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ORIGINAL ARTICLE

Laboratory monitoring of epstein-barr virus and cytomegalovirus in patients submitted to allogeneic hematopoietic stem cell transplant

Monitoramento laboratorial para epstein-barr vírus e citomegalovírus em pacientes submetidos ao transplante alogênico de células-tronco hematopoiéticas

Luciana Cristina Fagundes Gequelin[1], Maria C. D. Rossa[1], Irina N. Riediger[2], Luine R. Vidal[3], Ana Perola Drulla Brandão[4], Alexander W. Biondo[5]

[1] MSc - Central Laboratory of the State of Paraná, [2] PhD - Central Laboratory of the State of Paraná, [3] PhD - Virology Laboratory, Hospital de Clínicas - UFPR, [4] MSc student - University of São Paulo - USP, [5] PhD - Department of Cell Biology - UFPR

Central Laboratory of the State of Paraná, São José dos Pinhais - PR, Brazil; Laboratory of Virology, Hospital de Clínicas, Federal University of Paraná, Curitiba, Brazil, Sebastiana Santana Fraga St, 1001, São José dos Pinhais - PR, Brazil. CEP: 80560-500

ABSTRACT

The most common viral infections in transplant recipients are related to the Epstein-Barr virus (EBV) and to the Cytomegalovirus (CMV). EBV is associated with a condition known as post-transplant lymphoproliferative disorder (PTLD). CMV infection is a well-known cause of morbidity and mortality after allogeneic Hematopoietic Stem Cell Transplantation (HSCT). Real-time PCR and CMV antigenemia assay are currently the best tools for monitoring patients after HSCT. A total of 51 patients were monitored in Curitiba city, Southern Brazil, over a period of one year (July 2009 to July 2010). 601 samples were studied by PCR for EBV and CMV antigenemia assay. Overall, fifteen patients (29,4%) had detectable EBV and seventeen patients (33,3%) had detectable CMV in at least one sample. Determining the presence of active infection in immunosuppressed patients is essential in order to improve monitoring and prevent serious complications related to these viruses.

Keywords: Herpesvirus 4, Human; Cytomegalovirus; Hematopoietic Stem Cell Transplantation

RESUMO

As infecções virais mais comuns após transplante estão relacionadas ao Epstein-Barr vírus (EBV) e ao Citomegalovírus (CMV). O EBV está envolvido em uma complicação conhecida como desordem linfoproliferativa pós-tranplante (PTLD). E a infecção por CMV é ainda uma das grandes causas de mortalidade pós-tranplante de células-tronco hematopoiéticas (TCTH). O monitoramento dessas infecções por PCR em tempo real quantitativo (qPCR) para EBV e a antigenemia para CMV são atualmente as melhores ferramentas no manejo clínico dos pacientes imunossuprimidos. Durante o período de julho de 2009 a julho de 2010, um total de 51 pacientes foram monitorados prospectivamente. O qPCR e a antigenemia foram realizados em 601 amostras. Ao todo, 15 pacientes (29,4%) tiveram resultados detectáveis para o EBV em, pelo menos, uma amostra. Já para CMV, foram encontrados 17 pacientes (33,3%) com resultados detectáveis. É fundamental determinar a presença de infecção ativa em pacientes com imunidade comprometida para melhorar o acompanhamento e evitar complicações graves relacionadas a esses vírus.

Palavras-chave: Herpesvirus Humano 4; Citomegalovirus; Transplante de Células-Tronco Hematopoéticas

INTRODUCTION

Epstein-Barr virus (EBV), besides being the cause of infectious mononucleosis, is also associated with Hodgkin's and non-Hodgkin lymphoma, post-transplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma, gastric carcinoma and other epithelial malignancies[1,2,3,4]. It is important to remember that the role of EBV in associated diseases is not yet clearly know[5]. After initial infection, the virus remains latent in B cells, usually without posing risk for immunocompetent individuals. However, in immunocompromised patients, both primary infection and reinfection (or reactivation) may lead to severe complications[6].

PTLD usually occurs during the first postoperative year. Nevertheless, it may also occur later, even 10 years after the procedure[4,7,8]. A multicenter study of 26,901 patients undergoing hematopoietic stem cells transplant (HSCT) suggested a high incidence of PTLD in people older than 50 years who had undergone a second transplant operation. In individuals without risk factors, the incidence was 0.2%, while individuals with one, two or more risk factors showed an incidence of 1.1, 3.6 and 8.1%, respectively[9]. EBV DNA values often rise before the onset of an injury or before symptoms become evident. This justifies the monitoring of patients with multiple risk factors. Moreover, this "early warning" allows for preventive intervention, in order to reverse disease progression[10,11].

It is estimated that about 40 to 100% of the adult population has already had primary CMV (cytomegalovirus) infection[12]. After transplantation, CMV can cause subclinical or asymptomatic infections is can be detected with antigenemia and polymerase chain reaction (PCR). CMV may also cause serious complications, interfering with the functioning of various organs[13,14]. Several studies have shown a relationship between acute and chronic graft versus-host disease (GVHD) and risk for CMV infection[15,16,17,18]. In HSCT, the most common problems associated with CMV are pneumonia and gastrointestinal complications[12,19].

Serological tests should be made only in order to analyze the immune status of patients before transplantation[20]. CMV antigenemia assay remains the established method for diagnosis in patients after HSCT. However, the real-time quantitative PCR test is gaining prominence because it is easy and fast to perform[19].

With the aim of improving the monitoring of transplant patients, the target population chosen for this study was individuals who has undergone related or unrelated allogeneic HSCT. In addition, we assessed whether other factors such as gender, underlying disease, source of progenitor cells, are significant for emergence of EBV and CMV infections.

METHODS

The authors conducted a prospective cohort study with 51 patients: 19 were female and 32 were male, with a mean age of 16 years (range, 1 to 50 years). These patients had undergone allogeneic HSCT at the Bone Marrow Transplant (BMT) Service, Hospital de Clínicas, Federal University of Paraná (UFPR), Curitiba, Brazil, between July 2009 and June 2010. In total, 601 samples were collected (average, 11 samples per patient). The study was approved by the Ethics Committee on Human Research of the Federal University of Parana, Hospital de Clinicas (CAAE 0229.0.208.000-09, CEP. 313.EXT.019/2009-09.

The material was collected in 5-ml tubes containing EDTA anticoagulant (ethylenediaminetetraacetic acid),. Samples were collected after engraftment, during hospitalization and the period while patients remained in Curitiba.

CMV antigenemia assay

Polymorphonuclear leukocytes were isolated from whole blood collected with EDTA. Indirect immunofluorescence was performed using the suite of CMV BriteTM Turbo kit (IQR Products, The Netherlands) according to the manufacturer's instructions. Slides were mounted with glycerol buffer and visualized under epifluorescence microscopy. Only cells with nuclear staining were counted and a semi-quantitative result was expressed as the number of positive cells per 200,000 leukocytes.

EBV DNA extraction

After centrifugation of whole blood, the plasma from 200 µL of material was extracted using the easyMAG® automated platform (Biomerieux, Boxtel, The Netherlands), eluted in 60 µL elution buffer and processed for real-time PCR either on the same day or on the day after.

Real-Time PCR for EBV

An EBNA2 gene fragment was chosen as a target for the technique. The primers and and the probe were designed using the Primer Express® software (Applied Biosystems, Foster, CA USA). Forward primer: 5 'TGC TCT CTA GTT ACA GGG TGG AC 3'. Reverse primer: 5

'TGA CTG GTA TTC GTT YAG RGG ATT 3'. Probe: 5' FAM TGG AAA GTC CCC ACT CT NFQ MGB 3'. The qPCR reaction was carried out in 25µL reaction mixtures consisting of 12.5 µL of Universal Master Mix (Applied Biosystems, Foster, CA, USA), 300 nM of both primers, 200Nm of probe and 5 µL of extracted DNA. Each run had a negative control (water) and a four-point standard curve measured by a commercial calibration curve called OptiQuant® (AcroMetrix, Benicia, CA, USA). The sensitivity of the method was estimated at 88 copies/mL (58-430), and the detection limit was 88 to 4.46 x 10^5 copies/ml.

Real-time PCR equipment

We used the ABI 7500 system (Applied Biosystems, Foster, CA, USA) under standard cycling conditions (1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 45 cycles of 95°C for 15s and 1 cycle of 60°C for 1 min).

Statistical Analysis

Statistical analyses were performed using Fisher's exact test or chi-square test, GraphPad Prism software (version 3.00 for Windows, San Diego, CA, USA).

RESULTS

The most prevalent underlying diseases in the study population were severe aplastic anemia, Fanconi's anemia, acute myeloid leukemia and chronic myeloid leukemia.

532 of the 601 samples analyzed had undetectable EBV DNA. The 69 detectable samples belonged to 15 different patients. 39 samples had positive CMV antigenemia assays and 551 were nonreactive. 11 assays were not performed. With regard to risk factors for complications caused by EBV and CMV infections, the results of the multivariate analysis are shown in Table 1.

Table 1 - *Risk factors for complications caused by EBV and CMV infections*

		EBV			CMV		
RISK FACTOR		Detectable EBV viral load (n=15 patients)	Undetectable EBV viral load (n=36 patients)	p*	Positive CMV antigenemia assay (n=17 patients)	Negative CMV antigenemia assay (n=34 patients)	p*
Gender	Male	10	22	0.8	09	23	0.4
	Female	05	14		08	11	
DISEASES	ALD	01	01	0.5	01	01	1.0
	SAA	02	08	0.7	04	06	0.7
	FA	01	08	0.2	04	05	0.5
	ALL	03	02	0.1	01	04	0.6
	AML	02	06	1.0	02	06	0.7
	CML	02	05	1.0	03	04	0.7
	CHS	01	01	0.5	01	01	1.0
	WAS	03	01	0.07	01	03	1.0
	Other	00	04	-	00	04	-
CMV reactivation		07	09	0.2	-	-	-
EBV reactivation		-	-		05	09	1.0
related HSCT		02	23	0.002	09	16	0.8
unrelated HSCT		13	13		08	18	
Source of progenitor cells	BM	13	27	0.5	13	27	1.0
	UCPB	2	6	1.0	3	05	1.0
	PSC	0	3	-	1	2	1.0

ALD: Adrenoleukodystrophy, SAA: Severe aplastic anemia, FA: Fanconi's anemia, ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, CHS: Chediak-Higashi Syndrome, WAS: Wiskott-Aldrich syndrome, CMV: Cytomegalovirus, HSCT: Hematopoietic stem cell transplantation, BM: bone marrow, UCPB: Umbilical cord and placental blood, PSC: peripheral stem cells. *p: Calculated using Fisher's exact test or chi-square test, with a 95% confidence interval.

DISCUSSION

Viral load assays are currently the best tools for detecting and quantifying EBV DNA, although PTLD confirmation requires biopsy and histological examination[21]. In this study, although some biopsies displayed detectable EBV by qPCR, no case of PLTD was histologically confirmed. One explanation for the absence of this disease in the BMT Service is the fact that, prior to PTLD development, patients are preventively treated with anti-CD20 antibody (rituximab).

15 (29.4%) of the 51 patients assessed had detectable EBV. In their study, Omar et al.[7] reported that 30% of patients had at least one detectable viral load. Aalto et al.[22] found a smaller percentage: 14%, due to the larger number of samples and patients (5479 samples and 406 patients).

With regard to the risk factors for infection with/reactivation of EBV, only the type of transplant was statistically significant in the multivariate analysis ($p < 0.05$). The 13 samples with viral load above 1,000 copies/ml belonged to five different patients. Interestingly, all five patients were recipients of unrelated HSCT, four were under 17 years of age and had negative pretransplant serology for EBV, which confirms that high viral loads are more often found in young patients undergoing unrelated allogeneic HSCT and with a primary EBV infection post-transplant. 56 samples had EBV viral loads below 1000 copies/ml. This shows that post-transplant infections/reactivations may occur, often without any harm to the patient.

17 (33.3%) of the 51 patients who were tested for CMV infection had at least one positive result for antigenemia. Ruell et al.[23] found similar results: 30% of the patients in their study were tested positive for antigenemia at least once. In the study by Han et al.[24], 39% of patients were positive for antigenemia and another study found a higher value: 52.2%[25]. The reported CMV reactivation rates in the literature range from 30 to 70%[26].

Prophylactic and/or preventive antiviral treatment reduces the risk of CMV infection and its complications[27]. It is noteworthy that, in the multivariate analysis, no risk factor was significant for CMV infection.

The major limitation of this study was the sample size. Clearly, a greater sample would enable a more accurate analysis of the incidence of infections/viral reactivations and a more comprehensive assessment of risk factors for CMV and EBV infections.

CONCLUSION

This study concluded that it is important to determine the presence of active infection in patients with impaired immunity. The results found showed a fairly high incidence of EBV and CMV. Post-transplant monitoring helps decision making for clinical treatment and prevents serious complications related to these viral (CMV and EBV) pathogens.

REFERENCES

1. Lima MAP, Ferreira MVP, Barros MAP, Pardini MIMC, Ferrasi AC, Rabenhorst SHB. Epstein-Barr virus-associated gastric carcinoma in Brazil: comparison between in situ hybridization and polymerase chain reaction detection. Braz. J. Microbiol. 2012; 43:1.
2. Cohen JI. Epstein-Barr virus infection. New England Journal of Medicine 2000; 343(7):481-92.
3. Williams H, Crawford DH. Epstein-Barr virus: the impact of scientific advances on clinical practice. Blood 2006; 107(3): 862-869.
4. Gulley ML, Tang W. Using Epstein-Barr viral load assays to diagnose, monitor, and prevent posttransplant lymphoproliferative disorder. Clinical Microbiology Reviews 2010; 23(2):350-366.
5. Gequelin LCF, Riediger IN, Nakatani SM, Bomfim CM, Biondo AW. Epstein-Barr virus: general factors, virus-related diseases and measurement of viral load after transplant. Rev. Bras. Hematol. Hemoter. 2011; 33(5): 383-388.
6. Kullberg-Lindh C, Olofsson S, Brune M, Lindh M. Comparison of serum and whole blood levels of cytomegalovirus and Epstein-Barr virus DNA. Transplant Infectious Disease 2008;10(5):308-15.
7. Omar H, Hagglund H, Gustafsson-Jernberg A, Leblanc K, Mattsson J, Remberger M, et al. Targeted monitoring of patients at high risk of post-transplant lymphoproliferative disease by quantitative Epstein-Barr virus polymerase chain reaction. Transplant Infectious Disease 2009;11(5):393-9.
8. Styczynski J, Einsele H, Gil L, Ljungman P. Outcome of treatment of Epstein-Barr virus-related post-transplant lymphoproliferative disorder in hematopoietic stem cell recipients: a comprehensive review of reported cases. Transplant Infectious Disease 2009;11(5):383-92.
9. Landgren O, Gilbert ES, Rizzo JD, Socie G, Banks PM, Sobocinski KA, et al. Risk factors for lymphoproliferative disorders after allogeneic hematopoietic cell transplantation. Blood 2009; 113(20):4992-5001.

10. Gulley ML, Tang W. Laboratory assays for Epstein-Barr virus-related disease. Journal of Molecular Diagnostics 2008; n. 4, p. 279-92.
11. Catalán P, Alba A. Prophylaxis against Epstein Barr disease in pediatric and adult patients undergoing solid organ and hematopoietic stem cells transplantation. Rev. chil. infectol. 2012; 29:1.
12. Barron MA, Gao D, Springer KL, Patterson JA, Brunvand MW, Mcsweeney PA, et al. Relationship of reconstituted adaptive and innate cytomegalovirus-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2009; 49(12):1777-83.
13. Ljungman P, Griffiths P, Paya C. Definitions of cytomegalovirus infection and disease in transplant recipients. Clin Infect Dis. 2002; 34(8):1094-7.
14. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol Oncol Clin North Am. 2011; 25(1):151-69.
15. Machado CM, Menezes RX, Macedo MC, Mendes AV, Boas LS, Castelli JB, et al. Extended antigenemia surveillance and late cytomegalovirus infection after allogeneic BMT. Bone Marrow Transplant 2001; 28(11):1053-9.
16. Osarogiagbon RU, Defor TE, Weisdorf MA, Erice A, Weisdorf DJ. CMV antigenemia following bone marrow transplantation: risk factors and outcomes. Biol Blood Marrow Transplant 2000; 6(3):280-288.
17. Matthes-Martin S, Aberle SW, Peters C, Holter W, Popow-Kraupp T, Potschger U, et al. CMV-viraemia during allogenic bone marrow transplantation in paediatric patients: association with survival and graft-versus-host disease. Bone Marrow Transplant 1998; 21(2):53-6.
18. Meyers JD, Flournoy N, Thomas ED. Risk factors for cytomegalovirus infection after human marrow transplantation. J Infect Dis. 1986; 153(3):478-88.
19. Kuljic-Kapulica N, Stamatovic D, Savic D, Jovanovic D, Tukic L, Andjelkovic N. Monitoring of cytomegalovirus infection after allogeneic stem cell transplantation. Vojnosanit Pregl. 2010; 67(5):375-8.
20. Castagnola E, Cappelli B, Erba D, Rabagliati A, Lanino E, Dini G. Cytomegalovirus infection after bone marrow transplantation in children. Hum Immunol. 2004; 65(5):416-22.
21. Styczynski J, Reusser P, Einsele H, De La Camara R, Cordonnier C, Ward KN, et al. Management of HSV, VZV and EBV infections in patients with hematological malignancies and after SCT: guidelines from the Second European Conference on Infections in Leukemia. Bone Marrow Transplant 2009; 43(10):757-70.
22. Aalto SM, Juvonen E, Tarkkanen J, Volin L, Haario H, Ruutu T, et al. Epstein-Barr viral load and disease prediction in a large cohort of allogeneic stem cell transplant recipients. Clin Infect Dis. 2007; 45(10):1305-9.
23. Ruell J, Barnes C, Mutton K, Foulkes B, Chang J, Cavet J, et al. Active CMV disease does not always correlate with viral load detection. Bone Marrow Transplantation 2007; 40:55-61.
24. Han XY. Epidemiologic analysis of reactivated cytomegalovirus antigenemia in patients with cancer. J Clin Microbiol. 2007; 45(4):1126-32.
25. Bonon AHA, Rossi CL, Souza CA, Vigorito AC, Costa SCB. Comparison of serology, antigenemia assay and the polymerase chain reaction for monitoring active cytomegalovirus infections in hematopoietic stem cell transplantation. Rev. Inst. Med. trop. S. Paulo 2006; 48:5.
26. Asano-Mori Y, et al. High-grade cytomegalovirus antigenemia after hematopoietic stem cell transplantation. Bone Marrow Transplantation 2005; 36(9): 813-9.
27. Hebart H, AND Einsele H. Clinical Aspects of CMV Infection After Stem Cell Transplantation. Human Immunology 2004; 65:432-436.