Time Course and Frequency of Epstein-Barr Virus Reactivation after Kidney Transplantation: Linkage to Renal Allograft Rejection

Wolfram J. Jabs,[1] Susanne Maurmann,[1] Hans-J. Wagner,[2] Michael Müller-Steinhardt,[3] Jürgen Steinhoff,[1] and Lutz Fricke[1]

[1]Department of Medicine I, Division of Nephrology, [2]Department of Pediatrics, and [3]Institute of Immunology and Transfusion Medicine, University of Lübeck School of Medicine, Lübeck, Germany

The onset and frequency of Epstein-Barr virus (EBV) reactivation after kidney transplantation are unknown. By use of quantitative real-time polymerase chain reaction measurements, evidence of early EBV reactivation, occurring within the first week after the initiation of immunosuppressive therapy (median, 3 days), was observed in 13 of 23 patients, of whom 10 subsequently developed rejection episodes after 2–45 days (median, 5 days). By contrast, rejection was only diagnosed in 1 of 10 patients who did not show signs of viral reactivation. We suggest that EBV reactivation may induce a T cell response that, through the phenomenon of allo–cross-reactivity, could play a critical role in graft rejection.

Reactivation of the Epstein-Barr virus (EBV) periodically occurs in mucosa-associated oropharyngeal lymphoid tissues [1]. A diagnosis of EBV reactivation is usually made retrospectively by means of serologic testing. However, viral DNA quantitation by real-time polymerase chain reaction (PCR) has enabled a more precise definition of the actual reactivation event through the detection of plasma DNA (EB viremia) or significant increases in peripheral B cell viral load. In a recent study, we demonstrated an association between these new virologic parameters and serologic responses that are suggestive of reactivation occurring in healthy subjects [2]. Therefore, we became interested in the clinical setting of kidney transplantation, because the results of serologic studies supported the idea that EBV reactivation was associated with increased rejection rates [3], and a recent study suggested that the detection of EBV early antigen (EA) mRNA transcripts in peripheral blood B cells of long-term transplant recipients was associated with late allograft rejection [4]. However, neither study could reveal whether changes in these parameters of viral infection were a cause or consequence of rejection.

We therefore performed a longitudinal study on patients undergoing allogeneic kidney transplantation, to investigate the onset and frequency of virologically defined EBV reactivation after the initiation of immunosuppressive therapy. We correlated our findings to acute rejection episodes within the first 2 months after transplantation.

Materials and methods. Twenty-three patients undergoing allogeneic kidney transplantation were enrolled in the study. Patients were included irrespective of cytomegalovirus (CMV) donor/recipient status, history of previous transplants, numbers of major histocompatibility complex (MHC) mismatches, or initial immunosuppression. Patients' immunosuppressive regimens were assigned according to the immunologic risk of acute rejection. Depending on the CMV donor/recipient status, patients either received no antiviral medication or were treated orally with 400 mg of acyclovir twice a day or ganciclovir adjusted to renal function—500 mg every second day (patients with anuria) to 1000 mg 3 times/day for patients with a creatinine clearance >70 mL/min. Antiviral prophylaxis was assigned randomly as part of an open prospective study for the usage of acyclovir versus ganciclovir for the prevention of CMV reactivation and was discontinued 3 months after successful transplantation. Clinical details for all patients are given in table 1.

Rejection episodes were assumed in grafts with delayed function lasting for >4 days, with an unsatisfactory decrease of serum creatinine at the time of initial graft function, an increased vascular resistance index by means of Doppler ultrasound, or in functioning grafts with a significant increase in serum creatinine or a decrease in urinary output. For all grafts, other sources of graft deterioration, such as postrenal failure or dehydration, were excluded before biopsy or treatment. The diagnosis of rejection was confirmed histologically, except in patient 4, and was graded according to the Cooperative Clinical Trials in Transplantation classification [5]. Steroid pulse therapy was started before histologic confirmation in patients 2, 8, and 9. Histologic diagnosis of rejection and grading was done by an independent pathologist who was blinded to the EBV data. All patients gave informed

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Reprints or correspondence: Dr. Wolfram J. Jabs, Dept. of Medicine I, Div. of Nephrology, University of Lübeck School of Medicine, Ratzeburger Allee 160, 23538 Lübeck, Germany (jabs@innere1.uni-luebeck.de).

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Table 1. Patients' characteristics.

Patient	Age, years (sex)	No. of previous Tx	MHC mismatches[a]	Initial immunosuppression[b]	CMV status, donor/recipient	Antiviral drug[c]	Early EBV reactivation[d]		Rejection,[e] day after Tx
							First detection of plasma viremia, day after Tx	First increase in PBMC viral load, day after Tx	
1	32 (M)	…	2-2-2	CyA, MMF, MP	−/−	…	5	…	9
2	44 (F)	…	2-1-0	CyA, Aza, MP	−/+	Acy[f]	6	7	8
3	68 (F)	…	1-2-1	Bas, MMF, MP	−/+	Gan	0[g]	4	45
4	74 (M)	…	0-0-0	Bas, MMF, MP	−/+	…	…	3	5
5	36 (F)	1	1-2-0	FK, Aza, MP	+/+	Acy	3	…	13
6	68 (F)	…	0-0-2	Bas, MMF, MP	+/+	Gan	1	124	19
7	50 (M)	1	2-1-1	Bas, CyA, MMF, MP	+/−	…	0	4	5
8	33 (F)	1	1-1-1	Bas, CyA, MMF, MP	+/−	Gan	1	4	5
9	46 (M)	…	2-1-1	CyA, Aza, MP	−/−	…	4	12	21
10	62 (M)	2	1-0-1	FK, Aza, MP	+/+	Acy	…	5	8
11	53 (F)	…	1-1-1	CyA, Aza, MP	+/+	Acy	2	12	…
12	62 (M)	…	0-0-0	CyA, Aza, MP	+/+	Acy	3	…	…
13	59 (F)	…	0-1-1	CyA, Aza, MP	+/−	Acy	10	2	…
14	49 (M)	…	0-0-0	CyA, Aza, MP	−/+	Gan	…	…	10
15	67 (M)	…	1-2-0	CyA, MMF, MP	−/−	…	…	…	…
16	64 (M)	…	1-1-0	CyA, Aza, MP	−/+	Acy	…	…	…
17	26 (F)	…	1-2-0	CyA, Aza, MP	−/+	Acy	…	…	…
18	38 (F)	1	0-0-0	FK, Aza, MP	−/+	Acy	…	…	…
19	59 (M)	…	0-0-0	CyA, Aza, MP	+/+	Acy	…	…	…
20	58 (M)	2	1-1-1	ATG, Aza, MP[h]	+/+	Gan	…	…	…
21	45 (M)	…	0-0-0	CyA, Aza, MP	+/+	Acy	…	…	…
22	71 (F)	…	0-0-0	CyA, Aza, MP	+/−	Gan	…	…	…
23	52 (M)	…	0-0-0	CyA, Aza, MP	−/−	Acy	…	…	…

NOTE. Acy, acyclovir; ATG, antithymocyte globulin; Aza, azathioprine; Bas, basiliximab; CMV, cytomegalovirus; CyA, cyclosporine A; EBV, Epstein-Barr virus; F, female; FK, tacrolimus; Gan, ganciclovir; M, male; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; MP, methylprednisolone; PBMC, peripheral blood mononuclear cell; Tx, transplantation.

[a] MHC mismatches for class I, A and B locus, and class II, DR locus, between donor and recipient.

[b] Initial immunosuppressive medication was aimed to reach CyA trough levels of 180–250 ng/mL and FK trough levels of 10–15 ng/mL within the first 2 weeks. The MMF dose was 2 g/day maximum; Aza was administered at 2 mg/kg body weight. Bas was administered on day 0 and day 4, 20 mg each. ATG induction was administered perioperatively as a single dose of 2 mg/kg body weight. Therapy was subject to change on rejection episodes.

[c] Antiviral medication was administered randomly, depending of the degree of immunosuppression and the CMV status of the donor/recipient combination.

[d] Definition of early EBV reactivation is given in the Materials and Methods section.

[e] Days indicate first symptoms suspicious of rejection resulting in probatory steroid pulse therapy and/or biopsy. Rejection was confirmed histologically except in patient 4.

[f] Withdrawn on day 6.

[g] Day of Tx, before the initiation of immunosuppressive therapy.

[h] CyA was added on day 10 after Tx.

consent for providing additional blood samples. The study was approved by the local ethics committee.

By means of multiplex quantitative real-time PCR (ABI Prism 7700 Sequence Detection System; Applied Biosystems), patients were monitored for EBV plasma viremia (investigating DNA extracts from 2 mL of plasma), viral load in peripheral blood mononuclear cells (PBMCs), and mRNA transcripts of the immediate EA ZEBRA in PBMCs exactly as described elsewhere [2, 6]. All investigations included 3 no-template controls (water blanks), which were consistently negative for EBV-specific DNA. Furthermore, the risk of contamination of patients' samples was minimized by use of (1) the TaqMan PCR system, which has no need for any post-PCR handling; (2) DNA preparations of the Burkitt lymphoma cell line Namalwa for quantitative PCR standardization, avoiding high-copy preparations of EBV DNA–containing plasmids; and (3) preparation of blood samples in a separate room. Samples for the determination of virologic parameters were obtained before the first administration of any immunosuppressive drug and thereafter every second to third day until the time that the patient was discharged. Likewise, EBV antibody titers were measured before transplantation, after 1 week, and at the date of discharge by use of quantitative ELISA (Euroimmun). Materials for virologic examination were stored at −80°C until all patients had finished the study. Virologic parameters were analyzed thereafter to minimize assay-to-assay variations and to ensure an accurate quantification of viral loads. Early EBV reactivation was only diagnosed before any change in immunosuppression with (1) detection of plasma viremia, (2) conversion to detectable viral load in PBMCs, or (3) significant increase of viral load beyond

the upper 3-fold SD of individual viral load, if EBV DNA was detectable throughout in PBMCs [2]. The serologic criteria of EBV reactivation were as described elsewhere [3].

CMV serologic testing (for IgG and IgM antibodies) and the determination of pp67 mRNA and CMV DNA by PCR were performed for each patient weekly by the local Institute of Medical Microbiology. CMV infection or reactivation was considered to be present with the detection of pp67 mRNA in peripheral blood leukocytes or of CMV DNA in plasma samples.

The 2 groups of patients with and without early EBV reactivation after transplantation were compared for significant differences by means of the Mann-Whitney *U* test (age) or Pearson's χ^2 test (sex and antiviral medication). The 2 groups of patients with rejection versus nonrejection were compared by Pearson's χ^2 test (EBV reactivation and history of previous transplants) and by Student's *t* test (number of MHC mismatches) after the demonstration of normal distribution by the Kolmogorov-Smirnov test.

Results. According to the results of the virologic assays, we observed EBV reactivation in 13 of 23 patients during follow-up (table 1, top)—each reactivation occurred within the first week after transplantation (median, 3 days). Plasma viremia preceded increases in PBMC viral load in 7 patients. However, in 3 patients (1, 5, and 12), an episode of viremia and, in 2 patients (4 and 10), an increase in viral load, respectively, was the only signs of virus reactivation. Patients with or without reactivation showed no significant differences in terms of age (median ± SD, 51.5 ± 14 vs. 57 ± 13 years), sex, and the use of any antiviral drug. All patients who received basiliximab and all but 1 patient who received mycophenolat mofetil had reactivation of virus, although these patients also had an increased immunologic risk of rejection (due to omission of calcineurin inhibitors in recipients >65 years old, retransplantation, high levels of preformed cytotoxic antibodies, or living unrelated donation; table 1). However, no definite statement could be made as to whether any particular immunosuppressive medication correlated with the incidence of EBV reactivation because of the small study population.

Serologic testing did not help in the diagnosis of early EBV reactivation—significant increases in anti-EA titers and/or decreases in anti-EBNA1 titers were observed in 4 of 13 individuals who had virologic evidence of replication but also in 3 of 10 donors without viremia or changes in viral load. All patients had detectable anti–EBNA1 IgG titers before transplantation, indicating past virus infection. No patient developed symptomatic or asymptomatic CMV infection or reactivation that was temporally related to EBV reactivation during the first 4 weeks after transplantation.

In contrast to the results of Babel et al. [4], we were not able to demonstrate lytic mRNA transcripts during the course of EBV reactivation, neither in healthy [2] nor in the majority of

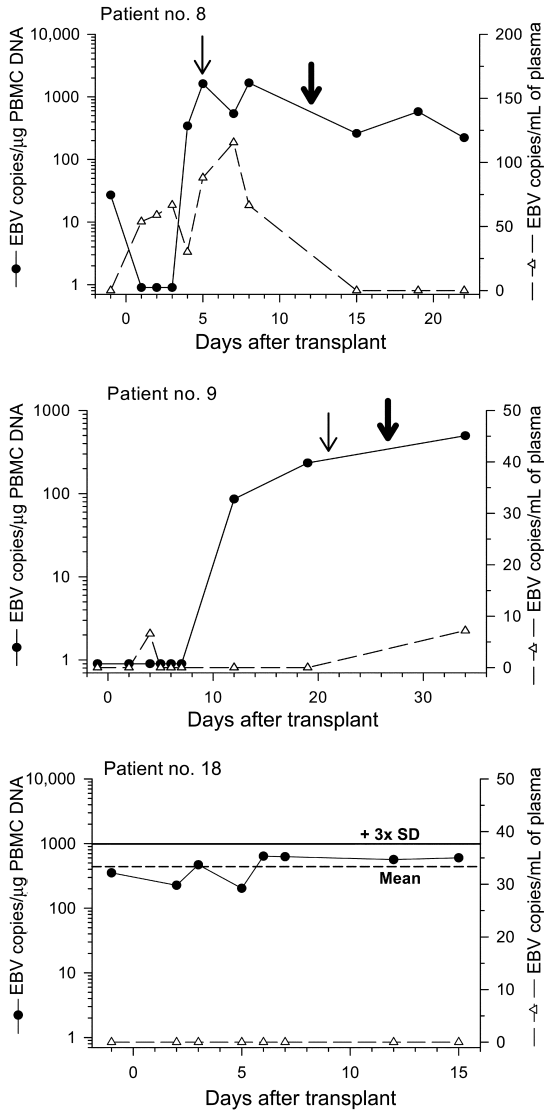

Figure 1. Assumption of rejection because of delayed graft function for patient 8 on day 5 (↓), when ultrasound revealed a swollen allograft and increased vascular resistance indices (RIs). Steroid pulse therapy was administered, which resulted in the prompt onset of diuresis. Because kidney function did not improve further, an allograft biopsy sample was taken on day 12 (↓) that confirmed the diagnosis of advanced tubulointerstitial rejection. The patient was switched to tacrolimus, which ultimately led to good graft function. Patient 9, who had initial graft function, had to provide a biopsy sample on day 22, because diuresis decreased, and empirically administered steroid therapy 1 day previously (↓) had had no effect on graft function. Histological results showed a tubulointerstitial rejection, which was treated with antithymocyte globulin. However, vascular RI dramatically increased, and a second biopsy confirmed severe arteritis on day 27 (↓). Treatment with OKT3 antibody could not avert graft loss caused by complete arterial thrombosis. Patient 18 had initial graft function with no clinical or ultrasound-based signs of rejection later on. She was discharged on day 13 after transplantation and, during 1 year of follow-up, was not readmitted or treated for assumed rejection. Mean and upper 3-fold SD for this patient's viral load in peripheral blood mononuclear cells (PBMCs) are given by dashed and solid lines.

immunosuppressed individuals. Only 3 patients in the present study (3, 5, and 9) became positive for ZEBRA mRNA and then only at their last follow-up visit. RNA detection of the immediate EA ZEBRA of EBV's lytic cycle, thus, is not sufficient for a valid diagnosis of EBV reactivation in the PBMCs of immunocompromised patients. However, more-sensitive assays perhaps applied to purified B cell preparations and that investigated different EAs may improve the diagnostic value of lytic mRNA detection [4].

We observed an intriguingly strong association between early EBV reactivation and acute rejection episodes ($\chi^2 = 9.00$; $P < .005$). Thus, 10 of 13 patients with virologic evidence of reactivation had graft rejection, but only 1 of 10 patients lacking early EBV reactivation (table 1) had graft rejection. Furthermore, in all 10 patients who had evidence both of reactivation and rejection, reactivation occurred before the diagnosis of rejection (median time difference, 5 days). Figure 1 (upper panels) shows data for 2 patients (8 and 9) who had characteristic time courses of early plasma viremia (1 and 4 days after transplantation, respectively), followed by increases in viral load in PBMCs (on days 4 and 11, respectively) and later by rejection. The lower panel gives representative results from a patient without rejection (18)—note that this patient had a significant viral load in PBMCs, but this remained stable throughout, and viremia remained undetectable. Only patient 14 had a rejection episode that was not preceded by reactivated EBV infection. It is of interest that this patient had EBV reactivation later on treatment with antithymocyte globulin for rejection. The 2 groups of rejection versus nonrejection showed a significant difference regarding the mean number of MHC mismatches (2.8 mismatches for patients with rejection and 1.3 for patients without rejection; $P < .05$). Regarding patients with a history of previous transplants (4 vs. 2 patients), no statistically significant difference between rejectors and nonrejectors was observed, because the number of patients was too small.

Discussion. The present study describes the time course and frequency of EBV reactivation after the initiation of immunosuppressive therapy in kidney transplant recipients. As opposed to CMV reactivation, which is unlikely to occur before week 4 after transplantation [7], EBV reactivation in our study population was observed immediately after transplantation and with an unexpectedly high frequency. A recent article [8] showed an incidence of EBV reactivation after allogeneic stem cell transplantation comparable to our data, although EBV reactivations in that study occurred remarkably later (median, ~2 months posttransplantation). This difference in detection of viral replication might be explained by the 2-week, rather than 2-day, intervals for monitoring, as well as by differences in immunosuppression regimen and the definition of EBV reactivation (plasma viremia alone). Indeed, the definition of viral reactivation remains the most important difference between the 2 studies. As we have already discussed elsewhere [2], we suggest that replicative EBV infection—apart from EB viremia—is associated with the infection of new B lymphocytes in the oropharyngeal tissue. Particularly during immunosuppressive therapy, this may result in prolonged EBV-driven B cell proliferation accounting for the remarkable increases in EBV load at the time of viral reactivation [1]. Therefore, our data may indicate that reactivation of EBV's replicative cycle is a common but asymptomatic event, which, in the presence of profound T cell suppression, is more likely to be detected than in healthy immunocompetent carriers.

Furthermore, we observed a novel link between early EBV reactivation after transplantation, as detected by viral DNA quantitation in PBMCs or plasma, and subsequent renal allograft rejection. Although the association of CMV infection with rejection episodes is well documented in the literature [3, 7, 9], a possible role for EBV in the context of early rejection events has not been systematically studied until now. As to the mechanism of this effect, it is clear that the EBV-induced cytotoxic T lymphocyte response contains clones that are reactive to self-MHC/peptide complexes that show strong allo–cross-reactivity against allo-MHC–presented peptides [10, 11]. Burrows et al. [12] convincingly argued that the alloreactive repertoire of an individual is dramatically influenced by past viral infections, such as herpesviruses, that elicit a strong memory T cell response. Indeed, the phenomenon of allo–cross-reactivity induced by viral infections was described in 1979 and confirmed later by Nahill and Welsh [13, 14]. Furthermore, Adams et al. [15] suggested recently that virally induced alloreactive memory provides a potent barrier to tolerance induction, as a certain threshold of alloreactive memory $CD8^+$ T cells is needed to promote rejection. Therefore, it is possible that EBV reactivation occurring during immunosuppression increases antigen load and induces the clonal expansion of EBV-specific T cell memory either to lytic or latent cycle antigens of the virus and that allo–cross-reactive components within this expanding response exceed the threshold needed for the promotion of rejection. To confirm this theory, future studies are warranted that involve quantitative determinations of EBV–allo–cross-reactive T cells together with viral load measurements in transplant recipients with MHC mismatches predisposing for EBV-induced allo–cross-reactivity.

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