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Human cytomegalovirus and Epstein–Barr virus infections occurring early after transplantation are risk factors for antibody-mediated rejection in heart transplant recipients

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Background: Antibody-mediated rejection (AMR) is a serious complication affecting the survival of patients receiving transplantation. Human cytomegalovirus (CMV) and Epstein–Barr virus (EBV) are common viral infections that occur after transplantation, frequently emerging as viral reactivation in donor grafts or transplant recipients. The present study aimed to investigate the association between CMV and EBV infections and early-onset AMR.

Materials and methods: This study was conducted at the Heart Transplantation Center of Padova General Hospital and included a cohort of 47 heart transplant recipients (HTxs), including 24 HTxs diagnosed with AMR and 23 control HTxs with no episodes of AMR. Only early cases of CMV and/or EBV infections (1–90 days after transplantation) were considered. Fisher's exact test and logistic regression analysis were used to statistically analyze the correlation and association between AMR and CMV or EBV infection.

Results: We observed a positive statistical association between CMV and EBV infections (two-sided Fisher's exact test, p = 0.0136) and between EBV infection and AMR (two-sided Fisher's exact test, p = 0.0034). Logistic regression analysis

revealed a direct statistical association between CMV and EBV infections and AMR risk (p = 0.037 and 0.006 and odds ratio = 1.72 and 2.19, respectively). AMR occurrence was associated with increased viral loads of both CMV and EBV early after transplantation.

Discussion: These findings suggest the role of CMV and EBV infections as relevant risk factors for AMR in HTxs for the first time.

KEYWORDS

human cytomegalovirus, Epstein Barr virus, heart transplantation, antibody mediated rejection, viral immunology

Introduction

Heart transplantation is a life-saving surgical procedure that is performed in the final stage of heart disease. Despite tremendous progress in transplant management, several post-transplant conditions, including acute and chronic graft rejection, still represent major complications that affect the functionality of durable and stable grafts (1, 2). In general, T-cell immune responses play a critical role in both acute and chronic rejection; however, antibody-mediated rejection (AMR) is one of the most insidious and abrupt complications possibly arising after transplantation. AMR is one of the most important reasons for the failure of heart transplantation and is associated with a worse prognosis and higher rates of cardiac allograft vasculopathy (CAV), hemodynamic complications, and death (3–11). AMR may develop not only in the early post-transplantation phase but also in the late post-transplant phase and is mediated by the presence of donor-specific anti-HLA antibodies (DSAs) (12–16). DSAs mediate graft tissue damage and rejection by mainly activating the complement system, although a study has also described a complement-independent mechanism (17). AMR ultimately leads to graft and endothelial damage, loss of graft function, and graft rejection (18). Nevertheless, the biological and pathological events that trigger AMR are either not known or only partially defined.

Active cytomegalovirus (CMV) and/or Epstein–Barr virus (EBV) infections are major threats to heart transplant recipients (HTxs) (19–21). Both these infections can occur owing to latent viral reactivation in seropositive donor grafts or transplant recipients. In HTxs, symptomatic CMV and EBV infections can lead to CMV disease, EBV-related post-transplant lymphoproliferative disease, and death (22, 23). CMVs and EBVs are large DNA viruses that modulate host cell gene expression via various piracy and decoy mechanisms (24). Therefore, concomitant EBV and CMV viral replication may synergistically affect the assets and stability of the immune system. Moreover, EBV has a well-defined tropism for B cells, and its genome can persist lifelong after lytic infection. The present study aimed to elucidate the potential role of early-onset active CMV and EBV infections as risk factors for AMR in a case–control cohort comprising 47 HTxs, including 24 HTxs with AMR and 23 controls.

Materials and methods

Patients and clinical definitions

This nested case–control study comprised a cohort of 47 HTxs. The study group included 24 patients diagnosed with AMR, and the control group included 23 patients without serologic/bioptic or clinical evidence of AMR. All 47 HTxs were seropositive for both CMV and EBV before transplantation. Patients were enrolled between June 2010 and June 2015 at the Heart Transplantation Center of Padova General Hospital. All transplant procedures and follow-ups were conducted at the Cardiothoracic Surgery Unit of Padova General Hospital. The Internal Review Board of Padova General Hospital approved all the medical procedures (protocol #NRC AOP0401). Patients were enrolled after obtaining informed consent to participate in the study. The participating patients were provided with a written informed consent form along with a letter to their primary care physician indicating the purpose of the study and collection and handling of patient data. Patients with pre-existing or acquired immunodeficiency were not included in the study. All patients with AMR presented *de novo* DSA. The participants in the control group were selected based on their CMV and EBV serostatus (R+) and similarities in the immunosuppressive regimen (Table 1). The control group was similar to the AMR group because it comprised participants selected within the same transplant center. Both groups were treated with similar standards of care protocols and procedures.

Criteria for AMR diagnosis and treatment

AMR was diagnosed according to the International Society for Heart and Lung Transplantation guidelines (7, 11, 25–31). The patients with AMR who were included in this study were pAMR1i+, pAMR1h+, pAMR2, and pAMR3. Twenty-two patients with asymptomatic AMR cases did not receive any specific treatment, whereas two with symptomatic AMR received plasmapheresis, intravenous immunoglobulin (Ig), and anti-CD20 antibodies.

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TABLE 1 Clinical and therapeutic conditions of the patients.

	AMR group		Control group		p-Value
	Absolute number	%	Absolute number	%	
Number of patients	24		23		
Age (median and range)	58 (4–77)		66 (35–75)		NS
Sex					
Male	20	83	23	100	NS
Female	4	17	0	0	NS
Immunosuppressive regimen					
CNI	21	88	23	100	NS
With MMF	11	52	10	43	NS
With AZA	2	10	3	13	NS
With mTOR inhibitors	5	24	2	9	NS
With steroids	5	24	4	17	NS
Acute rejection score (≥2R)	11.5		11		NS
CAV	6	25	7	30	NS
Active CMV infection	10	42	4	17	NS
Active EBV infection	13	54	2	9	0.001

CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; AZA, azathioprine; mTOR, mammalian target of rapamycin; CAV, cardiac allograft vasculopathy; NS, not significant; AMR, antibody-mediated rejection.

Patients with symptomatic AMR had reduced left ventricular ejection fraction and an abnormal electrocardiographic profile.

Evaluation of CMV and EBV DNAemia and CMV and EBV serology tests

Routine surveillance for viral reactivation or infection comprised weekly determination of CMV and EBV DNAemia during the first 100 days post-transplantation; this surveillance continued if there were clinical indications for infection. CMV and EBV DNAemia were evaluated by performing real-time polymerase chain reaction (PCR) on the Abi Prism 7900 HT system (Applied Biosystems, Carlsbad, CA, USA) using a system developed in-house (32, 33). The serologies of CMV IgG and IgM were assessed using diagnostic-grade IgG and IgM ELISA kits (Enzygnost, Dade Behring, Marburg, Germany). The EBV serology test was performed to evaluate IgG positivity for viral capsid antigen (VCA), Epstein–Barr nuclear antigen (EBNA) (Novagnost, Siemens, Marburg, Germany), and early antigen (EA) (Virion, Siemens, Marburg, Germany).

All transplant recipients received transplant conditioning therapy via the administration of anti-thymocyte globulin (1 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) for 4 days post-transplant. The immunosuppressive maintenance schemes are presented in Table 2. Transplant recipients underwent preemptive treatment for CMV and EBV infections once viral loads reached >5,000 copies/ml of whole blood. Interlaboratory quantitative PCR variability was determined as previously described (34). Preemptive treatment for CMV infection included oral administration of valganciclovir (Valcyte; Roche, Basel, Switzerland) at a standard dose (900 mg/BD) or intravenous administration of ganciclovir (5 mg/BD), corrected according to renal function. EBV infection was treated by administering valaciclovir (3,000 mg/BD); in cases of persistent EBV DNAemia, immunosuppressive treatment was tapered. Preemptive antiviral therapy was considered successful when two sequential CMV or EBV DNAemia test results were negative. No cases of CMV-resistant strains were detected among the transplant recipients.

TABLE 2 Fisher's exact test.

Variable pairs	Test and significance	Correlation
Early EBV/AMR	Two-sided Fisher's exact test, p = 0.0034	Positive
Early EBV/Early CMV	Two-sided Fisher's exact test, p = 0.0136	Positive

The two-sided pairwise correlation included the following variables: AMR, pre-transplant CMV/EBV seropositivity, and early (1–90 days after transplantation) infections. AMR is the dependent variable, whereas CMV or EBV infection is the explanatory variable. Only statistically significant associations are reported.
AMR, antibody-mediated rejection; CMV, human cytomegalovirus; EBV, Epstein–Barr virus.

Statistical analysis

Statistical analysis was performed using Stata 13 software (StataCorp, College Station, TX, USA). The variables considered for statistical analyses were CMV/EBV seropositivity before transplantation and early CMV and/or EBV DNAemia (occurring 1–90 days after transplantation). For Fisher's exact test, all variables were coded as categorical or binary (yes/no). Logistic analysis was performed to investigate the association between viral infection (predictor variables) and AMR (dependent variables). Statistical significance was set at a p-value of <0.05.

Results

The association between CMV and EBV infections and AMR was investigated in 24 HTxs with AMR. The control group comprised 23 adult HTxs without AMR. Table 1 presents the characteristics of the two groups. As seen in Table 1, the prevalence of active EBV infection was significantly higher in the AMR group than in the control group; however, no differences were observed in terms of age, sex, immunosuppression, acute rejection, and CAV. Moreover, neither immunosuppressive reduction nor total lymphocyte count was significantly associated with AMR (data not shown). Comparison of CMV and EBV viral loads (expressed as DNAemia levels) revealed that CMV and EBV infections frequently occurred simultaneously in the AMR group (Figure 1) and preceded and/or were concomitant with AMR events. This positive correlation between CMV and EBV infections in patients with AMR was also confirmed using the two-sided Fisher's exact test (p = 0.0136, Table 2). The two-sided Fisher's exact test also revealed a positive statistical correlation between early-onset EBV infection and AMR occurrence (two-sided Fisher's exact test, p = 0.0034, Table 3).

The association between early CMV or EBV infection and AMR was also investigated using logistic regression analysis, with CMV or EBV as predictors of AMR (dependent variable) (Tables 3, 4). Early-onset EBV and CMV infections were significantly associated with AMR (p = 0.006 and 0.037, with odds ratios of 2.19 and 1.72, respectively). CMV PCR and EBV PCR performed on AMR biopsies were positive only in cases of high viral load in the blood.

Conclusions

Active CMV and EBV infections are considered major risk factors for acute T cell-mediated graft rejection, contributing to the accelerated progression of atherosclerosis and allograft loss (35–37). The basic mechanisms have been widely investigated and involve complex immunomodulatory processes that ultimately lead to loss of graft function (38–41). In particular, both CMV and EBV contain large DNA genomes that produce various decoy molecules that act at both the intracellular and extracellular levels and interfere with several stages and critical points of immune regulation (42–45). In the present study, several statistical approaches were used to explore the association between CMV and EBV infections and AMR risk among HTxs. Fisher's exact test and logistic regression analysis revealed a strong statistically significant association between concomitant CMV and EBV infections and AMR, particularly in the presence of high viral loads. To the best of our knowledge, this strong relationship between EBV and CMV infections and AMR is a novel finding and has not been reported previously. We hypothesize that EBV infection plays a primary role in promoting AMR because EBV productively infects B cells, leading to abnormal B-cell proliferation and aberrant B-cell responses, which may ultimately increase the risk of developing DSAs and AMR in transplant recipients. In this speculative scenario, CMV may be a relevant cofactor because it is well established that CMV infection favors the emergence of other opportunistic infections, including EBV. Furthermore, CMV and EBV may be involved in AMR development by influencing both direct and indirect cellular pathways because AMR biopsies were positive for both CMV and

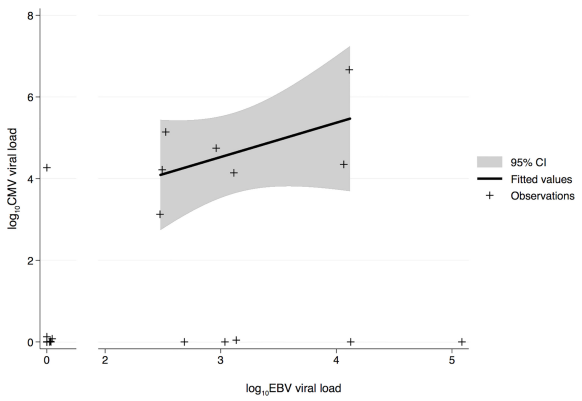

FIGURE 1
Log-transformed quantitative comparison of CMV and EBV viral load during the early post-transplant phase in transplant recipients with AMR. The solid line indicates the fitted values, gray area indicates the confidence interval, and crosses indicate the data points. CMV, human cytomegalovirus; EBV, Epstein–Barr virus; AMR, antibody-mediated rejection.

TABLE 3 Coefficients of the logistic regression model with AMR as the dependent variable and early EBV viremia as the predictor.

AMR	Odds ratio	Standard error	z	p	95% confidence interval	
Early EBV infection (log_{10} viral load)	2.1901	0.6294	2.73	0.006	1.2469	3.8467
Intercept	0.5642	0.2037	−1.58	0.113	0.2780	1.1450

The coefficients are presented in exponential form. EBV is a continuous variable, log_{10} EBV viral load. Log-likelihood = −26.9291. LR chi2(1) = 11.28. Prob chi2 = 0.0008. Pseudo R^2 = 0.1731. AMR, antibody-mediated rejection; EBV, Epstein–Barr virus.

TABLE 4 Coefficients of the logistic regression model with AMR as the dependent variable and early CMV viremia as the predictor.

AMR	Odds ratio	Standard error	z	p	95% confidence interval	
Early CMV infection (log_{10} viral load)	1.7207	0.4484	2.08	0.037	1.0325	2.8678
Intercept	0.7375	0.2593	−0.87	0.386	0.3703	1.4690

The coefficients are in exponential form. CMV is a continuous variable, log_{10} CMV viral load. Log-likelihood = −25.5624. LR chi2(1) = 7.00. Prob chi2 = 0.0081. Pseudo R^2 = 0.1205. AMR, antibody-mediated rejection; CMV, human cytomegalovirus.

EBV when blood viral loads were high. In the future, it would be interesting to assess whether EBV- and CMV-specific cell-mediated immunity (CMI) plays a role in preventing AMR because, in several transplant settings, virus-specific CMI plays an essential role in controlling viral replication (46–50). Overall, this is the first study to report a statistically significant correlation between CMV/EBV infection and AMR in HTxs.

This study has some limitations. It was a single-center study. Further, a small number of patients were enrolled in the study, and the primary focus was on patients with early post-transplant AMR. Nevertheless, AMR represents a rare event after heart transplantation. Moreover, other infectious agents may contribute to AMR onset; in the patients enrolled in this study, no clinical signs of herpes simplex virus 1/2 or varicella-zoster virus infection were observed. However, investigating the presence of other viral and non-viral infectious agents may provide critical insights into the microbial contribution to AMR initiation. Without a doubt, this finding needs to be further investigated in larger multicenter studies with more patients. We envision that if further studies confirm the association between early and overt EBV and CMV replication and AMR, strategies to prevent CMV and EBV infections using early post-transplant antiviral therapies may help reduce the incidence of AMR and improve the successful outcomes of heart transplantation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by NRC AOP0401. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DA, AS, GF, and CM collected and analyzed the data and wrote the manuscript. DS, GG, AG, and LB supervised the study. CM performed statistical analyses. MF, GF, and AA analyzed the AMR data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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