Glycoprotein B Genotyping of Human Cytomegalovirus Strains Isolated from Brazilian Patients with Sickle Cell Disease and Beta-Thalassemia Major

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Abstract

The role of the human cytomegalovirus (HCMV) infection in individuals with hemoglobinopathies is unclear. Our objective was to examine the molecular and genotypic characteristics of HCMV in patients with sickle cell disease, beta-thalassemia major, and volunteer blood donors by viral load quantitation, glycoprotein B (gB) genotyping, and phylogenetic analysis. The patients with sickle cell disease demonstrated the highest HCMV DNA prevalence (13.8%), followed by the patients with beta-thalassemia major (7.6%), and the blood donors (3%). The infection was characterized by a low mean viral load (3.8×10^3 copies/mL), but infections with higher copy numbers were also observed. Genotype gB2 was detected in the majority of cases (90.9%), followed by genotype gB1 (9.1%). No gB3/gB4 genotype was detected. No statistical significance was observed between HCMV DNAemia/gB genotype and hematological alterations or severity of the disease. The high number of sickle cell disease patients with HCMV DNAemia could be due to their partial immune dysfunction (multiple transfusions, spleen dysfunction, hydroxyurea treatment). The extensive HCMV gB2 prevalence in patients with hemoglobinopathies is probably due to HCMV epidemiologic characteristics in the examined region, and can be important during the clinical management of these patients.

Introduction

HUMAN CYTOMEGALOVIRUS (Human herpesvirus 5; HCMV), a ubiquitous viral agent, is the prototype member of the *Cytomegalovirus* genus (subfamily *Beta-herpesvirinae*) (13). After initial asymptomatic infection, HCMV remains latent in the immunocompetent host for a lifetime. Clinically important reactivation is observed in patients with immune suppression. In such cases, HCMV replication becomes uncontrollable and leads to high levels of morbidity and mortality (1).

In patients with inherited hemoglobinopathies (sickle cell disease, beta-thalassemia major), continuous therapy with blood derivatives can induce a state of moderate immune suppression, probably by impairment of the T_H -1 branch of the immune response. Thus, clinically important reactivation of HCMV may occur (3). Given this, HCMV impact on patients with hemoglobinopathies is largely unknown, and only sporadic studies have been performed. Patients with beta-thalassemia major, especially splenectomized ones, are

at high risk of transfusion-transmitted HCMV infection (15). Their high anti-HCMV IgG seroprevalence might be responsible for clinical consequences such as immunologic disturbances and susceptibility to other infections (30). High indices of HCMV DNAemia (17.5%) (12) have also been detected in patients with beta-thalassemia, which may lead to hepatitis, lymphadenitis, and upper respiratory tract infections (21). The mortality rates due to HCMV opportunistic disease in patients with beta-thalassemia receiving bone marrow transplants has been estimated as reaching 1.7% (2). Similarly, the HCMV impact on patients with sickle cell disease remains unclear. There are only two descriptions in the literature of HCMV infection in patients with sickle cell disease: one case of fatal pneumonia (18), and a case of fulminant hepatic failure (32).

Hence, the aim of this study was to examine different molecular and clinical characteristics of HCMV infection in patients with hemoglobinopathies (sickle cell disease and beta-thalassemia major) by HCMV viral load quantitation, gB genotyping, and phylogenetic analysis of the detected isolates.

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Materials and Methods

Subjects and specimens

From March 2010 to April 2012, 183 peripheral blood samples were obtained from 144 patients with sickle cell disease and 39 with beta-thalassemia major. One hundred volunteer blood donors were also included in the study as a control group. The demographic and clinical characteristics of the tested groups are shown in Table 1. The donors were seronegative for anti-HIV 1/2 (p24), anti-HCV, anti-HTLV-1/2, anti-*Treponema pallidum*, anti-*Trypanosoma cruzi* IgG, and HBcAg. The clinical records of the patients were revised by hematologist in order to register specific hematological alterations. All tested individuals were attended at the Regional Blood Center of Ribeirão Preto (Ribeirão Preto, Brazil), and they signed a written informed consent. The study (process no. 11741/2009) was approved by the Institutional Ethics Committee of the University Hospital at the School of Medicine of Ribeirão Preto, University of São Paulo.

DNA extraction, HCMV viral load quantitation, and gB genotyping

Four milliliters of total blood was collected in sterile tubes (Vacuette; Greiner Bio-One, Americana-SP, Brazil).

Plasma was separated by low speed centrifugation (1,426 g for 10 min) and was stored at -80°C until use. The buffy coat was separated as previously described (5). Plasma DNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, São Paulo, Brazil) and the buffy coat DNA using the Gentra Puregene Purification Kit (QIAGEN), respectively.

HCMV DNA was quantitated simultaneously in plasma and buffy coat by the use of in-house TaqMan® real-time polymerase chain reaction (PCR) amplifying 67 bp fragment from the UL97 gene. The forward UL97F (5'-ACC GTC TGC GCG AAT GTT A-3'), and reverse UL97R (5'-TCG CAG ATG AGC AGC TTC TC-3') primers, as well as the probe UL97P (5'-FAM-CAC CCT GCT TTC CGA C-3'-Q-MGB), were used in the 25 μL final volume reaction. HCMV quantitation was performed using a serially diluted at eight orders of magnitude (10^7 –0.5 copies/reaction) pCR® 2.1-TOPO vector (Life Technologies, São Paulo, Brazil) containing the 67 bp UL97 insert. For determining the analytical sensitivity of the reaction the probit algorithm was applied (SPSS Statistics for Windows v17; SPSS, Inc., Chicago, IL). The viral load was quantitated in ABI Prism 7500 equipment (Life Technologies) using standard amplification conditions. All samples were run in duplicate, and measures to prevent contamination were adhered to strictly.

The positive samples were genotyped using a semi-nested PCR for the gB (UL55) region. The first round PCR was

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF THE PATIENTS AND THE VOLUNTEER BLOOD DONORS

	Patient and control groups		
	Sickle cell disease	Beta-thalassemia major	Volunteer blood donors
Demographic and clinical characteristics	Number of patients: 144	Number of patients: 39	Number of donors: 100
	Mean age: 19.2 years (range 1–72 years)	Mean age: 23.6 years (range 3–45 years)	Mean age: 35.6 years (range 18–60 years)
	Ethnicity:	Ethnicity:	Ethnicity:
	Caucasians: 55.5%	Caucasians: 97.4%	Caucasians: 85%
	Afro-Brazilians: 44.5%	Afro-Brazilians: 2.6%	Afro-Brazilians: 15%
	Gender:	Gender:	Gender:
	Male: 48.6%	Male: 59%	Male: 69%
	Female: 51.4%	Female: 41%	Female: 31%
	Hemoglobin type of sickle cell disease:	Seroprevalence to infectious diseases:	Seroprevalence to infectious diseases:
	HbSS: 70.1%	Anti-HIV 1/2 IgG/p24: none	Anti-HIV 1/2 IgG/p24: none
	HbSC: 18.7%	Anti-HCV IgG: 25.6%	Anti-HCV IgG: none
	HbS β^+ : 10.3%	Anti- <i>Trypanosoma cruzi</i> IgG: none	HBcAg: none
	HbSD: 0.9%	Hematologic alterations:	Anti- <i>Treponema pallidum</i> IgG: none
	Seroprevalence to infectious diseases:	Thrombocytosis: 25.6%	Anti- <i>Trypanosoma cruzi</i> IgG: none
	Anti-HIV 1/2 IgG/p24: none	Leukocytosis: 20.5%	
	Anti-HCV IgG: none	Lymphocytosis: 15.4%	
	Anti- <i>Trypanosoma cruzi</i> IgG: 0.7%	Monocytosis: 10.3%	
	Hematologic alterations:	Thrombocytopenia: 10.3%	
	Leukocytosis: 38.2%	Leukopenia: 7.7%	
	Monocytosis: 34%	Lymphopenia: 5.1%	
	Thrombocytosis: 27.8%	Monocytopenia: 2.6%	
	Lymphocytosis: 22.2%		
	Thrombocytopenia: 11.8%		
	Neutrophilia: 8.3%		
	Neutropenia: 3.5%		
	Leukopenia: 1.4%		
	Monocytopenia: 1.4%		

performed by the primer pair gB-1319 (5'-TGG AAC TGG AAC GTT TGG C-3') (6) and gB-1676 (5'-TGA CGC TGG TTT GGT TGA ATG-3') (27), and the second one with the same forward primer and the reverse gB-1604 (5'-GAA ACG CGC GGC AAT CGG-3') (6).

Phylogenetic analyses

For phylogenetic analysis, the gB fragment obtained by semi-nested PCR was sequenced using Big Dye® Terminator Cycle Sequencing Kit v3.1 (Life Technologies). One hundred and sixty full and partial sequences corresponding to the examined region were retrieved from the GenBank by March 2014. The sequences were aligned using BioEdit v5.0.6 (Tom Hall, University of North Carolina, Chapel Hill, NC), and the identical ones were excluded by DAMBE software (29). The UL55 gene of the *Macacine herpesvirus 3* (RhUL55, GenBank GU552457) was used as an outgroup. Different approaches for phylogenetic tree reconstruction, including neighbor-joining (NJ) and maximum likelihood (ML), were applied using Phylip v3.69 (14). The final trees were visualized by TreeView v1.6.6 (22), and statistically supported by the bootstrap method (1,000 replicates). The detected HCMV isolates were deposited in the GenBank under the numbers KC990841–KC990855.

Statistical analysis

To compare the categorical variables, chi-square distribution was applied (GraphPad Software, La Jolla, CA). To evaluate the correlation between hematological alterations and HCMV viral load or gB genotype, the nonparametric Wilcoxon two-sample test was applied as implied by SAS v9.2 (SAS Institute, Cary, NC).

Results

We evaluated the prevalence of HCMV DNA in buffy coat/plasma and gB genotypes in patients with sickle cell disease, beta-thalassemia major, and healthy blood donors by a sensitive in-house-developed UL97 TaqMan® real-time PCR, gB sequencing, and phylogenetic analysis. Correlation between hematological parameters and molecular characteristics of HCMV infection was also performed. A summary of the results is shown in Table 2.

HCMV DNA was detected in the buffy coat of 13.9% ($n=20/144$, $p<0.05$) of patients with sickle cell disease. Of them, four also demonstrated HCMV DNA in plasma ($n=4/20$, 20%). Of the positive patients, nine (45%) were multiply

transfused, two (10%) were treated with hydroxyurea (1,000 mg/daily), and nine (45%) received no treatment. The majority of patients with DNAemia had the SS type of sickle cell disease (65%), while 35% had the SC type. Although a significant number of patients with sickle cell disease positive for HCMV DNA ($p<0.05$) had the SS type (the most severe one), no association between HCMV DNAemia prevalence and type of sickle cell disease could be made since this is a widespread sickle cell genotype in Brazil. From 39 patients with beta-thalassemia major, HCMV DNAemia was detected in three (7.7%). No plasma sample was found to be positive. All positive patients with beta-thalassemia major were children of preschool age. They were receiving packed red cell concentrates each month, which were obtained from Brazilian blood donors. From the patients with beta-thalassemia major, 25.6% were anti-HCV IgG seropositive, and no one was reactive for anti-HIV 1/2 IgG. The seroprevalence for anti-HCMV IgG of both patients with sickle cell disease and beta-thalassemia major was not evaluated due to this being almost universal in Brazil (8,31). Three blood donors ($n=3/100$, 3%), were found positive for HCMV DNA in the buffy coat, and in all cases plasma was negative. All donors were clinically asymptomatic and nonreactive serologically to anti-HIV 1/2/p24, anti-HCV, anti-HTLV 1/2, anti-*Treponema pallidum*, anti-*Trypanosoma cruzi* IgG, and HBcAg.

The in-house UL97 real-time PCR was sensitive (6.91 copies/reaction, confidence interval 95%) and with appropriate quantitation curve (slope -3.258 ; y intercept 37.665; R^2 0.997; efficiency 96.124%). The HCMV DNAemia in patients and blood donors was generally characterized by a low mean viral load in the buffy coat (median 3.8×10^3 copies/mL, range 4.7×10^2 – 3.8×10^5 copies/mL). Only four patients with sickle cell disease demonstrated a higher viral load ($>10^4$ copies/mL). In the cases of HCMV DNA plasma detection, the mean viral load was slightly lower (mean 3.1×10^3 copies/mL, 18.4%). In two patients with sickle cell disease, the higher viral load was accompanied by several clinical observations such as proliferative retinopathy, vascular eye affection, and thrombocytopenia (Table 3), but they could not be related solely to the HCMV DNA presence.

The detection of HCMV DNAemia coincided with various hematological alterations in patients with sickle cell disease and beta-thalassemia major as shown in Table 3. The performed statistical analysis, however, showed no significant correlation between hematologic alterations and presence of HCMV DNAemia (interquartile interval, $\times 10^3$, $p=n.s.$). Although some patients with beta-thalassemia major positive for HCMV DNA also demonstrated hematologic alterations (leukocytosis, thrombocytosis), similarly no statistical correlation was observed due to the small number of positive results.

To examine HCMV gB genotypes in Brazilian patients with hemoglobinopathies and blood donors, all PCR-positive samples were sequenced directly (26 isolates). However, four electropherograms were inappropriate for genotyping. Additionally, eight sequences belonging to gB2 and one to gB1 were eliminated by DAMBE software once they were highly identical. As a consequence, 13 sequences were maintained for the reconstruction of the final phylogenetic tree (Table 3). The phylogenetic analysis demonstrated the expected separation of the reference sequences into four clusters corresponding to the four main gB HCMV genotypes

TABLE 2. PREVALENCE OF HCMV DNAEMIA/gB GENOTYPES IN PATIENTS AND VOLUNTEER BLOOD DONORS

Tested group	Prevalence of HCMV DNAemia	HCMV gB prevalence
Sickle cell disease	13.9% ($n=20/144$)	90% gB2, 10% gB1
Beta-thalassemia major	7.7% ($n=3/39$)	100% gB2
Blood donors	3% ($n=3/100$)	100% gB2

gB, glycoprotein B; HCMV, human cytomegalovirus.

TABLE 3. CLINICAL AND VIROLOGICAL MARKERS OF THE POSITIVE PATIENTS/DONORS, WHOSE DETECTED HCMV STRAINS WERE USED FOR THE RECONSTRUCTION OF THE FINAL PHYLOGENETIC TREE

Tested group	Isolate name	Viral load in buffy coat (copies/mL)	Viral load in plasma	gB genotype	Hematological (clinical findings)	Type of sickle cell hemoglobin
Sickle cell disease	AF153Br	7.7×10^3	Negative	gB2	Monocytosis, thrombocytosis	SC
	AF155Br†	4.8×10^2	Negative	gB2	No hematological alterations	SC
	AF167Br‡	4.6×10^4	Negative	gB2	Leukocytosis	SS
	AF172Br	7.1×10^3	Negative	gB2	Leukocytosis, monocytosis	SS
	AF174Br	8.0×10^3	Negative	gB2	Leukocytosis, thrombocytosis	SS
	AF185Br†	3.8×10^2	Negative	gB2	Monocytosis	SS
	AF189Br	4.7×10^2	Negative	gB2	Neutrophilia, leukocytosis, monocytosis	SS
	AF195Br	6.8×10^2	Negative	gB2	Neutropenia	SS
	AF199Br†	1.3×10^3	Negative	gB2	No hematological alterations (<i>Vascular affection of eye</i>)	SS
	AF214Br‡	1.2×10^3	Negative	gB2	No hematological alterations	SC
	AF220Br*	3.7×10^4	1.7×10^3	gB1	Monocytosis	SS
	AF223Br*	3.8×10^5	1.5×10^3	gB2	Leukocytosis, neutrophilia, lymphocytosis, monocytosis	SS
	AF241Br	1.9×10^3	Negative	gB2	Leukocytosis, thrombocytosis, monocytosis	SS
	AF254Br	4.1×10^3	Negative	gB2	Leukocytosis, monocytosis	SS
	AF260Br*	6.9×10^3	Negative	gB1	Lymphocytosis, thrombocytosis, leukocytosis, monocytosis	SS
	AF268Br	1.0×10^3	Negative	gB2	Thrombocytopenia	SC
	AF278Br	6.9×10^4	8×10^3	gB2	Thrombocytopenia (<i>Retinopathy</i>)	SC
	AF280Br†	2.8×10^3	Negative	gB2	Leukocytosis, monocytosis, lymphocytosis	SS
Beta-thalassemia	TL11‡	5.0×10^2	Negative	gB2	Leukocytosis	None
	TL17‡	2.9×10^3	Negative	gB2	No hematological alterations	None
Blood donors	D18Br§	2.4×10^3	Negative	gB2	None	None
	D63Br§	2.1×10^3	Negative	gB2	None	None

Bold indicates representative sequence on the phylogenetic tree.

*Sequences identical for genotype gB1 (represented by the isolate AF260).

†Sequences identical for genotype gB2 (represented by the isolate AF199).

‡Sequences identical for genotype gB2 (represented by the isolate AF214).

§Sequences identical for genotype gB2 (represented by the isolate D18).

(Fig. 1). Two gB genotypes were determined in patients with hemoglobinopathies and volunteer blood donors: gB1 and gB2. Almost exclusively, with the exception of two sequences belonging to gB1 (9.1%), the remaining 20 belonged to genotype gB2 (90.9%). Genotypes gB3 and gB4 were not detected. The cluster comprised of gB2 sequences (reference isolates AD169/U11; Fig. 1) demonstrated higher bootstrap support (99%), and comprised isolates detected in different countries and types of specimens (urine, plasma, saliva). Our gB2 strains did not form one monophyletic cluster but were randomly distributed among published sequences from Brazil, China, South Korea, the United States, and India. In blood donors and in patients with thalassemia, the sequences were classified as gB2 (in a subcluster with strains from patients with sickle cell disease; Table 3). The sequences, identified as genotype gB1, were more divergent compared to other reference gB1 strains, and formed a separate lineage (Fig. 1). The almost complete prevalence of gB2 precluded the performance of statistic analysis correlating HCMV gB genotype to hematological alterations.

Discussion

This is the first study of its kind to examine HCMV gB genotypes in patients with sickle cell disease and beta-

thalassemia major. This survey is pertinent due to the serious HCMV impact on individuals with immune suppression and the new therapeutic approaches to treat sickle cell disease and thalassemia by hematopoietic stem cell transplantation.

In our study, HCMV DNAemia was detected generally at low viral load (mean 3.8×10^3 copies/mL, range 470–380,000 copies/mL) with some exceptions (3.7×10^4 , 4.6×10^4 , 6.9×10^4 , 3.8×10^5 copies/mL). The detected mean viral load was similar compared to that in immunosuppressed patients with solid organ transplantations (liver, kidney, heart) where the values determined were approximately 5×10^3 copies/mL for the primary HCMV infection and 3.6×10^3 copies/mL for the recurrent reactivation (19). HIV-infected patients with a median CD4⁺ number of 29 cells/ μ L also showed similar baseline HCMV loads in blood (3.4×10^3 copies/mL), but untreated patients with a viral load $> 5.1 \times 10^3$ copies/mL demonstrated 100% mortality (20). Therefore, we believe that HCMV load in combination with the level of immune suppression is responsible for the clinical outcome of the infection. Although the detected mean HCMV load in blood of patients with hemoglobinopathies was similar compared to groups with profound immunosuppression (HIV-infected patients, transplant recipients), the preserved cellular immunity in the case of the inherited hematologic disturbances could be responsible for their generally asymptomatic infection course.

FIG. 1. Phylogenetic analysis of the human cytomegalovirus (HCMV) isolates obtained from patients with sickle cell disease and volunteer blood donors. Neighbor joining phylogenetic tree based on the 296 bp partial nucleotide sequence of the HCMV glycoprotein B (gB; UL55) gene derived from 70 HCMV reference sequences, 13 new isolates, and one outgroup strain (*Macacine herpesvirus 1*) inferred by the Phylip v3.69 program. The bootstrap probabilities are expressed in percent and are indicated at important nodes. The sequence of the *Macacine herpesvirus 1* (GenBank CHU14664) was used as an outgroup. The tree, visualized by the TreeView program, was characterized by the classic separation of the reference nucleotide sequences and the examined isolates into four HCMV gB genotypes. The majority of the analyzed isolates belonged to HCMV genotype gB2. Only two sequences were characterized as HCMV genotype gB1. No HCMV genotypes gB3 and 4 were detected. Bar: 0.1 nucleotide substitutions per site per year. (Latin America map source: Archive of the Graphic Design Section, Regional Blood Center of Ribeirão Preto).

Although in patients with sickle cell disease the HCMV DNAemia was accompanied by various hematologic alterations (Table 3) and even some clinical symptoms (retinopathy, vascular eye affection), this could be result of steady-state anemia and impaired splenic function, and cannot be related to the presence of HCMV. This is further confirmed by the statistical analysis performed, which found no significant correlation between HCMV and hematologic alterations. Moreover, no clinical symptoms related to viral infection (fever, malaise, vomiting, prostration) were observed in the positive patients.

There are no studies in the literature examining the HCMV gB genotype frequency in patients with sickle cell disease and beta-thalassemia major. Almost all the detected HCMV strains (90.9%) belonged to genotype gB2, and only 9.1% to genotype gB1. The evaluation of HCMV gB dis-

tribution in various types of samples (or different disease entities) and in different regions of the world is important in order to determine the viral genotype frequencies in specific patient groups and if these frequencies are related to genetic variations/polymorphisms (9). Our results showing increased gB2 prevalence in patients with hemoglobinopathies are similar to the results obtained for HIV-infected patients in North America where gB2 is predominant (10,16,24). This similarity of gB prevalence in HIV-infected patients with patients with sickle cell disease may be due to similar routes of HCMV transmission or co-infection with multiple isolates, where the co-infection is masked by a more dominant isolate. On the contrary, in transplant recipients (or pediatric patients) where HCMV gB3 is more frequent, the infection is represented by reactivation of endogenous HCMV acquired early in childhood (28). However, our

findings could also demonstrate regional gB genotype circulation in this Brazilian state, with predominance of gB2. Since various studies (4,7,31) point out gB2 predominance in Brazil, it is quite possible that the examined region demonstrates extensive spread of this genotype that infects a higher proportion of the population, including patients with hemoglobinopathies. Another important point that cannot be ignored is the limitations of the direct sequencing to distinguish between viral subpopulations, representing only the predominant genotype (in the case of gB2). Such misdiagnosing has been frequently observed for important HIV-1 drug-resistant mutations tracked by direct sequencing (23). We believe that the detection of HCMV gB subpopulations in patients with hemoglobinopathies is pertinent and could be performed in further studies with higher numbers of tested patients.

HCMV DNA was detected in the buffy coat of three volunteer blood donors (3%), and all isolates were characterized as gB2 (Fig. 1). The infected donors did not report any symptoms attributable to viral infection, and this finding is compatible with asymptomatic reactivation (17). Once in Brazil, the anti-HCMV IgG seroprevalence is almost universal (89.4–96.4%) (8,25), the detection of a low-titer HCMV DNAemia in healthy individuals can be considered as a normal finding. The events leading to spontaneous HCMV reactivation in blood donors are unclear. Different factors such as high pollen emissions, stress, alcohol consumption, and oral herpes virus infection have been implicated (11,26) as possible stimulators for such a process. Nevertheless, more detailed studies are necessary to evaluate the frequency, seasonality, and impact of HCMV reactivation/reinfection on the healthy population and especially on blood donors and transfusion of blood derivatives.

Conclusion

In conclusion, the obtained results demonstrate for the first time the frequency of HCMV gB genotypes among patients with sickle cell disease and beta-thalassemia major. The presence of neither HCMV DNAemia nor specific gB genotype could be correlated to a specific hematological alteration or severity of the clinical condition. The obtained data add to the understanding of HCMV infection in patients with sickle cell disease and beta-thalassemia major, and could be useful during their clinical management.

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Author Disclosure Statement

No competing financial interests exist.

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