paciente.

ACKNOWLEDGEMENTS

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