4. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation.

Joost W.J. van Esser[1], Hubert G.M. Niesters[2], Steven F.T. Thijsen[3], Ellen Meijer[4], Albert D.M.E. Osterhaus[2], Katja C. Wolthers[2], Charles A. B. Boucher[3], Jan Willem Gratama[5], Leo M. Budel[6], Bronno van der Holt[7], Anton M. van Loon[3], Bob Löwenberg[1], Leo F. Verdonck[4], Jan J. Cornelissen[1].

Departments of Hematology[1], Virology[2], Medical and Tumorimmunology[5], Pathology[6] and Statistics[7], University Hospital Rotterdam / Daniel den Hoed Cancer Center, The Netherlands. Departments of Virology[3] and Hematology[4], University Medical Center Utrecht, The Netherlands.

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Abstract

Epstein-Barr virus lymphoproliferative disease (EBV-LPD) following allogeneic hematopoietic stem cell transplantation (allo-SCT) has a poor prognosis. We used a sensitive real-time polymerase chain reaction (PCR) assay for quantitative detection of EBV-DNA in plasma and serially measured EBV-DNA levels to assess the response to treatment in allogeneic hematopoietic stem cell transplantation recipients with EBV-LPD. Fourteen allogeneic hematopoietic stem cell transplantation recipients with EBV-LPD who had received a T-cell depleted (TCD) sibling (n=5) or matched unrelated donor (MUD, n=9) graft were monitored from the time of EBV-LPD diagnosis, during therapy and assessment of clinical response. Seven patients had complete responses of EBV-LPD to therapy of whom 21% (3 out of 14) survived beyond 6 months from EBV-LPD diagnosis. Clinically responding patients showed a rapid decline of EBV-DNA plasma levels within 72 hours (h) from the start of therapy. In contrast, all clinical non-responders showed an increase of EBV-DNA levels. Absolute EBV-DNA levels at the time of EBV-LPD diagnosis did not predict for response, but the pattern of EBV-DNA levels within 72 hours from the start of therapy (> 50% decrease versus increase) strongly predicted for clinical response (P = 0.001). In addition, lymphopenia ($\leq 0.5 \times 10^9/l$) at the time of EBV-LPD diagnosis was associated with non-responsiveness (P = 0.03) and poor outcome (P = 0.01). Quantitative monitoring of EBV-DNA levels from the start of and during therapy for EBV-LPD rapidly and accurately predicts for response to therapy as early as within 72 hours. It may thus provide a powerful tool to adjust and select treatment in individuals with EBV-LPD following allogeneic hematopoietic stem cell transplantation.

1. Introduction

Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD) is a serious complication of allogeneic stem cell transplantation (allo-SCT) and solid organ transplantation.[1,2] Although the incidence of EBV-LPD is generally less than 2% following allogeneic hematopoietic stem cell transplantation, it may increase up to 20% in patients with established risk factors, which include unrelated donor stem cell transplantation, the use of T-cell depleted allografts, use of anti-thymocyte globulin (ATG) and immunosuppression for prevention and treatment of graft-versus-host-disease (GVHD). [3] EBV-LPD is associated with a poor prognosis despite the recent introduction of new treatment modalities such as anti-B- lymphocyte monoclonal antibody therapy and donor lymphocyte infusion (DLI). [4-10] Most patients receive a combination of treatment modalities because the rapid and aggressive evolution of EBV-LPD does not allow for careful tailoring of therapy. Currently, accurate markers for monitoring response to therapy are lacking.

We recently developed a rapid, sensitive, specific and reproducible real-time polymerase chain reaction (PCR) assay for the quantitative detection of EBV-DNA in plasma. [11] EBV-DNA encoding for the non-glycosylated membrane protein BNRF 1 p143 is used as the target gene in this assay. No viral DNA was detected in plasma from healthy donors, while different levels of EBV-DNA were detected in plasma from patients with infectious mononucleosis and immuno-compromised patients with and without a diagnosis of EBV-LPD. [11] Using this quantitative PCR, we set out to assess the value of closely following EBV-DNA plasma levels for monitoring and predicting the response to treatment and subsequent survival in allogeneic hematopoietic stem cell transplantation recipients.

2. Patients and Methods

Patients

Fourteen consecutive EBV-LPD patients, who developed LPD between January 1997 and June 1999, were included in this study. These cases of EBV-LPD were diagnosed among a total of 193 allogeneic hematopoietic stem cell transplantation recipients in that time period, yielding a cumulative incidence of 7.3%. Indications for allogeneic stem cell transplantation were acute myeloid leukemia (AML, n=3), acute lymphoblastic leukemia (ALL, n=4), chronic myeloid leukemia (CML, n=3), multiple myeloma (MM, n=2), chronic myelo-monocytic leukemia (CMMoL, n=1) and severe aplastic anemia (SAA, n=1). Patients were transplanted either at the University Medical Center Utrecht (n=6) or at the University Hospital Rotterdam / Daniel den Hoed Cancer Center (n=8). The median age was 38 years (range, 18-55 years).

Table 1. Patient Characteristics

No	Age (yrs)	Sex (M/F)	Diagnosis	Conditioning Regimen	Donor Type	GVHD Prophylaxis	Graft Characteristics		
							MNC (10^7/kg)	CFU-GM (10^4/kg)	CD 3 (10^5/kg)
1	23	M	ALL CR2	Cy/TBI	Sib	TCD/CsA	1.2	13.0	2.3
2	21	F	ALL CR1	Cy/TBI/ATG	MUD	TCD/CsA	0.3	38.7	2.0
3	43	F	AML CR2	Cy/TBI/ATG	MUD	TCD/CsA	2.5	26.7	2.0
4	50	F	CML CP2	Cy/TBI	Sib	TCD/CsA	1.8	20.0	2.3
5	55	M	MM	Cy/TBI	Sib	TCD/CsA	0.2	21.4	2.0
6	45	F	CML CP3	Cy/TBI/ATG	MUD	TCD/CsA	1.8	34.4	2.1
7	23	M	ALL CR1	Cy/TBI/ATG	MUD	TCD/CsA	1.3	5.3	1.0
8	48	F	AML CR1	Cy/TBI	Sib	TCD/CsA	2.3	23.4	2.0
9	39	M	CMMol	Cy/TBI/ATG	MUD	TCD/CsA	0.6	3.3	1.0
10	18	F	SAA	Cy/TBI/ATG	MUD	TCD/CsA	7.0	30.0	6.8
11	25	F	AML CR2	Cy/TBI/ATG	MUD	TCD/CsA	3.2	23.1	1.0
12	22	M	CML CP1	Cy/TBI/ATG	MUD	TCD/CsA	2.0	23.8	1.3
13	37	M	ALL CR1	Cy/TBI/ATG	MUD	TCD/CsA	5.0	10.1	2.5
14	46	M	MM	Cy/TBI	Sib	TCD/CsA	0.1	6.7	2.0

Legend to Table 1.
M indicates male; F, female; ALL CR1/2, first respectively second complete remission acute lymphoblastic leukaemia; AML CR1/2, first respectively second complete remission acute myeloid leukaemia; CML CP 1/2/3, first resp second resp third chronic phase chronic myeloid leukaemia; MM, multiple myeloma; CMMoL, chronic myelo-monocytic leukaemia; SAA, severe aplastic anemia; Cy, cyclophosphamide; TBI, total body irradiation; ATG, anti-thymocyte globulin; Sib, matched related donor; MUD, matched unrelated donor; GVHD, graft-versus-host-disease; TCD, T-cell depletion; CsA, cyclosporin A; MNC, mononuclear cells; CFU-GM, colony forming units granulocytes-monocytes; CD3, T-cells.

Five patients received an allogeneic stem cell transplantation from a sibling donor (Sib) and 9 from a matched unrelated donor (MUD). Patient characteristics, donor type, GVHD prophylaxis and graft characteristics are presented in Table 1.

Transplantation Regimen

All patients received as a conditioning regimen cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (6 Gy on each of two successive days with partial shielding of the lungs for a total lung dose of 2 x 4.5 Gy). Horse or rabbit ATG (Imtix Sangstat, Amstelveen, The Netherlands) was added (15 mg/kg or 4 mg/kg, respectively, from day –7 through day –3) for patients who received an unrelated donor transplantation. Aciclovir prophylaxis (200 mg four times a day) was provided during neutropenia following stem cell transplantation for prevention of herpes simplex virus reactivation. Hematopoietic stem cells were obtained by bone marrow aspiration under general anesthesia. All grafts were partially depleted of T cells using erythrocyte rosetting (n=6), CD34 selection (n=3, CellPro, Brussels, Belgium) or immunorosetting using recipient erythrocytes and tetrameric antibodies to CD2 and CD3 (n=5, Sanquin, division CLB, Amsterdam, The Netherlands). [12-15] Partial T-cell depletion (TCD) resulted in a residual median number of 2.0 x 10^5 $CD3^+$ T-cells/kg (range, 1-6.8) in the bone marrow graft (Table 1). T-cell add back was performed if TCD resulted in less than 1.0 x 10^5 $CD3^+$ T-cells/kg in the graft. [13] All patients received additional GVHD prophylaxis with cyclosporin A (3 mg/kg/day) from day –3 till day +100 following stem cell transplantation.

EBV-LPD Diagnosis

EBV-LPD was diagnosed by histology and/or cytology and was classified according to the criteria of Knowles. [16] Immunohistology included antibody staining using CD19 (Becton Dickinson, San José**,** USA), CD20 (DAKO, Glostrup, Denmark) and EBV latent membrane protein 1 (EBV-LMP 1, DAKO) specific monoclonal antibodies. Furthermore, clonality was assessed using immunohistochemical staining with monoclonal antibodies to kappa and lambda light chains (DAKO). In situ hybridisation was performed to detect expression of Epstein-Barr virus-encoded small RNA molecules (EBV-EBER) using an

EBV-EBER probe (DAKO), and PCR was used for detection of EBV-DNA encoding for the *Bam*HI fragment. Furthermore, EBV-LPD-staging included physical examination, whole-body computer-tomography (CT) scanning and immunological analysis of blood and bone marrow using flow cytometry.

Quantitative EBV-specific PCR

Taqman PCR primers were selected from the EBV-DNA genome encoding for the non-glycosylated membrane protein BNRF1 p143 and generated a DNA product of 74 basepairs. As described before by Niesters et al. [11] A known EBV copy number based on a reference standard quantified by electron microscopy (ABI Advanced biotechnologies, Columbia, MD, USA) was used for standardization. Serial dilutions ranging from 10 to 10^7 genome equivalents per ml (geq/ml) were made to characterize linearity, precision, specificity, and sensitivity. The Taqman assay appeared to detect viral DNA in plasma over a linear span between 50 and 10^7 geq/ml with an average coefficient of variation of 1.56% (range, 0.7-7.0%). Test results below 50 geq/ml were considered negative. No viral DNA was detected in plasma of healthy EBV-seropositive individuals. [11] Blood samples were obtained frequently (sample every 2-3 days) commencing when patients were admitted and EBV-LPD was included in the differential diagnosis, until complete response to therapy for EBV-LPD or fatal outcome. The pattern of quantified EBV-DNA levels was retrospectively related to clinical response.

Statistical Analysis

Patients were analysed for response to treatment and for overall survival. Complete remission (CR) of EBV-LPD was defined as complete disappearance of lymphadenopathy on CT scanning and/or physical examination and disappearance of peripheral blood monoclonal B-cells. Overall survival was measured from diagnosis of EBV-LPD until death from any cause. Patients still alive at the time of analysis were censored at the last follow-up date. Patient characteristics and response were compared between subgroups using Fisher's exact test. Univariate survival analysis was performed using the logrank test to see whether there was a difference in survival between subgroups. All reported P-values are two-sided and a significance level of α=0.05 was used.

Table 2. EBV-LPD Characteristics

No	Donor Type	EBV-serology Prior to SCT (D/R)	Time SCT to EBV-LPD (d)	Pathology					Plasma EBV-BNRF (geq/ml)
				Morphology	Clonality	EBV-LMP	EBV-EBER	EBV-BamHI	
1	Sib	-/+	139	III	Mono	+	+	+	1,800
2	MUD	+/+	168	I	Poly	+	+	+	4,000
3	MUD	+/+	54	II	Mono	+	+	+	3,200,000
4	Sib	+/+	67	I/II	Mono	+	+	+	74,000
5	Sib	+/+	167	I/II	Mono	+	+	+	89,500
6	MUD	+/+	50	III	Mono	+	+	+	133,000
7	MUD	+/+	88	III	nd	+	+	+	300,000
8	Sib	+/+	81	II	Poly	+	+	+	13,300
9	MUD	+/+	49	II	Mono	+	+	+	4,157
10	MUD	+/+	61	nd	nd	nd	nd	nd	33,875
11	MUD	+/+	180	II	Mono	+	+	+	6,571
12	MUD	+/+	52	III	Mono	+	+	+	194,152
13	MUD	+/+	83	II/III	Mono	+	+	+	206,084
14	Sib	+/+	93	III	Mono	+	+	+	313,000

Legend toTable 2.
Sib indicates matched sibling donor; MUD, matched unrelated donor; D, donor; R, recipient; SCT, stem cell transplantation; EBV-LPD, Epstein-Barr virus associated lymphoproliferative disease; I. plasmacell hyperplasia; II, polymorphic hyperplasia; III, Non-Hodgkin's Lymphoma (criteria according to Knowles); nd=not determined; EBV-LMP, EBV latent membrane protein; EBV-EBER, EBV encoded RNA; EBV-BamHI, PCR for EBV-DNA encoding for the BamHI fragment; EBV-BNRF (geq/ml), plasma PCR with EBV membrane protein BNRF1 p143 as target (genome equivalent/ml) assessed at EBV-LPD diagnosis.

3. Results

Patient and Treatment Characteristics

Median time from stem cell transplantation to EBV-LPD diagnosis was 82 days (range, 49-180 days). All donor/recipient pairs were EBV-seropositive before stem cell transplantation, except one donor who was seronegative (Table 2). Thirteen patients (93%) presented with lymphadenopathy, 12 (93%) with fever, four patients (29%) had involvement of Waldeyer's ring and three (21%) had hepatosplenomegaly. Additional staging revealed that 4 out of 12 patients (33%) had bone marrow involvement (both morphological and immunological) and 4 out of 10 patients (40%) had monoclonal B-cells in their cerebrospinal fluid. A lactate dehydrogenase (LDH) level of more than 1.5 times the reference value was observed in nine patients (64%). EBV-LPD was diagnosed using histology and classified according to Knowles et al in 13 patients.[16] Cytology was performed in patient no 10. EBV as the definite cause of each LPD was confirmed either by the detection of EBV-LMP and/or by the detection of DNA (*Bam*HI) or RNA (EBER) in CD19- or CD20-positive B-cells in all but one patient (no 10). The latter patient was diagnosed with EBV-LPD based on the presence of monoclonal B-cells in cerebrospinal fluid, elevated LDH, stage IV lymphadenopathy (Ann-Arbor classification) and a high quantitative plasma EBV-DNA level. Monoclonal B-cell proliferation was detected in 10 patients and polyclonal disease in two patients.

EBV-LPD was considered stage IV in 10 patients as a result of monoclonal B-cells in bone marrow and/or peripheral blood or diffuse infiltration of extranodal tissue. Several treatment modalities were applied (Table 3) including: interruption of immunosuppression (SI, n=9), aciclovir (ACV, 3 times 10 mg/kg/day, n=7), chemotherapy [CTX, cytarabin two times 1 g/m^2 q 12 hours, n=1 or cyclophosphamide 750 mg/m^2 (day 1) , doxorubicin 50 mg/m^2 (day 1), oncovin 1.4 mg/m^2 (day 1), prednisone 100 mg (days 1-5), n=1], anti-CD20 monoclonal antibody (anti-CD20, 375 mg/m^2, Rituximab, Mabthera® , Roche Pharma, Basel, Switzerland, n=7) and donor lymphocyte infusion (DLI) 1 x 10^6 CD3$^+$ T-cells/kg (n=8).

Table 3. EBV-LPD Therapy

No	Donor type	Therapy					Outcome		
		SI	ACV	CTX	Anti-CD20	DLI	Response	Survival	COD
1	Sib	+	+	-	-	+	PD	Dead	EBV-LPD
2	MUD	+	+	-	+	-	CR	Alive, 466+	-
3	MUD	+	+	+	+	+	CR	Dead	GVHD
4	Sib	+	-	-	+	-	CR	Dead	GVHD
5	Sib	+	-	-	+	+	PD	Dead	EBV-LPD
6	MUD	-	+	-	+	+	PD	Dead	EBV-LPD
7	MUD	-	+	+	-	+	PD	Dead	EBV-LPD
8	Sib	+	-	-	+	-	CR	Alive, 76+	-
9	MUD	-	-	-	-	+	PD	Dead	EBV-LPD
10	MUD	-	-	-	-	+	CR	Dead	GVHD
11	MUD	+	-	-	-	-	CR	Dead	AML relapse
12	MUD	+	+	-	-	+	PD	Dead	EBV-LPD
13	MUD	-	+	-	-	-	PD	Dead	EBV-LPD
14	Sib	+	-	-	+	-	CR	Alive, 188+	-

Legend to table 3.
Sib indicates matched related donor; MUD, matched unrelated donor; SI, stop immune suppression; ACV, aciclovir; CTX, chemotherapy; Anti-CD 20, anti-B-cell monoclonal antibody therapy (Rituximab); DLI, donor lymphocyte infusion; PD, progressive disease; CR, complete remission; Survival, alive (d); COD, cause of death; EBV-LPD, Epstein-Barr virus associated lymphoproliferative disease; GVHD, graft-versus-host-disease.

Complete clinical responses were observed in seven patients. Non-responding patients (n=7) all showed rapidly progressive disease and died as a result of EBV-LPD. Responders were evaluated at a median of 17 days (range, 5-51 days) and non-responders at a median of 11 days (range, 4-29 days). Clinical response evaluation was performed by physical examination and by computerized tomography in seven patients, at autopsy in six patients and by physical examination alone in one patient. Considering each treatment modality separately, seven patients received aciclovir and two of these patients developed a response; seven were treated with anti-B-lymphocyte monoclonal antibody immunotherapy followed by a response in five patients; DLI was given to eight patients and two of them responded; immunosuppression was stopped in nine patients of whom six ultimately responded. Cessation of immunosuppression combined with anti-CD20 were the principal treatment strategies in responding patients. Three responders survived, these surviving patients received anti-CD20 as part of their treatment and no DLI. GVHD grade II-IV following DLI was observed in two patients. GVHD was considered the primary cause of death in three patients. One patient died from recurrent acute myeloid leukemia.

Quantitative EBV-specific PCR

All patients showed highly elevated EBV-DNA levels at EBV-LPD diagnosis. The median EBV-DNA level was 82,000 geq/ml (range, 1,800-3,200,000 geq/ml) at start of therapy. Patients with a complete clinical response to therapy ultimately became negative for plasma EBV-DNA, whereas all non-responders showed a progressive increase of their plasma EBV-DNA levels (Figure 1).

Plasma EBV-DNA disappeared at a median number of 17 days from the start of therapy (range, 5-51 days). A decrease of viral load could be observed early after initiation of therapy. Therefore, we asked the question whether an increase or decrease of the viral load as measured within 72 hours (h) from the start of therapy would predict for the ultimate clinical response. Individual EBV-DNA levels at the start of therapy, at 48-72 hours from the start, and at the time of clinical response evaluation are presented in Figure 1. All clinically responding patients showed a reduction of at least 50% of their viral load within 72 hours of start of therapy, in contrasts to the progressive increase of EBV-DNA levels in clinical non-responders. The difference was highly significant ($P = 0.001$).

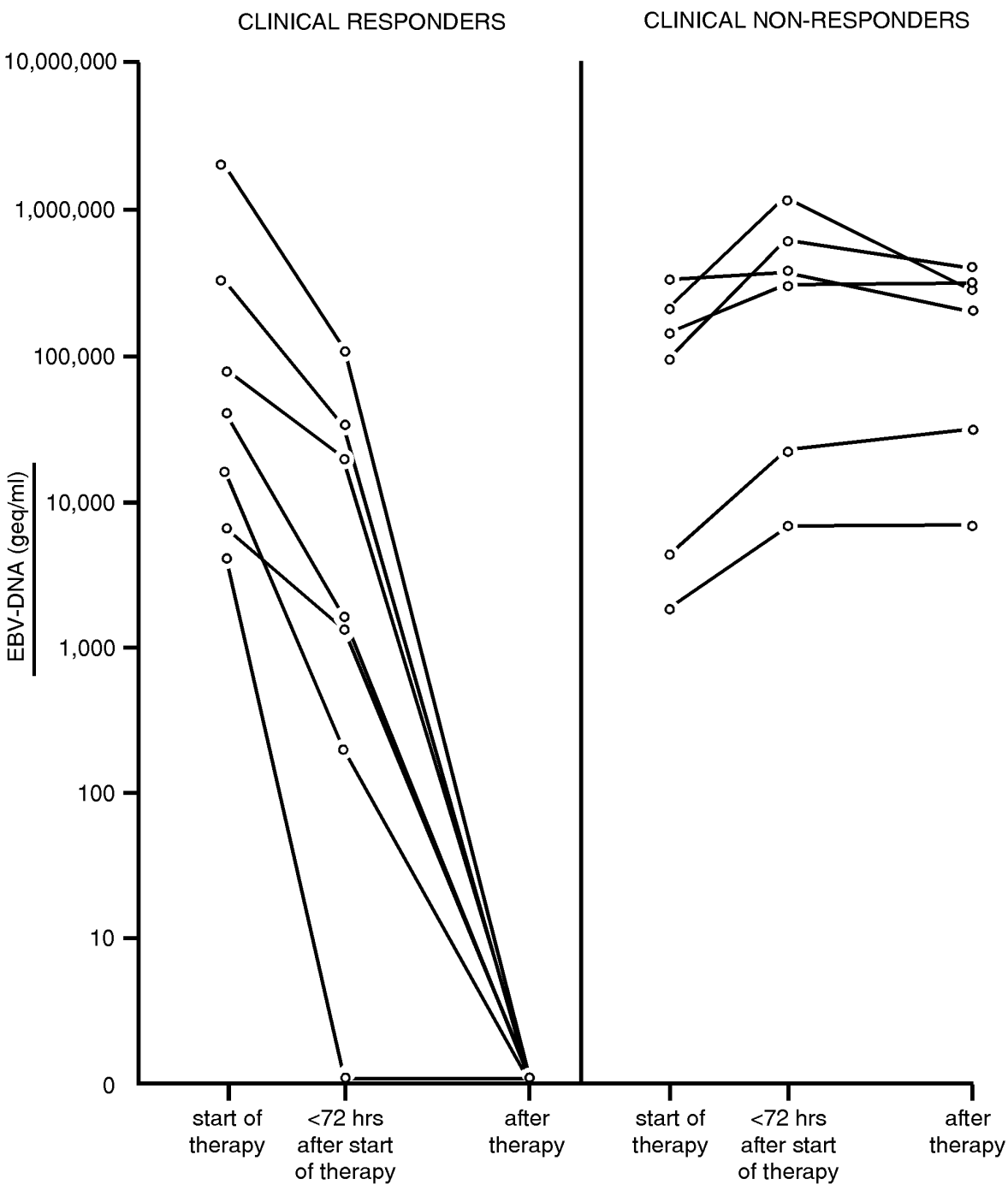

Figure 1. EBV load following therapy for EBV-LPD. Individual EBV-DNA levels for clinical responders (left) and clinical non-responders (right) at the start of therapy, after 72 hours and at clinical response evaluation. Responders were evaluated at a median of 17 days (range, 5-51 days) and non-responders at a median of 11 days (range, 4-29 days)

An early decline of EBV-DNA levels, however, did not predict for survival. EBV-DNA levels at the time of EBV-LPD diagnosis between clinically responding and clinically non-responding patients overlapped, respectively 4,000-3,200,000 geq/ml (median 34,000 geq/ml) versus 1,800-300,000 geq/ml (median 133,000 geq/ml). Absolute EBV-DNA plasma levels did not predict for response or survival.
All patients showed an elevated LDH at the time of EBV-LPD diagnosis. We additionally evaluated whether a decrease of LDH within 72 hours from the start of therapy would also predict for response. However, LDH levels increased in two out of seven clinically responding patients, decreased in four and remained unchanged in one other responding patient. No association was observed between the pattern of LDH and subsequent response. Additionally, the predictive value of granulocytopenia ($\leq 0.5 \times 10^9$/l) at the time of EBV-LPD diagnosis was evaluated.

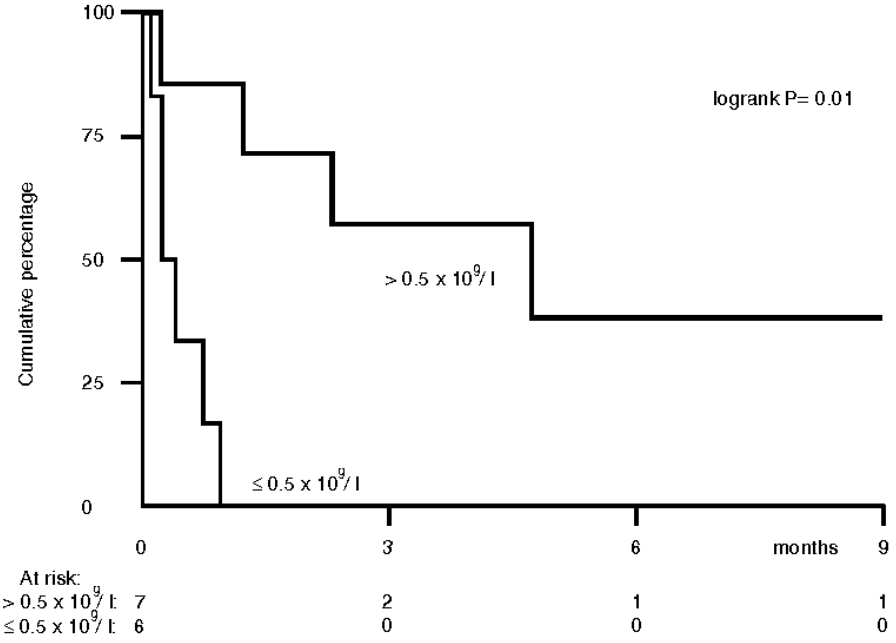

Figure 2. Lymphocyte count at diagnosis of EBV-LPD and overall survival. Overall survival (n=13) in relation to lymphocyte number (> 0.5 x 10^9/l versus ≤ 0.5 x 10^9/l) at diagnosis of EBV-LPD.

However, none of our patients showed granulocytopenia at the time of EBV-LPD diagnosis. In contrast, lymphopenia ($\leq 0.5 \times 10^9$/l) was present in six patients and was a poor prognostic marker for survival in this group of patients (Figure 2) (P = 0.01). In addition, lymphopenia at EBV-LPD diagnosis was also associated with non-

responsiveness (P = 0.03). Patient no 12 was excluded from this analysis because of lymphocytosis as a result of leukemic EBV-lymphoma (monoclonal B cells).

Stepwise Therapy and Selection of Treatment

Following the observation of an association between response to therapy and fast decrease of EBV viral load, one recent patient (no 14) received step-wise therapy guided by intensive monitoring of his viral load using quantitative PCR. The patient had received a sibling stem cell transplantation for multiple myeloma in first partial remission and developed EBV-LPD at day 93. At that time 313,000 geq EBV-DNA/ml were measured and the lymphocyte count was 1.2 x 10^9/l. Immunosuppression (cyclosporin A) was discontinued and he received one infusion of anti-CD20 (Rituximab, Mabthera ®, 375 mg/m^2).

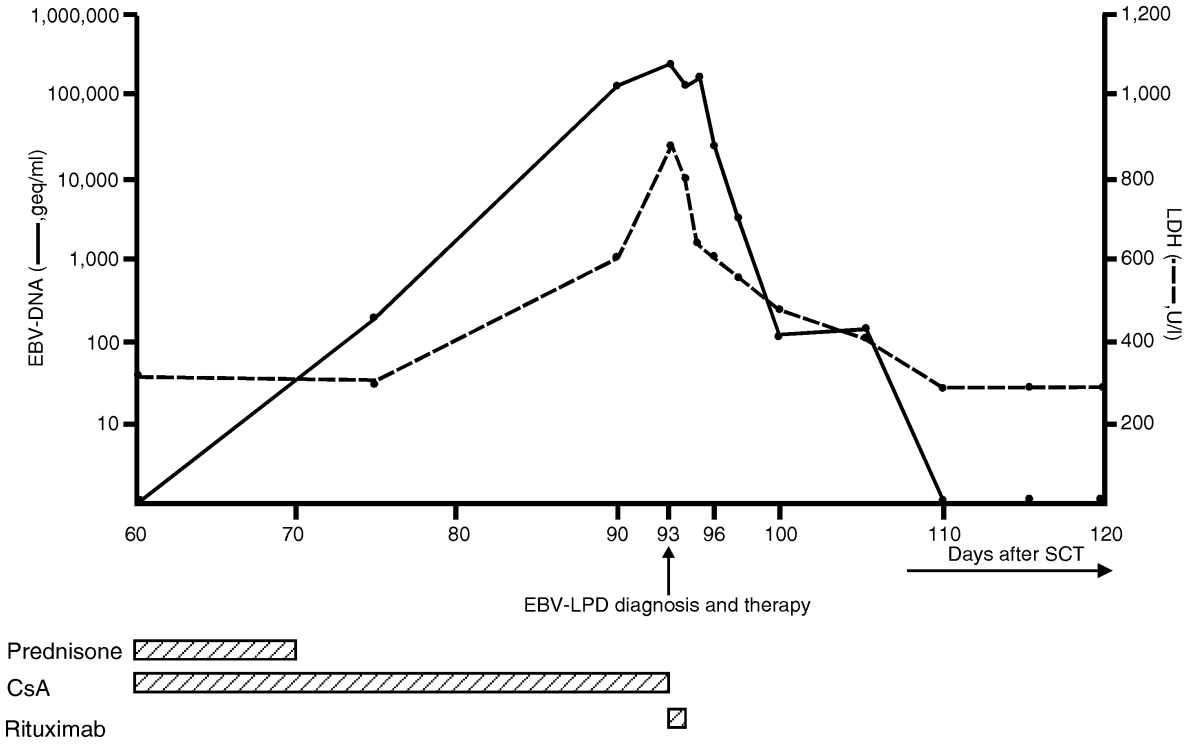

Figure 3. Therapy for EBV-LPD guided by EBV load. EBV-DNA levels and lactate dehydrogenase concentration (LDH) in patient no 14 who developed EBV-LPD at day 93 after allogeneic hematopoietic stem cell transplantation for multiple myeloma, and was treated using anti-CD20 immunotherapy and cessation of immunosuppression.

During the following days, fever increased, and serum LDH continued to rise. Because disease progression seemed imminent, his EBV seropositive donor was asked to donate lymphocytes. Meanwhile daily monitoring of viral load showed a decrease to 31,000 geq/ml within 72 hours after anti-CD20 infusion. Therefore, DLI was withheld and the patient developed a complete clinical response at day 129 after stem cell transplantation with undetectable plasma EBV-DNA levels at day 110 after stem cell transplantation (Figure 3). No relapse of EBV-viremia or EBV-LPD has occurred until recent follow-up at day 245 after stem cell transplantation.

4. Discussion

EBV-LPD is a serious complication of stem cell and organ transplantation and is associated with high morbidity and mortality. [1-3] Recently, new treatment modalities have been reported for patients with EBV-LPD following allogeneic hematopoietic stem cell transplantation.[4-10,17-19] However, the effect of these therapies varies greatly among individual patients. Sensitive and, particularly, early markers for evaluation of response are urgently needed in order to select and adjust treatment with minimal delay. Here, we have shown that the close monitoring of EBV-DNA in plasma using a quantitative real-time PCR provides a powerful tool for predicting clinical response within 72 hours after initiation of therapy.

Following a primary infection in healthy individuals, EBV infects and immortalizes B-lymphocytes, which is followed by a lifelong viral latency. [1,2] Proliferation of EBV-infected B-cells is prevented and controlled by an adequate T-cell dependent specific immune response.[19] However, strong reduction in the numbers of EBV-specific T lymphocytes, such as following allogeneic hematopoietic stem cell transplantation, may result in reactivation of the virus and, ultimately, the development of EBV-LPD. Currently, new methods are being developed to monitor, on the one hand, the EBV viral load sensitively and semi-quantitatively and, on the other hand, the EBV-specific immune response.[10,17,18,20-26,30] PCR-based assays to detect viral DNA use either DNA extracted from peripheral blood mononuclear cells or DNA directly extracted from plasma. [24,26-29,33] We applied a real-time-based PCR assay for quantifying EBV-DNA extracted from plasma, that appeared very rapid, sensitive and accurate over a five-log linear range. [11] Plasma as the source of viral DNA was preferred over DNA extracted from cells, because it has been shown that EBV-DNA can be detected in DNA extracted from leucocytes from patients with viral latency, while the presence of EBV-DNA in plasma is diagnostic of active EBV replication. [21,26,30,31] Our results compare well with these earlier findings, as all responding patients showed a complete clearance of EBV-DNA in plasma. Monitoring viral load in plasma rapidly and accurately reflected the response to therapy in our group of patients. All clinically responding patients showed a rapid and complete clearance of viral DNA, whereas all clinically non-responding patients showed a progressive increase of their

viral load (Figure 1). Notably, the distinction between responders and non-responders could already be made 72 hours after initiation of therapy. That early distinction indicates a fast clearance of EBV-DNA from plasma and early control of viral replication. It may be explained by efficacious therapy immediately resulting in interruption of viral replication and by rapid revival of residual EBV-specific immunity because of discontinuation of immune suppression. Monitoring of viral load before the onset of EBV-LPD was not performed in most of our patients. However, prediction of impending EBV-LPD by increasing EBV-DNA levels might allow for the initiation of pre-emptive therapy. A study addressing the predictive value of viral load in plasma is currently being performed. Results of that study may indicate which patients may be eligible for pre-emptive therapy at a certain threshold of quantified viral load.

In 13 out of the 14 patients presented, we were able to evaluate whether lymphopenia as a general marker for the patients' immune recovery, would predict for response and survival. Lymphopenia (lymphocyte count $\leq 0.5 \times 10^9$/l), was significantly associated with a poor outcome (Figure 2) and non-responsiveness as well. Lymphopenia following allogeneic hematopoietic stem cell transplantation may reflect an impaired immune reconstitution. Patients receiving a T-cell depleted graft from an unrelated donor, in particular, may suffer from an impaired reconstitution. [32] In addition, the use of ATG may considerably contribute to the immune deficiency, as was recently shown by Curtis et al, who reported the relative weight of several risk factors for the development of EBV-LPD. [3] Our patients showed varying levels of EBV-DNA at the time of LPD diagnosis. Absolute EBV-DNA levels at the time of diagnosis did not predict for response nor for survival, but lymphopenia did appear as a strong predictor. These results may suggest that, apart from viral load, outcome may be more determined by the patients' immune status.

DLI has been shown to effectively transfer EBV-specific immunity and, thereby, rapid clearance of EBV-viremia and resolution of lymphoma. [8] However, DLI may be associated with the development of GVHD, which may impair the patients' immune response and is associated with considerable morbidity and mortality, as was evident in two of our patients. The use of EBV-specific T-cells, such as developed by Rooney et al, seems very promising, but is still difficult to implement on a wider scale. [17,18] Alternatively, EBV-LPD may effectively be treated by B-cell targeted immunotherapy. [5-7] Seven patients received anti-CD20 immunotherapy in our study, including three patients who also received DLI (Table 3). Although the combination of several treatment modalities does not allow us to draw firm conclusions, the rapid clearance of EBV-DNA in patients who received anti-CD20 without DLI (as illustrated by patient no 14, Figure 3) suggests that DLI may be withheld initially and then selectively given to patients with an increase of viral load. Therefore, the adoptive transfer of donor leucocytes or EBV-specific T-cells, may preferably be considered in patients who do not show a rapid reduction of their viral load and/or in patients, who lack residual (EBV-specific) immunity.

In conclusion, quantitative monitoring of EBV viral load in the plasma of allogeneic hematopoietic stem cell transplantation recipients with EBV-LPD rapidly predicts for a response to therapy. It may thus provide an important tool to adjust and select treatment for individual patients with established EBV-LPD.

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