Epstein-Barr Virus Lymphoproliferative Disease following Allogeneic Hematopoietic Stem Cell Transplantation: Prediction and early Intervention

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Epstein-Barr Virus Lymphoproliferative Disease following Allogeneic Hematopoietic Stem Cell Transplantation: Prediction and early Intervention

Epstein-Barr virus geïnduceerde lymfoproliferatieve ziekte na allogene hematopoëtische stam cel transplantatie: voorspelling en vroegtijdige interventie

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De wereld gaat en gaat, als lang na dezen
mijn roem verging, mijn kennis hooggeprezen.
Wij werden vóór ons komen niet gemist,
na ons vertrek zal het niet anders wezen.

Uit de Rubaijat
J.H. Leopold

Voor mijn vader

Contents and publications

Abbreviations

ATG	Anti-Thymocyte Globulin
Allo-SCT	Allogeneic hematopoietic Stem Cell Transplantation
BM	Bone Marrow
Bu	Busulphan
CFU-GM	Granulocyte-Macrophage Colony Forming Units
CMV	Cytomegalovirus
COD	Cause of Death
CR	Complete Remission
C_t	Cycle threshold
CTL	Cytotoxic T-cells
Cy	Cyclophosphamide
CyA/CsA	Cyclosporin A
D	Donor
DNA	Deoxyribonucleic Acid
DLI	Donor Lymphocyte Infusion
EBV	Epstein-Barr Virus
EBNA	Epstein-Barr virus Nuclear Antigen
EBER	Epstein-Barr virus Encoding RNA
EM	Electron Microscopy
h/hrs	hour(s)
HLA	Human Leucocyte Antigen
HIV	Human Immunodeficiency Virus
IEA	Immediate Early Antigens
IM	Infectious Mononucleosis
LMP	Latent Membrane Protein
LP	Leader Protein
LPD	Lymphoproliferative Disease
MNC	Mononuclear Cells
MUD	Matched Unrelated Donor
NK-cell	Natural Killer cell
NHL	Non-Hodgkin's Lymphoma
OKT3	Anti-T-cell antibody Ortho-Klone
PB	Peripheral Blood
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PR	Partial Remission
PTLD	Post-Transplant Lymphoproliferative Disease
$PV^{+/-}$	Positive/negative predictive value
R	Recipient

Abbreviations (continued)

RNA	Ribonucleic Acid
SCT	Stem Cell Transplantation
Sib	HLA-identical family donor
TRM	Treatment-Related mortality
TCD	T-Cell Depleted
TBI	Total Body Irradiation
Tx	Transplantation

1. Introduction

1. Introduction

Since its discovery, Epstein-Barr virus (EBV) has been associated with a variety of both infectious and malignant human diseases. EBV was first detected by electron microscopy in cultured Burkitt's lymphoma cells in 1964 by Epstein, Achong and Barr.[1] EBV is a DNA virus with a linear genome of 172 kb and belongs to the genus lymphocryptovirus within the family of gamma herpesviruses. These viruses are characterized by (B-cell) lymphotropism, their ability to establish latent infection in host cells and to induce proliferation of these latently infected cells.[2] EBV was distinguished from other, at the time known human Herpes viruses, because of its strong association with Burkitt's lymphoma and its potent growth-transforming ability for B-lymphocytes in vitro. Approximately 90% of humans will become infected with EBV, generally without clinical evidence of disease. Primary infection usually occurs asymptomatically in childhood and results in a lifetime carrier state with periodic release of infectious virus into saliva which may cause infection of naive individuals.[2] Sometimes later, e.g. during adolescence, the latter type of infection may be referred to as kissing disease or Pfeiffer's disease.[3] EBV causes various benign syndromes, such as infectious mononucleosis, chronic active EBV infection, X-linked lymphoproliferative disease and oral hairy leukoplakia. EBV has also been associated with malignant diseases including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's lymphoma, and post-transplant lymphoproliferative disease (PTLD).[4-7] This thesis deals with the development of molecular monitoring of EBV-DNA in plasma of recipients of allogeneic hematopoietic stem cell grafts and the introduction of such monitoring in therapeutic and preventive approaches. In this chapter we shall briefly review the pathogenetic role of EBV in infectious and malignant diseases. Subsequently the clinical presentation, diagnosis, incidence, risk factors and treatment of EBV associated lymphoproliferative disease (LPD) in recipients of hematopoietic stem cell transplants is discussed followed by an outline of this thesis.

2. Infection, immunity, and malignancy

EBV-infection

EBV shedding in the oropharynx occurs intermittently in EBV-seropositive individuals, during such periods of EBV shedding other persons may become infected. When EBV enters the oropharyngeal cavity it penetrates local lymphoid tissue (lingual-, palatine-, and pharyngeal tonsils). Squamous epithelium covering the lymphoid tissue dips into the tonsillar crypts where B-cells are densely situated. Following primary infection these B-cells are the first to be infected. [8] Although earlier studies have discussed elaborately whether EBV replicates in epithelium [9-14], a recent study identified EBV replication in epithelial cells in vitro.[15] Following contact between virus and B-cells, EBV enters the B-cell by binding its major viral envelope glycoprotein 350/220 to the EBV receptor CD21

(receptor for complement C3d) on the surface of the B-cell. Binding of glycoprotein 42 to major-histocompatibility-complex class II (co-receptor) and binding of glycoprotein 350/220 to its receptor facilitate fusion of the viral envelope with the B-cell during endocytosis.[15-18] Receptorbinding results in activation of the B-cell, which may further favor penetration of the virus into the cell. The de-enveloped capsid subsequently travels into the nucleus, degrades and releases viral DNA.

Immortalization of B-cells

Linear EBV-DNA is being transported to the cell-nucleus, where transcription and translation of Epstein-Barr virus nuclear antigen (EBNA)-2 and EBNA-leader protein (LP) start within 4 hours following infection. EBNA-2 is essential for B-cell transformation and is an activator of EBV-latent membrane protein (LMP)-1 and LMP-2 and genes involved in growth and transformation of the infected B cell. EBNA-LP augments the ability of EBNA-2 to up-regulate LMP-1.[2,8] LMP-1 contributes to the growth and transformation of B-cells and is the major transforming gene of EBV.[19,20] LMP-1 mimics in this respect the function of CD40, a receptor constitutively present on the surface of B-cells.[21] B-cell activation leads to differentiation of the B-cell to a B-blast, that expresses besides EBNA-2, LMP-1 and EBNA-3A, 3B, 3C and EBNA-4. The circularisation of linear EBV-DNA to episomal EBV-DNA (circular form of EBV-DNA) in the B-cell nucleus is completed twenty hours after infection, and with the expression of EBNA-1 the transformation of the B-cell to an immortalized B-cell is completed. LMP-2 is only transcribed once the circular viral episome is formed and prevents B-cell activation stabilising the latent state. [20] The non-translated types of EBV-encoded RNA (EBER) do not encode proteins but may be important for oncogenesis and resistance to programmed cell death, or apoptosis. [22]

Latency and reactivation

EBNA-1 plays a pivotal role in the maintenance of EBV in dividing latently infected B-cells.[23-25] EBV-infected B-cells in vivo can express four different programmes of gene usage depending on the location and differentiation stage of the infected B-cell. One of these programmes is used to produce infectious virus, the other three are all associated with latent infection, in which no virus is produced. These latent growth programmes are known as the growth programme, the default programme and the latency programme (Table 1).[26,27] During the growth programme EBV infects resting naive B-cells ($CD27^-$, $sIgD^+$) in tonsils and drives these cells out of their resting state to become activated proliferating lymphoblasts.[28] Three viral proteins are expressed in the default programme, of which EBNA-1 ensures replication of the viral genome during cell division.[2,27] Physiologically, B-cells that encounter antigen become activated and migrate into the follicle of a lymph node where they form germinal centers. Following proliferation and somatic hypermutation, cells that have mutated their immunoglobulin genes become antibody producing plasma cells or memory B-cells, remaining cells that are not selected

go into apoptosis.[27] Signals through the B-cell receptor and from T helper cells are essential for B-cell survival during this period. EBV mimics these pathways to rescue an activated B-lymphoblast into the memory B-cell pool and create a state of latency. The key proteins to achieve that goal are LMP-1 and LMP-2A.

Table 1. Latency transcription Programmes (adapted from [27])

Programmes	Gene expression	Proposed function
Growth programme	EBNA 1-6, LMP-1, LMP-2A, LMP-2B	Activates resting B-cell to become a proliferating lymphoblast
Default programme	EBNA-1, LMP-1, LMP-2A	Provides necessary survival signals for: 1. infected lymphoblasts to differentiate into memory B-cells, 2. maintenance of persistently infected memory B-cells
Latency programme	None (LMP-2A)	Persistence of virus in resting recirculating memory B-cells

EBNA indicates Epstein-Barr virus nuclear antigen; LMP, latent membrane protein.

First, LMP-1 interacts with a set of molecules that act as intermediary in signalling by tumour necrosis-factor-receptor-associated factors (TRAFs).[29] CD40 is a member of TRAF and situated on germinal centre B-cells. Following stimulation by CD154, situated on T helper cells, CD40 can deliver a survival signal for the B-cell, rescuing it from apoptosis and driving proliferation.[30] LMP-1 acts as a functional homologue of CD40 and in addition induces cellular bcl-2 providing apoptosis resistance.[21] Secondly, receptors for LMP-2A and the B-cell receptor contain a common pathway (immunoreceptor tyrosine based activation motifs, ITAM).[31] In the absence of antigen, the B-cell receptor delivers a non-proliferative signal that is essential for survival of B-cells. This is the signal that LMP-2A mimics. During this process the immunodominant EBNA-2, EBNA-3A, 3B, 3C are not expressed. Analogously to normal B-cells encountering an antigen, EBV-infected B-cells enter the follicles and undergo germinal centre differentiation to become memory B-cells. When the EBV-infected B-cell ultimately leaves the tonsil into the peripheral circulation as resting memory B-cell ($CD27^+$, Ig^+, $CD23^-$, $CD5^-$) it will switch off all latent gene expression.[32] In this way the virus can persist in a benign state and will not be recognized by the immune system as the main targets for cytotoxic T-cell response lack expression.[33,34]

In a small proportion of latently infected B-cells, EBV eventually may undergo lytic replication. This lytic replication is accompanied by considerable nuclear and cytoplasmic

changes of infected cells, because new virus particles need to be produced. Production of new virus includes, synthesis of viral DNA, assembly of nucleocapsids, and transport of the virus through the nuclear membrane followed by cytoplasmic transport of tegumented capsid and envelopment through budding into Golgi vesicles followed by release of enveloped virions by exocytosis at the plasmamembrane.[35-38] The switch from latency to lytic replication involves the expression of the immediate early genes BZLF1 and BLRF1 followed by the coordinate expression of a cascade of about 80 early and late genes. Early genes are essential for viral replication. BHRF1, most abundantly expressed during this cycle, shows structural and functional homology to host-encoded bcl-2 having anti-apoptotic activity also.[39,40] Other important early genes are, BALF1 which regulates the function of BHRF1, BALF2 being important in DNA replication, and BALF5, encoding the DNA polymerase protein.[26,39] Lastly, viral genome replication, production of structural proteins and subsequent virion assembly takes place. These processes finally lead to donor cell death and release of viral progeny. Important genes in this stage are the BLLF1 gene, which encodes for the glycoprotein 350/220 (capsid antigen) that mediates binding to CD21, and BCRF1, a viral homologue of interleukin-10, which may stimulate B cell proliferation and inhibit the function of cytotoxic T-cells.[41,42]

Immune response to EBV-infection

Cellular immunity

Initial cell mediated defense against EBV consists of direct cytolysis by non-specific T-cell or natural killer cell responses. These are followed by the development of EBV-specific $CD4^+$ and $CD8^+$ T-cell responses. Cytotoxic T-cells especially recognize epitopes from the EBNA-3 family of latent proteins.[43,44] Less common, subdominant, reactivities against EBNA-2, EBNA-LP, LMP-1 and -2 have been described. Cytotoxic T-cells may become memory cells after clearance of infection (1/1000 T-lymphocytes versus 1/100,000 EBV infected B-cells), that persist for live and may respond rapidly upon reactivation of the virus.[45,46] EBNA-1 may escape the immune response by virtue of impaired major histocompatibility complex presentation, as its Glycine/Alanine repeat domain prevents proteasome-dependent processing for presentation by major histocompatibility complex class I.[47,48] Reactivation of EBV from latently infected B-cells generally results in lytic antigen expression.[35] Several immediate early (BZLF1, BRLF1) and early (BMLF1, BMRF1, BALF2) antigens serve as targets for specific $CD8^+$ cytotoxic T-cells.[49,50] These lytic-antigen specific T-cells can mount up to 40% of the total $CD8^+$ T-cell population during acute infection, whereas only 2% is targeted to a latent EBV protein sequence.[51,52]

Humoral immunity

The antibody response to primary EBV infection is characterized by substantial immunoglobulin (Ig) M titres against viral capsid antigen and rising IgG titres to early antigen. Functionally, antibodies to viral membrane antigen (glycoprotein 350) can both neutralize infective free virus and direct antibody-dependent cellular cytotoxicity against

productively infected cells.[53,54] Such neutralising antibodies develop late after primo-infection and persist lifelong, but they are not able to control the proliferation of latently infected cells, and may only limit infection and prevent superinfection by new incoming strains.[55] Anti-EBV antibody titres are characterised by a lifelong persistence at a specific level in one patient.[56] The serologic hallmarks of a non-immuno-compromised EBV seropositive person are: Ig G antibodies to EBNA-1, Ig G antibodies to viral capsid antigen and Ig G antibodies to Zebra (BZLF1, lytic gene).[16,53,54,56,57]

EBV-induced infectious syndromes

Infectious Mononucleosis

Most primary EBV infections occur in infancy and are asymptomatic. In adolescence and in adults however up to 50% of EBV infections may result in symptomatic infectious mononucleosis.[2] A recent study showed that expansion of T-cells differs depending on whether the infection progresses without clinical symptoms or develops into infectious mononucleosis.[58] The classical triad consists of fever, lymphadenopathy, and pharyngitis. Most patients have lymphocytosis with cytologically atypical lymphocytes, heterophilic serum antibodies, and elevated serum aminotransferase levels. In general, symptoms subside within a few weeks without sequelae. Complications may include splenic rupture, hepatitis, myocarditis, encephalitis, meningitis, aplastic anemia, and thrombocytopenia. To date no specific therapy has been proven to be effective in infectious mononucleosis.

Chronic active EBV infection

Chronic active EBV-infection starts as a primary EBV infection but lasts for more than 6 months and is accompanied by abnormally elevated EBV antibodies titres (anti-viral capsid antigen IgG, anti-early antigen IgG) and low titres to EBNA. In addition, histologic evidence of major organ involvement (pneumonitis, uveitis, hepatitis, splenomegaly, bone marrow hypoplasia) and increased viral load in affected tissues may be present.[59,60] Although the exact pathogenesis of chronic active EBV-infection is not clear, clonal expansion of EBV-infected T or natural killer (NK) cells may be observed.[61,62] The T-cell type of disease is characterised by fever, and high titres of EBV antibodies, whereas hypersensitivity to mosquito bites and high IgE may prevail in patients with the NK-cell type of chronic active EBV infection. The cytotoxic T-cell response in chronic active EBV infection is restricted to a few epitopes.[63] Furthermore, this limited immune response and the low antigenicity (expression of latency genes EBER and EBNA-1 only) of EBV infected T- and NK cells in chronic active EBV-infection may explain the aggressive clinical course. Although allogeneic bone marrow transplantation and cytotoxic T-cell infusion have been reported successful, to date, no specific treatment for chronic active EBV infection has been established.[64-66]

X-linked lymphoproliferative disease
X-linked lymphoproliferative disease is an inherited disease only affecting males, which results from a mutation in the signalling lymphocyte activation molecule (SLAM) gene located on the X-chromosome. The absence of a functional SLAM gene may impair normal T and B cell interaction, which results in unregulated growth of EBV-infected B-cells.[67] Most patients die of infectious mononucleosis (57%), furthermore, frequent other complications are hypogammaglobulinemia (29%) and malignant lymphomas (24%).[68]

Oral hairy leukoplakia
Oral hairy leukoplakia especially occurs in immunocompromised patients (transplant recipients, human immunodeficiency virus (HIV)-infected patients). It presents as white wartlike epithelial lesions of the oral mucosa, especially the lateral border of the tongue, which contain EBV-DNA and Herpesvirus like particles. Multiple strains are often present in one lesion, showing active viral replication and expression of lytic viral proteins.[69,70] Aciclovir may be an effective treatment modality, however, frequent recurrences occur after therapy has been stopped.[71]

Malignancies associated with EBV

Hodgkin's Disease
Hodgkin's disease is a malignant lymphoma characterised by the presence of mononuclear Hodgkin cells and their multinucleated variant, the Sternberg-Reed cell. The background of these cells consists of lymphocytes, plasma cells, histiocytes and eosinophils. Hodgkin cells and Sternberg-Reed cells are derived from B-cells in most cases.[72] EBV-DNA (*Bam*HI-W region of the EBV genome) can be detected in 40-60% of patients with Hodgkin's disease, especially in the lymphocyte depleted subtype and mixed cellularity subtype. [6] The EBV genome may be present in the Hodgkin's and Reed-Sternberg cells, and is monoclonal.[6,73,74] Individuals with a history of infectious mononucleosis carry a 2-4 fold increased risk of developing Hodgkin's disease, the risk being most pronounced during the first 3 years following primary infection.[75] Antibody titres to viral capsid antigen- and early antigen-proteins may be high at diagnosis of Hodgkin's disease.[76] The latter 3 observations suggest a causative link between EBV and Hodgkin's disease.

Burkitt's Lymphoma
Burkitt's lymphoma is a high-grade lymphoma of small, noncleaved B-cells. The endemic (African) form is EBV DNA positive in 90% of cases.[77] Endemic Burkitt's lymphoma is characteristically located in the jaw or abdomen and preferentially occurs during childhood. [2] In Africa infection with Plasmodium falciparum is thought to play a role in the pathogenesis of Burkitt's lymphoma because it stimulates B-cell proliferation and diminishes T-cell control of proliferating EBV-infected B-cells favoring Burkitt's lymphoma.[78] Children with elevated antibodies to EBV are at risk for developing Burkitt's lymphoma in endemic areas.[79] Sporadic Burkitt's lymphoma, occurring in Europe and the

United States, is associated with EBV in only 20% of cases. It most commonly presents with an abdominal mass and is seen at older age. HIV infected individuals are at risk for Burkitt's lymphoma. The clinical presentation resembles that of the sporadic form.[2] Burkitt's lymphoma cells often contain chromosomal translocations involving chromosomes 8 (c-myc oncogene), 14 (immunoglobulin heavy chain), or chromosomes 22 and 2 (immunoglobulin light chain). The translocation t(8;14) is apparent in 80% of patients with Burkitt's lymphoma. The variant translocations, t(2:8) or t(8:22), are evident in 20% of cases. The translocations result in juxtapositioning of the c-myc oncogene to the constant region of the heavy or light chain.[80] Overexpression of c-myc results in increased tumorigenicity of EBV-immortalized B-cells.[81]

T-cell non-Hodgkin's lymphoma

Although EBV is considered a B-lymphotropic virus, EBV may also infect T-cells and NK-cells. To date, 3 distinct categories of T-cell non-Hodgkin's lymphomas (NHL) have been associated with EBV. These include: 1. virus-associated hemophagocytosis associated T-cell lymphocytosis/lymphoma, 2. nasal T-NHL, and 3. peripheral T-NHL.[2] It has been postulated that EBV may enter the T-cell during the process of B-cell kill by an activated cytotoxic T-cell.[82] As T-cells may exhibit a very low expression of CD21, EBV may enter the T-cell via CD21, if present and functional, or an alternative receptor or mechanism.[83-85] Despite intensive chemotherapy outcome of EBV related T-cell NHL is poor. Especially, fulminant T-cell NHL occurring in the setting of a chronic active EBV-infection has a dismal outcome.[86]

Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma is especially prevalent in southern China, northern Africa and Alaskan Eskimos. The incidence approaches 50 per 100,000 persons per year in southern China as compared to <1 per 100,000 in other parts of the world.[87] Almost 100% of anaplastic or poorly differentiated nasopharyngeal carcinoma contain EBV and express EBV proteins, whereas the majority of keratinizing squamous-cell and non-keratinizing nasopharyngeal carcinoma express EBV.[88] The EBV genome is present in transformed epithelial cells but not in the lymphocytes of the tumor. Clonal EBV is found in the early dysplastic lesions or carcinoma in situ, indicating that EBV infection precedes the development of nasopharyngeal carcinoma.[89] The detection of EBV specific IgA antibodies in nasopharyngeal carcinoma patients may be useful in screening individuals for nasopharyngeal carcinoma and monitoring patients during follow-up after treatment of established disease.[90,91]

Gastric Carcinoma

Approximately 10% of gastric carcinomas are associated with EBV worldwide.[92,93] The expression pattern is exceptional as only EBNA-1, EBER and LMP-2 are detected, and LMP-1 and EBNA-2 are not expressed. Transcription of the transforming and immortalizing EBV gene BARF1 (*Bam*HI A rightward open reading frame) may be

detected in addition which suggests that it may act as an alternative viral transforming factor instead of LMP-1.[94]

3. Post-transplant lymphoproliferative disease

Introduction

Most solid organ and bone marrow recipients/donors carry latent EBV at the time of transplantation, as a result of the widespread distribution of EBV. Uncontrolled outgrowth of EBV infected B-cells may occur in patients with a severe immune deficiency, as may occur following allogeneic hematopoietic stem cell transplantation, solid organ transplantation, and congenital/acquired immune deficiencies.[16,17,59,68,95-97] Uncontrolled B-cell proliferation may result in B-cell lymphoproliferative disease, which is associated with considerable morbidity and mortality.[17] Post-transplantation lymphoproliferative disorders (PTLD) refer to a range of hyperplastic to neoplastic lymphoid proliferations which occur after solid organ- or bone marrow transplantation, they mostly are of B-cell origin, and commonly (90%) contain EBV.[98] We shall refer to PTLD as EBV-lymphoproliferative disease (EBV-LPD) only if the presence of EBV was unequivocally demonstrated in malignant lymphocytes, thereby showing the causative role of EBV. Crawford et al were the first to demonstrate the presence of EBNA in a PTLD occurring in a renal transplant recipient.[99] The majority of PTLDs is EBV positive (EBV-LPD), as has been concluded from immunohistological and molecular studies. EBV-DNA has been found in PTLD tumor tissue by in situ hybridisation and Southern blotting.[17,100-102] Furthermore, EBV-specific proteins have been detected by immuno-histochemistry and Western blotting.[102,103] The incidence of PTLD varies among different transplant groups. The incidence is ± 1% among recipients of renal grafts, ± 2% after liver transplantation, 3-4% after heart transplantation, 8% after lung transplantation, 28.5% in recipients of intestinal transplants. The incidence of PTLD after allogeneic hematopoietic stem cell transplantation may vary between 2-25%.[104-112] Differences of incidence may reflect the intensity of immunosuppressive measures applied to prevent rejection or graft-versus-host disease after transplantation.

Clinical presentation

The clinical presentation of PTLD may vary considerably as B-cell lymphoproliferation may present in nodal or extranodal site(s) and also as a B-cell leukemia. Generally, 3 characteristic clinical presentations may be distinguished.[17,98,113-116]:

1. A mononucleosis-like syndrome with fever, sore throat, myalgia, tonsillar hypertrophy and cervical lymphadenopathy.
2. A tumorous form with symptoms secondary to the presence of lymphoid tumours including pain, obstruction or perforation.

3. Disseminated disease with monoclonal B-cells in blood and bone marrow, high fever, and/or multiorgan failure.

Most commonly, the first 2 presentations of PTLD are seen following solid organ transplantation, whereas the third form is more frequently encountered following allogeneic hematopoietic stem cell transplantation. The more aggressive clinical presentation of the latter may be explained by an immediate and severe immunopromised condition in the first months following allogeneic hematopoietic stem cell transplantation. PTLD is localised within the allografted organ in 36-100% of recipients of a renal or lung graft. In contrast, patients allografted with a liver or heart rarely experience PTLD at the site of their donor organ [17,104,116] Both origin (donor versus recipient) of the B-cell and virus may differ comparing PTLD following allogeneic hematopoietic stem cell transplantation and solid organ transplantation. PTLD generally develops in donor B-cells in the setting of an allogeneic hematopoietic stem cell transplantation, whereas recipient B-cells usually cause PTLD following solid organ transplantation.[117-119] PTLD most commonly originates from recipient EBV if the recipient has been EBV-seropositive before transplantation. [119] EBV seronegative recipients of a solid organ transplant may acquire donor EBV and subsequently be at risk of developing PTLD caused by donor EBV.[118,120-122]

Diagnosis of PTLD

A diagnosis of PTLD is usually made on the basis of lymph node histology. However, thorough histological examination, including morphology, immunohistochemistry to assess clonality and presence of LMP, and molecular evaluation to detect EBER or EBV-DNA should be performed in order to assess clonality and the causal relation with EBV (EBV-LPD). Investigations required for staging include whole body computer tomography, flow cytometric or molecular detection of monoclonal B-cells in blood, bone marrow, and cerebrospinal fluid (if indicated), or T-cell receptor rearrangement studies in case of a T cell lymphoma. Serology is of little value in the diagnosis of PTLD, and negative serology may even be misleading due to the severe immune dysfunction of patients.[123]

Pathological classification systems of PTLD distinguish 3 subtypes.[105,116,124-128]

1. Plasmacytic hyperplasia. The underlying architecture of the lymph node is preserved but is diffusely infiltrated by plasmacytoid lymphocytes with some plasma cells and sporadic immunoblasts. B-cells are preferably polyclonal. It furthermore lacks oncogene and tumor suppressor gene rearrangements, such as N-ras, c-myc, p-53, bcl-2 or bcl-6.[124]
2. Polymorphic B-cell hyperplasia/lymphoma. These tumors arise at nodal and/or extranodal sites and mostly show monoclonal lymhocyte infiltration. Histological examination shows a combination of plasmacytic lymphocytes / plasma cells and immunoblasts without atypia and only mild necrosis. No oncogene and tumor

suppressor gene rearrangements are present. When the transition to lymphoma occurs plasmacytoid differentiation is lacking, and atypical immunoblasts prevail in the presence of necrosis.

3. Immunoblastic lymphoma. Histologically, large monomorphic atypic immunoblasts are present with abundant necrosis. It may present as a disseminated monoclonal lymphocyte neoplasm, containing only one EBV-strain with alterations in one or more proto-oncogenes or tumor suppressor genes, such as N-ras, c-myc, p-53, bcl-2 or bcl-6. [124]

From a clinical point of view, the distinction between polymorphic hyperplasia and immunoblastic lymphoma has proven to be rather artificial, because both histological subtypes can be present concomitantly and they do not predict a different clinical course and response to therapy. Lesions may contain monoclonal and/or oligoclonal lymphocyte components, and may or may not express oncogene alterations. The term polymorphic PTLD has been introduced to refer to those PTLDs with characteristics of both immunoblastic and polymorphic B-cell hyperplasia.[105,128,129]
Immunohistology may include antibody staining with CD19, CD20, LMP-1, EBNA-1, and EBNA-2. In contrast to LMP, both EBNA-1 and –2 are expressed very heterogeneously in PTLD lesions.[126] In order to differentiate between poly- and monoclonal disease staining with monoclonal antibodies to kappa and lambda can be performed. Alternatively, immunoglobulin gene rearrangement studies can be done. Assessing clonality, however, may be of limited value because both poly- and monoclonal proliferations may coexist in one PTLD lesion.[130,131] Using molecular diagnostic techniques, such as in situ hybridisation and polymerase chain reaction, EBER and EBV-DNA can be detected.[132] These assays are important for a diagnosis of EBV associated disease.[100] Karyotypic analysis of biopsy material from PTLD lesions is usually normal, however, deletions or translocations of chromosome 6, trisomy 11, and t(8;14) can be found.[116] Untill recently, no systematic analysis has been performed to assess whether the presence of certain oncogenetic mutations in PTLD predicts clinical outcome. In a recent study, the prognostic value of mutations in the proto-oncogene BCL-6 in 33 solid organ recipients with histologically proven PTLD has been reported.[133] Mutations in BCL-6 appeared to predict failure of response to reduction in immunosuppression.

EBV load
EBV serology is the gold standard for diagnosing EBV-infection in immunocompetent individuals, but is of only limited value in immuno-compromised individuals.[134] Therefore, various authors have explored other ways to monitor EBV-infection in immunodeficient individuals. The diagnosis of EBV infection depends to a large extent on assaying viral nucleic acids (DNA) in conditions of immunodeficiency. Viral nucleic acids can be measured quantitatively in different compartments using various methods. Viral load can be assessed in plasma, mononuclear cells (MNC), or in whole blood samples.[135-147] Comparative polymerase chain reaction (PCR) assays with end-point dilution, quantitative competitive PCR assays as well as the more recent real-time quantitative PCR assays have

been used.[144,145,148,149] Real-time PCR monitoring of EBV DNA has emerged as a sensitive and reproducible test.[144,150,151,152] High levels of EBV DNA in peripheral blood may be associated with a diagnosis of EBV-LPD. However, a high viral load may also be present in patients with infectious mononucleosis and in transplant recipients without definite EBV-LPD.[142,150,153-155] On the other hand, a gradually increasing EBV load is an important risk factor for impending EBV-LPD.[135,138,143,149,155-157] In case of established EBV-LPD, EBV load may be used to monitor response to therapy.[139,151,153,156-159] A recent report suggested that plasma viral load may be more specific for a diagnosis of EBV-LPD following renal transplantation as compared with measurement of EBV-DNA in MNC.[151] Furthermore, plasma PCR may reflect more closely the response to therapy for EBV-LPD both following solid organ transplantation and allogeneic hematopoietic stem cell transplantation.[139,151,159]

Detection of EBV specific immunity

By using tetramers of specific HLA-class I molecules loaded with viral peptides, it has become possible to detect peptide-specific T-cells in the setting of cytomegalovirus (CMV)-disease and EBV-lymphoproliferative disease.[160-163] Marshall et al studied the development of EBV-specific $CD8^+$ T-cells using the tetramer technique following unmanipulated- and T-cell depleted allogeneic hematopoietic stem cell transplantation. They showed that EBV-specific T-cells formed a substantial percentage of CD8 T-cells within one month of unmanipulated transplantation. A correlation between T-cell numbers and viral load was demonstrated.[164] The recovery and function of these EBV-tetramer-binding T-cells following T-cell depleted allogeneic hematopoietic stem cell transplantation is currently unknown, but might greatly add to our understanding of the pathogenesis of EBV-LPD following allogeneic hematopoietic stem cell transplantation. Humoral immunity is of little value in the management of patients with PTLD following allogeneic hematopoietic stem cell transplantation and will therefore not be discussed.[123]

Risk factors for developing PTLD

The presence of a sufficient number of EBV-specific T-cells with potent anti-EBV activity is pivotal for prevention of PTLD.[52,98] Therefore, especially factors, which adversely affect the presence and maturation of T-cell-dependent immune responses and generation of cytotoxic T-cells, may become risk factors for EBV-LPD.

Risk factors in recipients of solid organ transplants.

Table 2 shows results observed in large studies performed in at least 100 recipients. Opelz et al assessed the incidence of PTLD among 52,775 solid organ transplant recipients (kidney and heart). They showed that the risk of PTLD correlated with the cumulative intensity of the immunosuppressive regimen applied for prevention of transplant rejection.[104]

Table 2. Risk factors for developing PTLD following solid organ transplantation *

Reference	Type of Tx	No of patients	Risk factor	No of patients with risk factor	No of patients at risk developing PTLD	Relative Risk (*range*)
Swinnen [165]	Heart	154	OKT3 dose (> 75 mg)	14	5	9.5
Opelz [104]	Kidney, heart	52,775	ATG/OKT3	17,355	117	1.8 (*1.3-2.5*)
			CyA+AZT	27,456	145	1.5 (*1.03-2.08*)
Walker [167]	Liver,heart, lung, kidney-pancreas	381	EBV-serostatus recipient (-)	18	9	24
			OKT3	177	12	6
			CMV-serostatus recipient (-)	66	6	4
Shpilberg [177]	Liver, lung, kidney, heart	303	$CD56^{+}DR^{+}$ count	nr	32	6.9
Swerdlow [174]	Heart,lung, heart-lung	1,563	EBV-serostatus recipient (-)	127	23	10

Tx indicates transplantation; OKT3, anti-T-cell antibody Ortho-Klone; ATG, Anti-Thymocyte globulin; CyA, cyclosporin A; AZT, azathioprin; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; nr, not reported; (-), negative; PTLD, post-transplant lymphoproliferative disease.

**) only risk factors that appeared significant were included in this table.*

Patients at highest risk were those, who had received immuno prophylaxis with anti T-cell antibodies (Relative Risk (RR): 1.8) and patients, who received a combination of the immunosuppressive agents cyclosporin A and azathioprine (RR: 1.5).[104,165] Swinnen et al evaluated the effect of the use of the anti-T-cell antibody Ortho-Klone (OKT3) on the incidence of PTLD in heart transplant recipients.[165] OKT3 is a murine monoclonal antibody reactive with the human CD3-receptor-T-cell complex and exerts broad anti-T-cell activity.[165,166] The incidence of PTLD was 1.4% in patients without OKT3 treatment as compared to 11.4% in patients who had been treated with OKT3 (RR: 9.5). Successive treatment courses with OKT3 further increased the cumulative incidence of PTLD to 35%.[165,167] The immunosuppressive drug mycofenolate mofetil in combination with cyclosporin A did not increase the incidence of PTLD in renal allograft recipients as compared to azathioprine/cyclosporin A or cyclosporin A alone.[168,169] Both cyclosporin A and tacrolimus primarily inhibit T-helper cell activation through inhibition of interleukin-2 production.[116] Mycofenolate mofetil exerts reversible inhibition of the enzyme inosine monophosphate dehydrogenase required for purine synthesis during cell division. Mycofenolate mofetil inhibits proliferation of both T and B cells and thereby the production of antibodies and generation of cytotoxic T-cells. Mycofenolate mofetil may therefore have a beneficial effect on prevention of proliferation of EBV infected B-cells.[170]

Apart from the intensity of the immunosuppressive regimen, the serostatus of the recipient also emerged as an important risk factor. The incidence of PTLD in EBV seronegative recipients may increase more than 20-fold as compared to EBV seropositive patients, which may be explained by the lack of EBV-specific cellular immunity of the recipient. [17,171-175] In addition, CMV serostatus also proved an adverse risk factor if the recipient had been CMV seronegative prior to transplantation and the donor CMV seropositive. [167]
That association is not entirely clear, but may be explained by reactivation of CMV in the absence of an adequate immune response and subsequent CMV-induced immune suppression.[167,176] A high absolute count of activated NK-cells ($CD56^+DR^+$), as assessed prior to transplantation, may also increase the risk for PTLD.[177] It may be explained by chronic antigenic stimulation, which especially occurs in patients with prior autoimmune diseases and an immune deficiency prior to transplantation.

Risk factors in recipients of an allogeneic hematopoietic stem cell graft
Table 3 shows results observed in large studies performed in at least 100 recipients. As in recipients of solid organ transplants, recipients of allogeneic hematopoietic stem cell grafts may become severely immunocompromised by immunosuppressive agents needed for prevention of rejection and graft-versus-host disease. In contrast to solid organ recipients, recipients of allogeneic hematopoietic stem cell grafts regenerate a new immune system, which originates from the donor. Well known factors impairing immune reconstitution following allogeneic hematopoietic stem cell transplantation include the degree of human leucocyte antigen (HLA-)mismatching between donor and recipient, higher age of the recipient, more intensive radiotherapy applied before transplantation, presence of graft-versus-host disease (GVHD) and T-cell depletion (TCD) of the donor stem cell graft.

Table 3. Risk factors for developing PTLD following Allogeneic hematopoietic stem cell transplantation *

Reference	No of Patients	Risk factor	No of Patients with risk factor	No of patients at risk developing PTLD	Relative Risk
Zutter [122]	2,475	OKT3	24	4	nr
		TCD	64	2	nr
		aGVHD II-IV	2,031	6	nr
Witherspoon [179]	2,246	TCD	nr	2	12.4
		OKT3	nr	3	15.6
		ATG	nr	7	4.9
		HLA mismatched donor	336	8	3.8
Gerritsen [115]	142	Campath+αCD11a	25	9	nr
		HLA TCD mismatched donor	65	9	nr
Hale [111]	2,582	TCD	20	5	15
Micallef [110]	428	TCD	44	5	30.5
		ATG	45	5	12.7
		aGVHD II-IV	221	7	7.7
		TBI+ATG	38	5	nr
Curtis [112]	18,014	HLA mismatched donor	3,390	30	4.1
		TCD	2,521	42	12.7
		ATG	1,101	25	6.4
		Anti-CD3	21	3	43.2
		aGVHD II-IV	7,063	42	1.9
		TBI	13,025	74	2.9
		Extensive cGVHD	3,872	16	4.0
Gross [109]	2,395	TCD	247	13	5.4
		HLA mismatch	315	11	2.2

TCD indicates T-cell depletion; aGVHD, acute Graft-versus-Host Disease; HLA, Human Leucocyte Antigen; TBI, total body irradiation; ext cGVHD, extensive chronic GVHD ; nr, not reported.

**) only risk factors that appeared significant were included in this table*

All these factors impair reconstitution of a new B-cell and T-cell repertoire following allogeneic hematopoietic stem cell transplantation and thus are also implicated as potential risk factors for PTLD.[109-112,115,122,175,176] Several studies have evaluated whether these factors would predict for PTLD and compared them to established risk factors in the setting of solid organ transplantation. The use of anti-thymocyte globulin (ATG) and especially OKT3, as has been observed after solid organ transplantation, were also strongly associated with a considerable risk for PTLD after hematopoietic stem cell transplantation.[110,112,122] Furthermore, the degree of T-cell depletion of the donor graft also emerged as an important risk factor. Stringent depletion enhanced the risk of PTLD in several studies.[109-112,115,122,175] The coexistence of more than 1 risk factor further enhances the risk. The incidence of PTLD rose to 24% in patients, who received a T-cell depleted stem cell graft from a HLA-mismatched donor in a study by Gerritsen et al.[115] The use of unrelated donor stem cell grafts, but especially the use of HLA mismatched donor grafts were also associated with an increased risk of PTLD, although the relative weight was less than the risk associated with the use of OKT3 or T-cell depletion.
A greater incidence of PTLD was also observed in patients developing graft-versus-host disease requiring treatment with anti-T-cell immuno-therapy such as anti-thymocyte globulin. [112]

Therapeutic approaches

One of the major problems in evaluating treatment of PTLD is the lack of prospective randomised trials. Furthermore, patients often receive multiple therapy modalities concomitantly with a reduction of immunosuppression. Therefore, it has been difficult to assess the merits of each intervention individually. However, various treatments have been evaluated and reported in literature.

Reduction of immunosuppression
Reduction of immunosuppression may induce complete remissions of PTLD after solid organ transplantation.[177] Especially patients with localized disease may respond, as patients with disseminated PTLD often show progression despite reduction of immunosuppression.[177,178] Reduction of immuno-suppression as such has not been studied following allogeneic hematopoietic stem cell transplantation. A retrospective analysis in heart-transplant recipients revealed that the majority of PTLDs occurring in the first year following transplantation respond favorably to reduction in immunosuppression.[179] If PTLD develops more than 1 year after transplantation, responses tended to be rare.[178-181] Histological subtype of PTLD did not affect the response rate, as responses were readily observed in each subtype and in polyclonal as well as monoclonal PTLDs.[129,133] A significant disadvantage of reducing immunosuppression is the obvious risk of organ graft rejection or acute graft-versus-host disease in case of allogeneic hematopoietic stem cell transplantation.[115,182-185] Therefore, immunosuppression is often interrupted temporarily or the dose reduced only partially. In conclusion, various retrospective studies have shown

that reduction of immune suppression is an important part in the treatment of patients with PTLD.

Antiviral drugs

To date no randomised studies have been published addressing the role of antiviral drugs in patients with PTLD. Also no clinical studies have shown that aciclovir and/or ganciclovir may induce durable responses in PTLD. In addition, PTLD has been described consistently in patients receiving both aciclovir and ganciclovir prophylaxis, again indicating the low therapeutic efficacy of these antiviral drugs.[153,180] Although EBV shedding from the oropharynx has been reported to be reduced by both aciclovir and ganciclovir, numbers of EBV-infected B-cells were not affected by these drugs.[186,187] The lack of efficacy of aciclovir and ganciclovir may be explained by the absence of EBV-induced thymidine kinase in latently infected B-cells, which is needed for both aciclovir and ganciclovir to exert their cytotoxicity.[188] Thymidine kinase is expressed during lytic cycle of infection, but not during latency.[188] At present it is unknown whether or when B-cells in PTLD lesions express thymidine kinase. It has been suggested to combine ganciclovir with arginine butyrate, as the latter compound may induce the expression of thymidine kinase.[188,189] However, no clinical studies have been reported recently. In conclusion aciclovir and ganciclovir cannot be recommended as therapy of PTLD.

Chemotherapy

In analogy with the treatment of malignant lymphoma in general, chemotherapy may also induce complete remissions in a high proportion (33-100%) of patients with PTLD. However, long term outcome has been rather disappointing due to high toxicity and sometimes transient duration of responses.[190-193] Septic complications in neutropenic patients accounted for the majority of treatment related mortality.[185,190,192,194] In order to prevent cardiac- and infectious toxicity, low-dose cyclophospamide, doxorubicin, oncovin and prednisone (CHOP) like regimes have been studied by Gross and Oertel.[195,196] They showed complete remission rates of 75% (6/8) and 100% (3/3), respectively, without severe toxicity in limited numbers of patients. Reduced treatment related mortality was also reported following ProMACE-CytaBOM chemotherapy in cardiac transplant recipients.[194] Complete remission rate measured 75% (6 out of 8 patients) and no patient relapsed after a median follow up of 64 months.[194] Only case-reports, no larger studies have been published with regard to the use of chemotherapy in PTLD following allogeneic hematopoietic stem cell transplantation. In conclusion, the reduced toxicity profile and high response rates of new chemotherapy schemes may favour the use of chemotherapy in PTLD following solid organ transplantation. Chemotherapy should be applied very cautiously after allogeneic hematopoietic stem cell transplantation as chemotherapy may adversely affect the immune system. Furthermore, the fact that hematopoietic stem cell recipients have often been treated with intensive radio-/chemotherapy prior to transplantation places them at an elevated risk of cumulative toxicity.

Interferon-α

Interferon-α is a cytokine with antiviral- and anti-neoplastic activity.[197-199] Several case reports have described complete remissions in patients with PTLD following solid organ transplantation treated with a combination of interferon-α and intravenous immunoglobulin.[198,200-203] Liebowitz studied 18 solid organ transplant recipients with PTLD, who were treated with a reduction of immunosuppression and interferon-α. Fifteen patients obtained complete or partial remissions, but median survival was short and measured approximately 6 months.[204] Three studies evaluated the use of interferon-α for PTLD following allogeneic hematopoietic stem cell transplantation. Durable responses were reported in several patients, however, the specific value of interferon-α could not be assessed in these studies as most patients received additional treatment modalities.[109,205,206] Toxic side effects of interferon-α were noted in a considerable proportion of patients. Interferon-α may also negatively contribute to induction of graft rejection or induction of graft-versus-host disease, which may preclude a wider use of interferon-α in PTLD.[109,204] In conclusion, although initial reports on interferon-α are promising, larger prospective trials are needed.

Anti B-cell immunotherapy

Monoclonal antibodies with specificity for the B-cell surface molecules CD21, CD24 and CD20 have been successfully applied in the treatment of PTLD (Table 4, Table 5).[185,207-220] These monoclonal antibodies may exert anti B-cell cytotoxicity by opsonization and antibody-dependent cellular cytotoxicity. Anti-CD21 may also block the receptor by which EBV intrudes the B-cell.[221] Recent reports showed that especially complement mediated cytotoxicity plays an important role when CD20 is used.[222,223] High remission rates (50-80%) were reported in the first two prospective clinical studies using anti-CD21 and anti-CD24 in solid organ- and bone marrow recipients with established PTLD.[207,208] Relapse rates in those studies were approximately 10% and survival at 12 months from immunotherapy was ± 50%. Comparable response rates were observed in studies using anti-CD20 immunotherapy using the humanized anti-CD20 monoclonal antibody rituximab (± 70%).[211] Both recipients of solid organ transplants and hematopoietic stem cell grafts showed high response rates and overall survival probabilities of 65 to 75%. Mortality from progressive PTLD was mainly observed in patients with multivisceral disease, central nervous system involvement, or late onset PTLD (> 1 year following transplantation). [207,221] No serious adverse effects have been described following anti B-cell immunotherapy. Mild neutropenia may occur in 40% of patients receiving anti-CD21 and anti-CD24, and hypotensive reactions secondary to cytokine release have been reported in 10% of patients with bulky disease.[221] B-cell lymphopenia of 6-9 months duration may occur following monoclonal anti-CD20 immuno therapy, which as yet has not been associated with a significant increase in the susceptibility to infections nor with decreased serum immunoglobin levels.[224]

Table 4. Response of PTLD to anti B-cell immunotherapy following allogeneic hematopoietic stem cell transplantation

Reference	No of patients	Therapy	Remission		Relapse	Survival (%) at 1 year
			Complete	Partial		
Fischer [208]	10	αCD21+αCD24	9	1	3	58
Benkerrou [207]	28	αCD21+αCD24	16	1	2	38
Milpied [211]	6	αCD20	5	-	1	66
Kuehnle [215]	3	αCD20	3	-	0	nr
Faye [225]	12	αCD20	8	-	0	88

α CD20/21/24 indicates, monoclonal antibody therapy against CD20/21/24, respectively; nr, not reported.

In conclusion, anti-B-cell immuno therapy has been established in several phase II studies as an effective and non-toxic therapy for PTLD in both solid organ- and allogeneic stem cell transplantation. Since anti-CD20 monoclonal antibody therapy has been licensed (rituximab), that type of immunotherapy will be applied most frequently. The dose and scheme of administration of rituximab may be subject of further study.

Adoptive transfer of T-cell immunity

Infusion of donor lymphocytes from an EBV-seropositive donor has been proven to be effective in the treatment of established PTLD following allogeneic hematopoietic stem cell transplantation (Table 6). [109,148,228-231] A major drawback, however, is graft-versus-host disease ensuing following donor lymphocyte infusion (30-60%), often resulting in considerable morbidity and mortality. To avoid graft-versus-host disease induced by allo-reactive T-cells, Rooney et al infused polyclonal EBV-specific cytotoxic T-cells in patients who had evidence of uncontrolled EBV replication, either as an elevated EBV load (> 20,000 EBV genomes per μg mononuclear cell DNA) or frank EBV-LPD, following allogeneic hematopoietic stem cell transplantation. Two of 3 patients obtained complete remission, the third patient had a complete remission of a histologically proven PTLD. No patient developed graft-versus-host disease. Functional EBV-specific cytotoxic T-cells could be traced by gene-marking until 18 months following initial infusion.[232,233] Recently, these results were confirmed in a larger study. Anti-EBV cytotoxic T-cell infusion were administered prophylactically at a median of 3 months following allogeneic hematopoietic stem cell transplantation in 39 patients.

Table 5. Response of PTLD to anti B-cell immunotherapy following solid organ transplantation

Reference	Tx	No of patients	Therapy	Remission		Relapse	Survival (%) at 1 year
				Complete	Partial		
Fischer [208]	Heart, heart-lung, liver, kidney, kidney-pancreas	8	αCD21+αCD24	7	1	0	58
Leblond [185]	Heart, lung, kidney	10	αCD21+αCD24	8	1	1	50
Benkerrou [207]	Heart, kidney, lung, heart-lung	31	αCD21+αCD24	20	3	2	55
Cook [212]	Lung	3	αCD20	2	1	1	nr
Zompi [216]	Liver	3	RI+ αCD20	2	-	0	nr
Milpied [211]	Liver, kidney, heart, lung, kidney-pancreas, liver-kidney	30	αCD20	15	2	0	77
Oertel [213]	Heart, Liver, kidney	6	αCD20	4	-	0	nr
Caillard [226]	Kidney	13	RI+ αCD20	7	-	nr	nr
Verschuuren [227]	Lung	3	αCD20	3	-	1	66

Tx indicates transplantation; α CD20/21/24, monoclonal antibody therapy against CD20/21/24; RI, reduction of immune suppression; nr, not reported.

In these patients no PTLD occurred, whereas 11% of historical controls (7 out of 61) developed PTLD.[234] PTLD following solid organ has also been treated with EBV-specific cytotoxic T-cells. Complete remissions were observed in several cases without the occurrence of rejection.[235-237] Although the treatment strategy is highly specific and without major side-effects, preparation of EBV-specific cytotoxic T-cells is time-consuming and technically elaborate. Furthermore, additional drawbacks may include a diminished effectivity during use of steroids, occurrence of exaggerated inflammatory response in case of bulky disease, and unresponsiveness to EBV-specific cytotoxic T-cells as a result of mutation of EBV epitopes.[215,234,238] In conclusion, treatment of PTLD following allogeneic hematopoietic stem cell transplantation by donor lymphocyte infusion is effective, but may be complicated by severe graft-versus-host disease. The preparation of EBV-specific cytotoxic T-cells seems promising, but requires rather elaborate technical preparations.

Table 6. Response of PTLD to donor lymphocyte infusion or EBV specific CTLs following allogeneic hematopoietic stem cell transplantation

Reference	No of patients	Therapy	Complete Remission	Relapse	Survival (%) at 1 year
Papadopoulos [228]	5	DLI	5	0	60
Rooney [233]	3	CTL	3	0	nr
Lucas [148]	5	DLI	1	0	0
	1	CTL	1	0	nr
Rooney [234]	6	CTL	6	0	nr
Gross [109]	3	DLI	0	0	nr

DLI, indicates donor lymphocyte infusion; CTL, cytotoxic T-lymphocyte infusion; nr, not reported.

Prevention of PTLD following solid organ transplantation and allogeneic hematopoietic stem cell transplantation

As morbidity and mortality of established PTLD following solid organ transplantation and allogeneic hematopoietic stem cell transplantation is high, the emphasis should be on prevention of PTLD. The therapeutic approaches described above may also be applied for prevention. To date, no trials have been reported specifically addressing prophylaxis with antiviral agents such as aciclovir or ganciclovir. A reduced incidence of PTLD following solid organ transplantation was suggested in patients treated prophylactically with aciclovir or ganciclovir. However, these studies mostly had a non-randomized design without a

control group or PTLD was a secondary endpoint.[239-244] In the setting of allogeneic hematopoietic stem cell transplantation, no benefit has been reported of aciclovir or ganciclovir prophylaxis with respect to PTLD.[122,178] B-cell depletion of the hematopoietic stem cell graft using monoclonal anti B-cell antibodies was reported very effective in the prevention of PTLD following allogeneic hematopoietic stem cell transplantation as compared to historical controls.[245,246] Furthermore, B-cell depletion did not delay engraftment, or increase incidence of infection. Rooney et al. evaluated the prophylactic infusion of EBV-specific cytotoxic T-cells in patients with high risk features such as HLA mismatch and T-cell depletion. A significant reduction in the incidence of PTLD was observed as compared to historical controls (0/39 versus 7/100).[234] Gustaffson et al. prophylactically administered EBV-specific cytotoxic T-cells following allogeneic hematopoietic stem cell transplantation guided by viral load and observed 1 PTLD out of 4 patients treated.[246] In contrast to the experience with CMV, prophylaxis guided by viral load as yet has gained little interest in the prevention of PTLD sofar.

4. Outline of this thesis

Until recently, EBV-LPD could only be diagnosed in recipients of an allogeneic hematopoietic stem cell transplantation with overt established EBV-LPD, usually presenting as a critical illness in patients with generalized lymphadenopathy. The diagnosis was made on the basis of lymph node histology, since early and sensitive markers of EBV infection and reactivation were lacking. Recently the development of PCR-based assays has created the possibility to sensitively and quantitatively monitor EBV-DNA in peripheral blood samples and to evaluate their diagnostic value in immunocompetent patients with EBV infection and in immuocompressed patients with (impending) EBV-LPD.

The development of a real-time quantitative PCR assay for detection of EBV-DNA is described in chapter 2. The assay was evaluated in-vitro using an EBV standard determined by electron microscopy and in-vivo using plasma samples of patients with EBV-infection or EBV-LPD. Using that assay, we then retrospectively assessed the predictive value of the assay using plasma samples from a large cohort of recipients of an allogeneic hematopoietic stem cell transplantation. One hundred and fifty-two patients of whom 2-weekly plasma samples were available, were examined for predictive parameters as regards the incidence of EBV reactivation and EBV-disease. In addition, risk factors for reactivation and disease were assessed and correlated to transplant outcome measures (chapter 3).

In a cohort of 14 patients presenting with EBV-LPD, we next asked the question whether quantitative follow-up levels of EBV-DNA would predict response to therapy and survival. Quantification of EBV-DNA appeared as a very accurate and sensitive marker of response (chapter 4). Moreover, quantification of EBV-DNA before the onset of clinical overt EBV-

LPD appeared to accurately predict impending EBV-LPD. High positive and negative predictive values of EBV-DNA were established. Based on these results a prospective clinical study was designed aiming at the prevention of EBV-LPD and EBV-LPD-mortality. This prospective phase II study included 49 recipients of an allogeneic hematopoietic stem cell transplantation, and 15 patients received pre-emptive treatment when EBV load in plasma exceeded a threshold level of 1,000 EBV genome equivalents per ml. Comparison of prospectively followed patients to a recent historical cohort showed effective prevention of EBV-LPD and EBV-LPD-mortality (chapter 5).

Although the positive predictive value of quantified EBV-DNA appeared relatively high, most patients with viral reactivation were able to mount an immune response and clear their viral reactivation. Sofar, little was known with respect to the recovery of EBV specific cellular immunity following allogeneic hematopoietic stem cell transplantation. The recent introduction of HLA-class I tetramers presenting viral peptides has enabled the monitoring of peptide specific cytotoxic $CD8^+$ T-cells in peripheral blood samples following allogeneic hematopoietic stem cell transplantation. We were able to use several EBV peptide specific tetramers and monitor the recovery of EBV specific $CD8^+$ T-cells. The question whether impaired recovery of cellular T-cell immunity would identify patients at serious risk of EBV reactivation and progression to EBV-LPD was addressed in 61 recipients of a T-cell depleted allogeneic hematopoietic stem cell transplantation. Results described in chapter 6 suggest a pivotal protective role of T-cell immunity against EBV in recipients of an allogeneic hematopoietic stem cell graft. Finally, the overall results of our studies and future issues with respect to diagnosis, prevention and treatment of EBV-LPD are discussed in the general discussion (chapter 7).

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2. Development of a Real-Time Quantitative Assay for detection of Epstein-Barr Virus.

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Abstract

Using a real-time polymerase chain reaction (PCR), we developed and evaluated a rapid, sensitive, specific and reproducible method for the detection of Epstein-Barr virus (EBV) DNA in plasma. This method allowed us to screen plasma and serum samples over a range between 100 and 10^7 genome equivalents of EBV-DNA per milliliter (geq/ml) using two sample preparation methods based on absorption. A precision study yielded an average coefficient of variation for both methods of less than 12 %, with a coefficient of regression for the standard curve of a minimum of 0.98. We detected EBV-DNA in 19.2% of plasma samples from immunosuppressed solid organ transplant patients without symptoms for EBV infections, with a mean load of 440 geq/ml. In all transplant patients diagnosed with EBV related lymphoproliferative disease (EBV-LPD), EBV-DNA could be detected with a mean load of 544,570 geq/ml. No EBV-DNA could be detected in healthy individuals, and a mean of 6,400 geq/ml could be detected in patients with infectious mononucleosis. Further studies revealed that the inhibitory effect of heparinized plasma could be efficiently removed by use of an extraction method with Celite as the absorbent.

1. Introduction

Epstein-Barr virus (EBV) is the etiological agent of infectious mononucleosis (IM) and is etiologically associated with Burkitt's lymphoma and nasopharyngeal carcinoma. Usually, the virus produces a mild and self-limiting primary infection in childhood. However, as a gamma herpesvirus, it persists for life by a combination of latency in B-lymphocytes and chronic replication in oropharyngeal epithelial cells.[1] A serious complication after allogeneic hematopoietic stem cell transplantation and solid organ transplantation is the development of EBV-related lymphoproliferative disease (EBV-LPD) due to immunosuppressive therapy. [2] The condition can be rapidly fatal if it is not diagnosed and treated in an early stage.

Recently, it has been observed that there is a relation between EBV-LPD and the EBV load in plasma or infected peripheral blood lymphocytes, as measured by semiquantitative or competitive polymerase chain reaction (PCR) assays. [3-10] With the advent of real-time Taqman quantification and improved sample preparation techniques, the whole process from sample retrieval to quantitative result can be reduced. Furthermore, the dynamic range in which samples can be analyzed quantitatively without dilution has improved considerably. [8,12,13]
In this paper, we describe the validation of a Taqman based assay for the quantification of EBV-DNA in plasma. The assay is based on the linearity, takes into account intra- and inter assay variability, as well as detection limits, and can be performed in a routine setting, providing quantitative results within less than 6 hours. Furthermore, we have evaluated two different extraction methods, not only for EDTA-treated plasma, but also for heparin-treated plasma, because heparin is known to be inhibitory for PCRs. [14,15]

2. Materials and methods

Patients and samples

Serum samples from patients with a clinical suspicion for a primary EBV-infection (infectious mononucleosis, n=22) and a serological profile positive for IgM viral capsid antigen (VCA) and negative for anti-EB nuclear antigen (EBNA), were used for this analysis. These samples were kindly provided by dr Peter Schroder (Groningen Public Health Laboratory). Plasma samples from recipients of an allogeneic hematopoietic stem cell graft (n=5) and solid organ transplant patients (n=5), who were diagnosed clinically and histologically with EBV-LPD, were also enrolled in the present evaluation study. Samples were taken before start of treatment (including before the start of reduction of immunosuppressive treatment) was initiated. Lymph node biopsy specimens were also obtained from these patients and the diagnosis was confirmed with the EBER probe. Furthermore, samples from a cohort of randomly selected, EBV-seropositive, solid organ

transplant recipients (kidney, heart and liver, n=109) were included for cross-sectional analysis. These patients had no EBV- related disease and were routinely screened for hepatitis markers. Healthy individuals and blood donors (n=100) without any sign of infectious mononucleosis or EBV-LPD were used as a control group. From all patients, EDTA plasma samples were aliquoted and frozen at -80ºC within 2 hours after collection. Only serum was available from the 22 patients with a primary EBV infection.

Nucleic acid extraction

For the isolation of EBV-DNA from plasma or serum samples, two protocols were used. The first protocol was essentially based on the method described by Boom and coworkers. [16] Briefly, 100 µl of plasma was added to 1 ml of buffer 1 (120 g guanidinium isothiocyanate in 100 ml 0.1 M Tris [pH 6.4], 22 ml 0.2 M EDTA [pH 8.0] 2.6 g Triton X-100). After the addition of 50 µl Celite solution, the mixture was incubated for 10 minutes at room temperature and subsequently centrifuged for 10 s at full speed in a tabletop centrifuge. The pellet was washed twice with buffer 2 (identical to buffer 1 but without Triton X-100 solution and EDTA), twice with 70 % ethanol, and once with aceton. The silica pellet was dried at room temperature in a vacuum exsiccator for 10 min, after which the DNA was eluted from the silica by adding 100 µl RNAse and DNAse-free water and was incubated for 10 min at 56°C. After centrifugaton at 12,000g for 2 min, the supernatant contained the DNA and was ready for use.

The second and commercially available protocol was essentially based on the High Pure Viral Nucleic Acid kit protocol (Roche Diagnostics, Almere, The Netherlands). To compare this method directly with the above described procedure, a 100 µl plasma sample is added to the mixture provided with the kit, and finally, the same volume of 100 µl is eluted. Briefly, a 100 µl plasma sample was added to 100 µl 6 M guanidine-HCl-10 mM urea-10 mM Tris-HCl-20% (vol/vol) Triton X-100 supplemented with carrier RNA and 800 µg of proteinase K. After incubation for 10 min at 72ºC, 50 µl isopropanol was added and the mixture transferred onto a High Pure filter tube combined with a collection tube. The filter tube was centrifuged at 12,000g for 1 min in a standard tabletop centrifuge at room temperature. The filter was washed twice with 450 µl of buffer (20 mM NaCl and 2 mM Tris-HCl [pH 7.5] in ethanol). After placement of a new collection tube under the filter, 100 µl Rnase- and Dnase-free water was added to elute the DNA. To reduce the detection level of the assay, the input and elution volumes compared to those used in the original procedure can be changed, that is, the input volume can be increased to 200 µl of plasma and the elution volume can be decreased to 50 µl.

Real-Time Taqman assay

The PCR primers for the Taqman assay were selected from the EBV-DNA genome and encode the nonglycosylated membrane protein BNRF1 p143. [17,18] The forward and reverse

primers and the probe were designed using Primer Express software (PE Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), and generated a DNA product of 74 basepairs.

The primers used were EBV/p143 forward primer (5'-GGA.ACC.TGG.TCA.TCC.TTG.C) and the reverse primer (5'-ACG.TGC.ATG.GAC.CGG.TTA.AT), which were synthesized at Isogen Biosciences (Maarssen, The Netherlands). A fluorogenic probe (5'-CGC.AGG.CAC.TCG.TAC.TGC.TCG.CT) was synthesized by PE Biosystems with a FAM reporter molecule attached to the 5' end and a TAMRA quencher linked at the 3' end. The PCR amplification was performed in a 50-µl volume containing 2x Taqman universal master mixture, 45 pmol of forward primer per µl, 2.5 pmol of reverse primer, 5 pmol of the Taqman probe, and 10 µl of isolated DNA. All reactions were performed in duplicate. After preparation of the reaction tubes, the whole plate holder was centrifuged at 1,000g for 1 min at room temperature in a swingout rotor (Hettich, Rotina 48R, Tuttlingen, Germany) to remove small air bubbles in the vessels. The amplification and detection was performed with an ABI Prism 7700 Sequence Detection System (PE Biosystems). After incubation for 2 min at 50 °C with uracil *N'*-glycosylase to inactivate possible PCR contaminants from former reactions, the reaction tube was incubated for 10 min at 95°C to inactivate the uracil *N'*-glycosylase and to release the activity of the AmpliTaq Gold DNA polymerase. The PCR cycling program consisted of 42 two-step cycles of 15 s at 95°C and 60 s at 60°C. Real-time measurements were taken, and a threshold cycle (C_t) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit of 0.04. Each run contained several negative controls (no template), a positive control containing a known EBV copy number based on a standard for which the EBV copy number was counted by electron microscopy (EBV EM standard), and a standard dilution curve for plasmid DNA containing the PCR product as insert (see below). Each specimen was run in duplicate and was considered positive only if both replications were above the threshold limit.

Standardization

For the standardization of the assay, a standard containing 6.68 x 10^9 EBV particles per ml (EBV B95-8, Advanced Biotechnologies Incorporated, Maryland, U.S.A.), as determined by electron microscopy, was used. Serial half-log dilutions of this standard, ranging from 10^7 down to 10 geq/ml, were made to characterize linearity, precision, specificity and sensitivity of the Taqman assay.
For the preparation of the standard curve for the routine Taqman runs, the PCR product of 74 basepairs was directly cloned into a pCRII vector (InVitrogen, Leek, The Netherlands) and transformed into the appropriate bacterial strain. The colonies were prescreened by PCR to confirm the size of the insert. Plasmid DNA was isolated on the Vistra Labstation (Amersham Pharmacia Biotech, The Netherlands) and was isolated in bulk. The standard curve made from the plasmid was calibrated using the EBV EM standards and was

routinely made in duplicate with a range equivalent to from 100 up to 10^7 geq/ml. It was shown that the slope for the plasmid standard was not significantly different from the slope obtained with the EBV EM standard ($P < 0.0001$) (data not shown).

Statistics

The standard curve was created automatically by the ABI 7700 Sequence Detection System software by plotting the C_t values against each standard of known concentration. This C_t value was also used for calculation of the intra- and interassay coefficients of variation for the technique. Logarithmic transformation of the readings of the different assays was carried out for the comparison of the isolation procedures. *x*-*y* scatter diagrams were drawn, and the correlation coefficients (r^2) or Spearman correlation (r) was determined and linear regression analysis was done by using the statistical functions of SPSS (version 8.0) software. Student *t* test was used for comparison of EBV-DNA copy numbers in each group analyzed.

3. Results

The limit of detection by both extraction methods was determined with half-log dilutions of the EBV EM standard. Both assays were used in an identical format, in which 100 µl of patient material was used as input, while the DNA was eluted in 100 µl RNAse- and DNAse-free water. Both assays were able to detect viral DNA over a linear span of between 100 and 10^7 geq/ml (Figure 1). Statistical analysis of the standard curves over this range showed that both methods were linear with an r^2 value of a minimum of 0.98. Furthermore, the slopes of both standard curves were not significantly different. However, the average C_t values obtained by the extraction method described by Boom et al. were 1.44 lower over the whole linear range ($P < 0.0001$) than those obtained with the High Pure Viral Nucleic Acid extraction kit.[16] This shows that the efficiency of the extraction step was better for the method described by Boom et al., as also indicated by the fact that 50 geq/ml of the dilution were detected in all eight replicates, whereas 50 geq/ml were detected in only one of eight replicates with the adapted High Pure Viral Nucleic Acid extraction kit.[16] Both methods were unable, however, to detect 10 geq/ml of the EBV EM standard per ml used in the formats described above.[16]

A precision study for both extraction methods was performed by evaluating serial half-log dilutions of the EBV EM standard ranging from 50 to 10^7 geq/ml in originally EBV-seronegative serum. The C_t values obtained were used for the calculation. The study was carried out over 3 consecutive days and two sets of independent isolations were performed.

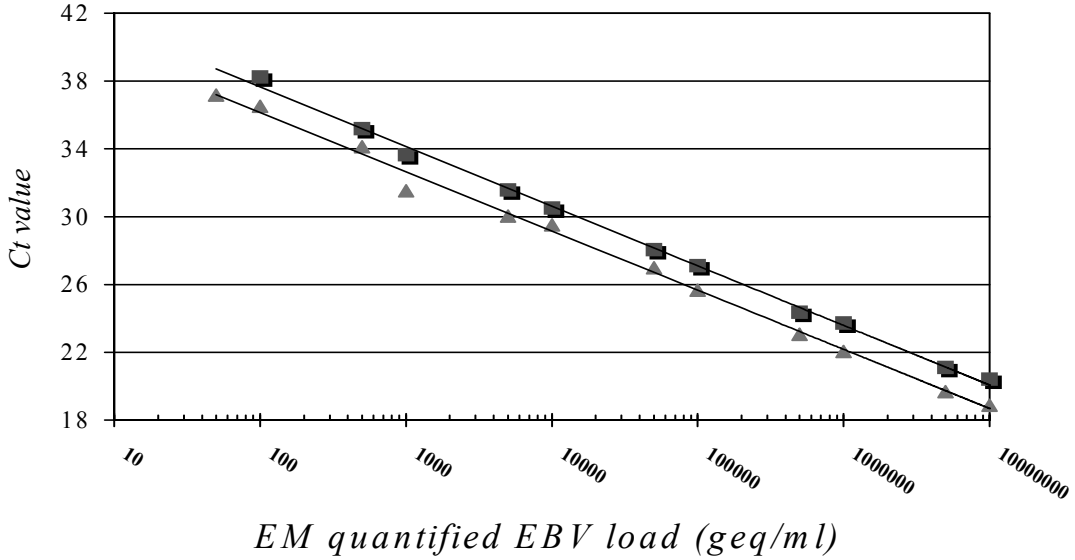

Figure 1. Standard curve for Taqman PCR. Serial dilutions of the EBV EM standard ranging from 50 to 10^7 geq/ml were made. Extraction was performed by the method of Boom (▲, Spearman correlation coefficient, 0.997) or the method with the High Pure Viral Nucleic Acid kit (■, Spearman correlation coefficient, 0.998) as matrix. Equal volumes of input and output material (100 µl) were used. The C_t values, which correspond to the PCR cycle number in which the value is above the threshold limit, are plotted against the calculated number of particles counted by electron microscopy.

A total of eight replicates of the 12 dilutions for both extraction methods were tested on each day. Again, only the method of Boom et al. was able to detect EBV in the sample with 50 geq/ml. [16]

The assay exhibited a very good total precision throughout the range of the numbers of EBV-DNA copies in the EBV-seropositive samples, with coefficients of variation ranging from 0.7 to 11.7%. The relatively high coefficient of variation (11.7%) was due to the inability to detect 100 geq/ml in one of the eight replicates by the method with the High Pure Viral Nucleic Acid extraction kit. The average coefficient of variation for the High Pure Viral Nucleic Acid extraction kit was 2.37% (range, 1.1-11.7%), and for the method of Boom et al. 1.56% (range, 0.7-7.0). There was no difference in between-day variation and within-run variation, or within the independent isolations (data not shown).

We furthermore evaluated whether both extraction methods were able to remove efficiently the inhibitory effect of heparin on the PCR-based assay. Therefore, four dilutions of the EBV EM standard were made in heparin- or EDTA-treated plasma, the dilutions ranged from 500 to 10^7 geq/ml. The efficiencies of isolation and amplification by the method of Boom et al were almost identical whether EDTA-treated or heparin-treated plasma was used (Figure 2), with variation being less then twofold.[16]

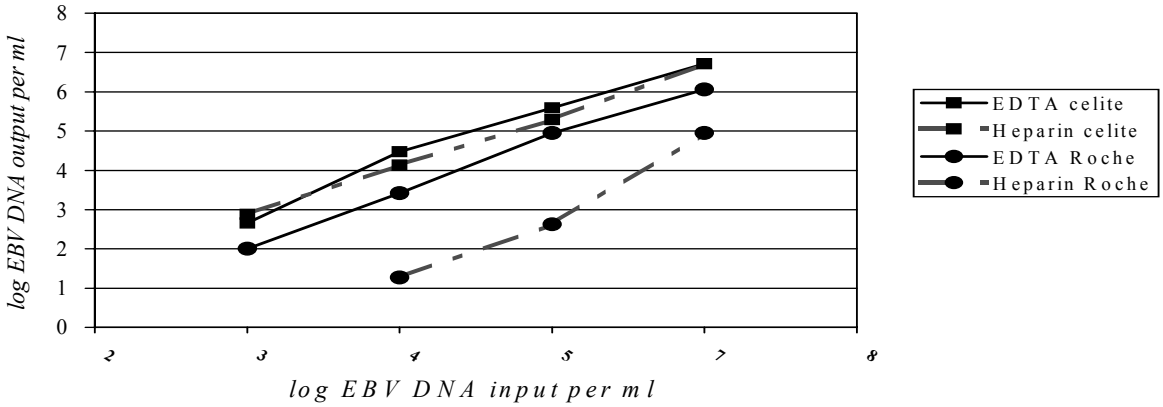

Figure 2. Effect of heparin on real-time Taqman PCR. Detection of EBV EM standard dilutions made in heparin-treated plasma (dashed line) or EDTA-treated plasma (solid line). From these EBV dilutions, ranging from 500 to 10^7 geq/ml, DNA was isolated by the extraction method of Boom et al., which uses Celite (■) to absorb the DNA, as well as by the method with the High Pure Viral Nucleic Acid kit (Roche Diagnostics) (●). The extracted DNA was quantified by the Taqman assay.

However, with the High Pure Viral Nucleic Acid extraction kit, the efficiency was reduced between 12- and 200-fold when heparin-treated plasma samples were compared to EDTA-treated plasma samples. EBV could not be detected in the heparin-treated plasma sample containing 500 geq/ml. Use of EDTA is much easier than the use of heparinase I, which degrades heparin and involves another incubation step. [14] This experiment also confirms again that the method of Boom et al. is able to extract the EBV DNA more efficiently than the High Pure Viral Nucleic Acid extraction kit, as indicated by the lower C_t value.
The analytical specificity of the assay was determined by analyzing DNAs from other human herpesviruses (Herpes simplex virus types 1 and 2, Varicella zoster virus, Cytomegalovirus, and human Herpesviruses 6, 7 and 8), as well as from other viruses routinely used in the laboratory for DNA or RNA analysis (Hepatitis B, C, and G viruses and Human Papilloma virus). All these samples yielded results below the detection level of 100 geq/ml or a C_t value of 42.

4. Discussion

To demonstrate that the Taqman-based assay described here could be used to detect EBV-DNA in clinical samples and to determine a baseline value for plasma EBV-DNA levels in different groups, we evaluated clinical samples from 100 healthy individuals, 22 patients with infectious mononucleosis, a cohort of 109 asymptomatic immunosuppressed solid organ transplant recipients, and 10 patients with a confirmed diagnosis of EBV-LPD. The results are summarized in Table 1. For this evaluation, samples were analyzed in duplicate

using the High Pure Viral Nucleid Acid extraction kit, which has a cutoff value of 100 geq/ml. As expected, EBV-DNA could not be detected in the plasma of any of the 100 healthy individuals.

We were able to detect low levels of EBV-DNA in 21 of 109 solid-organ transplant patients (19.2%), with a mean of 440 geq/ml (range, <100-12,000 geq/ml). This was, however, not statistically different from the value for the control group (P = 0.19). We could detect a signal for EBV-DNA in 16 of 22 samples (72.7%) from infectious mononucleosis patients, with a mean value of 6,400 geq/ml (range, <100-45,000 geq/ml). This EBV- DNA load was significantly higher as compared to the control group (P < 0.006). It has been shown previously, that the presence of EBV-DNA in plasma is diagnostic for a clinical EBV infection.[5,6,19] EBV-DNA should be absent in the plasma of healthy individuals.

Table 1. Quantification of EBV-DNA by real-time Taqman analysis

Patient Group	No. of Patients	% positive	Mean EBV DNA load[a] (geq/ml, range)
Healthy Donors	100	0	<100
Solid-Organ Transplant Patients	109	19.2	440 (<100 – 12,000)[b]
Infectious mononucleosis	22	72.7	6,400 (<100 – 45,000)[c]
EBV-LPD	10	100	544,750 (74,000 – 3,200,000)[d]

a *All values are averages of two independent experiments*
b *Not statistically different from healthy control group (P = 0.19)*
c *Statistically different from control group (P < 0.006) and Solid-Organ Transplant Group (P < 0.0001).*
d *Statistically different from all other groups studied (P < 0.0001).*

Using a sensitive detection method like PCR, however, one is able to detect viral genomes in peripheral blood mononuclear cells (MNCs) of healthy controls.[8,19] In the study of Kimura et al., a viral load of 315 copies per µg of MNC DNA was set as a criterion for distinguishing a latent infection from a symptomatic EBV-infection or EBV-related disease.[7]

EBV-DNA could be detected in 16 of 22 samples from infectious mononucleosis patients with primary infection. It can be concluded from the data of Yamamoto et al. that this is due to the time point of sampling.[19] Also, the group of Kimura et al. did not find a positive

EBV-DNA signal in MNCs from all of their IM patients analyzed.[8] We expected to be able to find active replication of EBV in the group of immunocompromised solid-organ transplant recipients due to immune suppression. We were able to detect EBV-DNA in plasma of 19.2% of patients analyzed. None of these patients had clinical signs of active EBV-infection or EBV-related disease. However, one could expect specifically that EBV reactivation is more likely to occur in this group than in healthy individuals. Our findings indicate that plasma EBV loads of up to 12,000 geq/ml can easily be detected in our cohort without any evidence of EBV related disease. However, no data are available on the longitudinal follow- up period required to determine whether several EBV reactivation periods can be detected in this group. We could confirm the data from Kimura et al, who also detected EBV-DNA in MNCs from 14% of posttransplant patients without signs of EBV-related disease. [8] The group for which the use of a quantitative PCR should be most useful are patients with a diagnosis of EBV-LPD. In the group of 10 transplant patients diagnosed with EBV-LPD but for whom treatment such as reduction of immunosuppressive therapy, or the initiation of antiviral treatment was not yet initiated, the mean EBV load in plasma was 544,750 geq/ml (range, 74,000- 3.2 x 10^7 geq/ml), which is significantly higher than in the groups mentioned above ($P < 0.0001$). However, there is a difference between a clinical diagnosis of EBV-LPD and the viral load at which one should be aware that EBV-LPD is developing. Therefore, we suggest that routine monitoring of patients at risk for EBV-LPD will allow determination of whether there is a progression of this life-threatening disease from a virological point of view.

In summary using real-time PCR technique, an easy-to-use and highly reproducible technique is available for evaluation of the significance of EBV-DNA in plasma samples of imunosuppressed patients. Depending on the isolation method used, inhibition by heparin of the amplification reaction can be eliminated. In this study we also confirm data presented by others that there is a relation between plasma EBV-DNA levels and EBV-related diseases. However, active replication could also be detected in patients without clinical EBV-related disease. Future studies must define cutoff levels at which treatment of patients at risk for EBV-LPD should be initiated. The technique can then be used to monitor the effect of antiviral therapy on EBV, whether this is by infusion of donor T-cells, a change of immunosuppressive therapy, or provision of nucleoside analogues to inhibit EBV replication.

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3. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic hematopoietic stem cell transplantation and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted stem cell transplantation.

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Abstract

Reactivation of the Epstein-Barr virus (EBV) after allogeneic hematopoietic stem cell transplantation (allo-SCT) may evoke a protective cellular immune response or may be complicated by the development of EBV-lymphoproliferative disease (EBV-LPD). So far, very little is known about the incidence, recurrence, and sequelae of EBV reactivation following allogeneic hematopoietic stem cell transplantation. EBV reactivation was retrospectively monitored in 85 EBV-seropositive recipients of a T-cell- depleted (TCD) allogeneic hematopoietic stem cell transplantation and 65 EBV-seropositive recipients of an unmanipulated allogeneic hematopoietic stem cell transplantation. Viral reactivation (more than 50 EBV genome equivalents geq/ml) was monitored frequently by quantitative real-time plasma polymerase chain reaction (PCR) until day 180 after stem cell transplantation. Probabilities of developing viral reactivation were high after both unmanipulated and TCD-allogeneic stem cell transplantation (31% ± 6% versus 65% ± 7%, respectively). A high $CD34^+$ cell number of the graft appeared as a novel significant predictor ($P = 0.001$) for EBV reactivation. Recurrent reactivation was observed more frequently in recipients of a TCD-graft, and EBV-LPD occurred only after TCD- stem cell transplantation. High-risk status, TCD, and use of ATG were predictive for developing EBV-LPD. Plasma EBV-DNA quantitatively predicted EBV-LPD. The positive and negative predictive values of a viral load of 1,000 geq/ml were, respectively, 39% and 100% following TCD. Treatment-related mortality did not differ significantly between TCD and non-TCD transplants, but the incidence of chronic graft-versus-host disease was significantly less in TCD-patients. It is concluded that EBV reactivation occurs frequently after TCD and unmanipulated allogeneic hematopoietic stem cell transplantation, especially in recipients of grafts with high $CD34^+$ cell counts. EBV-LPD, however, occurred only after TCD and EBV viral load quantitatively predicted EBV-LPD in recipients of a TCD graft.

1. Introduction

Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD) is a serious complication of allogeneic hematopoietic stem cell transplantation (allo-SCT) and solid organ transplantation.[1-3] Although the incidence of EBV-LPD is generally less than 2% after allogeneic hematopoietic stem cell transplantation, it may increase to 20% in patients with established risk factors, such as unrelated donor stem cell transplantation, the use of T-cell-depleted (TCD) allografts, use of antithymocyte globulin (ATG) and immunosuppression for prevention and treatment of graft-versus-host disease (GVHD).[4-8] EBV-LPD is associated with a poor prognosis despite the use of anti-B-lymphocyte monoclonal antibody therapy, donor lymphocyte infusion (DLI) and infusion of EBV-specific cytotoxic T cells (CTL).[9-15] Therefore, early diagnosis and preventive measures such as B-cell depletion of the donor graft, and pre-emptive therapy may be clinically useful.[4,7,16-24] We developed a real-time polymerase chain reaction (PCR) assay for the quantitative detection of EBV-DNA in plasma.[25] The assay accurately monitors viral load in plasma from patients with infectious mononucleosis and immunocompromised patients at risk of EBV-LPD or with established EBV-LPD.[25,26] In contrast to cytomegalovirus (CMV) antigenemia after allogeneic hematopoietic stem cell transplantation and the risk of developing CMV-disease, little is known about reactivation of EBV during the first 3 to 6 months after allogeneic hematopoietic stem cell transplantation and the predictive value of EBV reactivation for subsequent EBV-LPD. Although several studies have shown an association of viral load and a diagnosis of EBV-LPD, no study has longitudinally followed a larger cohort of allogeneic hematopoietic stem cell transplantation recipients with multiple risk factors.[27-43] We set out to monitor EBV reactivation by real-time PCR at regular time intervals after allogeneic hematopoietic stem cell transplantation. Incidences, risk factors, and sequelae of EBV reactivation were compared between patients receiving a TCD- stem cell transplantation and patients having transplantation with an unmanipulated stem cell graft. We show that subclinical EBV reactivation is a very frequent event after allogeneic hematopoietic stem cell transplantation and that quantification of EBV DNA appears useful to identify patients at risk of progression to overt EBV-LPD.

2. Patients and methods

Patients

The study population consisted of 152 consecutive patients treated at 4 transplant centers, who received stem cell transplants between March 1996 and June 1999. Patients underwent allografting at the department of hematology of the university hospitals of Utrecht (TCD stem cell transplantation) or Rotterdam (TCD stem cell transplantation), the Netherlands; Essen (non-TCD-stem cell transplantation), Germany; or Genoa (non-TCD-stem cell transplantation), Italy. Transplant protocols were approved by local institutional review

boards and all patients provided informed consent. Patient characteristics are presented in Table 1. Eighty-five patients received a TCD stem cell transplantation and 67 patients received a non-TCD stem cell transplantation. Median age was 41 years (range, 17-55 years) in the TCD group and 31 years (range, 17-56 years) in the non-TCD group ($P < 0.01$). Standard-risk patients had a diagnosis of acute lymphoblastic leukemia (ALL) in first complete remission (CR1), acute myeloid leukemia (AML) in CR1, chronic myeloid leukemia (CML) in first chronic phase and untreated (very) severe aplastic anemia (SAA), all other diagnoses were considered high risk. The non-TCD group included more patients with CML, and fewer patients with lymphoma, multiple myeloma or high risk disease ($P = 0.001$). Unrelated donor grafts were used more frequently in the non-TCD group ($P = 0.001$). The use of ATG added to the conditioning regimen for prevention of rejection was confined to patients having transplantation with TCD grafts from unrelated donors.

Transplantation

The conditioning regimen preceding a TCD-SCT consisted of cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (12 Gy in 2 fractions). Rabbit ATG (Imtix Sangstat, Amstelveen, The Netherlands) was given for prevention of rejection prior to SCT in recipients of a TCD unrelated donor graft. If patients had previously been treated with locoregional irradiation, the conditioning regimen consisted of oral busulfan (4 mg/kg on each of 4 successive days) and cyclophosphamide (120 mg/kg). The conditioning regimen in case of an unmanipulated SCT consisted of cyclophosphamide (120 mg/kg) and TBI (10 Gy in 4 fractions or 10 Gy in 3 fractions).

Partial T-cell depletion was performed using sheep erythrocyte rosetting (n=53) or CD34 selection (CellPro, Wezembeek, Belgium) (n=32). Median T-cell numbers differed more than 2 logs between TCD and unmanipulated grafts (2.0×10^5/kg versus 510×10^5/kg), but numbers of granulocyte-macrophage colony-forming units (CFU-GM) and $CD34^+$ mononuclear cells (MNCs) did not differ significantly between the groups of patients. Peripheral blood-derived stem cells were used relatively more often than bone marrow-derived stem cells in patients receiving a TCD-graft as compared with patients receiving an unmanipulated graft ($P < 0.01$). Graft-versus-host (GVH) prophylaxis was cyclosporin A (3 mg/kg) from day –3 until day +100 after TCD stem cell transplantation, and the combination methotrexate (15 mg/m^2 on day 1; 10 mg/m^2 on day 3, 6 and 11) and cyclosporin A was used in recipients of an unmanipulated stem cell transplantation.

All patients received ciprofloxacin and fluconazole for prevention of infection during neutropenia, and cotrimoxazole was given after neutrophil recovery until day 180 to 360 after stem cell transplantation. Patients having transplantation in Utrecht (TCD stem cell transplantation) and Genoa (non-TCD stem cell transplantation) received long-term aciclovir prophylaxis from day 0 until day 360. Erythrocyte and platelet products for transfusion were filtered to remove leucocytes and subsequently irradiated (25 Gy).

Table 1. Patient characteristics

Characteristic	Allogeneic T-cell-depleted SCT (n=85)	Allogeneic non-T-cell-depleted SCT (n=67)	P-value
Sex male/female (n)	48/37	50/17	0.02
Age, y (median, range)	41 (17-55)	31 (17-56)	< 0.01
Diagnosis (n):			
AML CR1	11	3	
AML >CR1	8	8	
ALL CR1	5	6	
ALL >CR1	7	2	
ALL CR1 Ph^+	5	-	
MDS	3	1	
CML CP1	8	28	
CML >CP1	5	16	
SAA	5	-	
MM	15	1	
M. Hodgkin	2	-	
NHL	10	2	
CLL	1	-	
Risk status: SR/HR (n)	25/60	37/30	0.001
Donor type (n)			
Sib	61	30	0.001
MUD	24	37	
Conditioning regimen (n)			
Cy/TBI	59	67	
Cy/TBI/ATG	23	-	
Bu/Cy	2	-	
Bu/Cy/ATG	1	-	

Table 1. Patient characteristics (continued)

Characteristic	Allogeneic T-cell-depleted SCT (n=85)	Allogeneic non-T-cell-depleted SCT (n=67)	P-value
Graft characteristics: (median, range)			
MNC x 10^8/kg	0.13 (0.01-9.32)	3.43 (0.13-14.0)	
CD3 x 10^5/kg	2.0 (1.0-7.5)	510 (7.4-2195)	< 0.001
CFU-GM x 10^4/kg	16.7 (1.9-85.9)	14.1 (4.0-132)	0.6
CD34 x 10^6/kg	1.25 (0.06-6.43)	2.2 (0.04-14.1)	0.7
EBV-serology (n)			
D-R-	-	2	0.2
D+R-/D+R+/D-R+	85	65	
Stem cell source (n)			
BM	66	63	< 0.01
PB	19	4	

AML1 CR1 or >CR1 indicates acute myeloid leukemia in first or subsequent complete remission; ALL CR1 or >CR1, acute lymphoblastic leukemia in first or subsequent CR; ALL CR1 Ph^+ , ALL CR1 philadelphia chromosome-positive; MDS, myelodysplastic syndrome; CML CP1 or >CP1, chronic myeloid leukemia in first or subsequent chronic phase; SAA, severe aplastic anemia; MM, multiple myeloma; NHL, Non-Hodgkin's lymphoma; CLL , chronic lymphocytic leukemia; SR, standard risk; HR, high risk; Sib, HLA identical family donor; MUD, matched unrelated donor; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; ATG, anti-thymocyte globulin; MNC, mononuclear cells; CFU-GM, granulocytes-monocyte colony-forming units; D+/-, EBV-seropositive / seronegative donor; R+/-, EBV-seropositive/seronegative recipient; BM, bone marrow; PB, peripheral blood.

Patients were hospitalized in reverse isolation and rooms with high-efficiency particulate-filtered air. All patients received food with a low microbial count until discharge, and parenteral alimentation was given in case of severe mucositis.

Real-time Taqman Assay

Taqman PCR primers were selected from the EBV-DNA genome encoding for the nonglycosylated membrane protein BNRF1-p143 and generated a DNA product of 74 basepairs, as described before. [25] A known EBV-DNA copy number based on a reference standard quantified by electron microscopy (ABI Advanced Biotechnologies, Columbia, MA, USA) was used for standardization. Serial dilutions ranging from 10 to 10^7 EBV-DNA 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. [25] EBV reactivation was defined as a plasma EBV-DNA level exceeding 50 geq/ml. Recurrent reactivation was defined by a positive PCR (more than 50 geq/ml) after (at least) two consecutive negative PCR results following a preceding episode of reactivation. Viral load was monitored in blood samples drawn at 2-week intervals starting at stem cell transplantation until day 180 after stem cell transplantation.

EBV-LPD diagnosis

A diagnosis of EBV-LPD was preferably based on lymph node histology or cytology and was classified according to the criteria of Knowles et al.[44] Immunohistology included antibody staining with CD19-specific (Becton Dickinson, San José, CA, USA), CD20-specific (DAKO, Glostrup, Denmark) and EBV latent membrane protein-1-specific (DAKO) monoclonal antibodies. Furthermore, clonality was assessed using immunohistochemical staining with monoclonal antibodies to kappa and lambda light chains (DAKO). In situ hybridization was performed to detect EBV-encoded small RNA molecules (EBV-EBER) using an EBV-EBER probe (DAKO) and PCR for detection of EBV-DNA encoding for the *Bam*HI fragment. EBV-LPD staging included physical examination, whole-body computed tomography scanning (CT) scanning, and flow cytometric detection of monoclonal B lymphocytes in blood, bone marrow, and, if indicated, cerebrospinal fluid.

Endpoints and statistical analysis

The data were analyzed as of January 2000. Patient characteristics of non-TCD patients and TCD-patients were compared using Fisher exact test or Pearson chi-square test, whichever was appropriate, in case of discrete variables, or the Wilcoxon rank-sum test in case of continuous variables. End points of the study included time to EBV reactivation, EBV-LPD, acute GVHD grades II to IV, chronic GVHD and treatment-related mortality (TRM). Time to first EBV reactivation was determined from the date of transplantation until day 180, and patients were censored at the date of last serum sample if this sample had been taken before day 180. Time to EBV-LPD was measured from SCT until EBV-LPD. Patients who died without EBV-LPD were censored at the date of death. Patients still alive at the date of analysis were censored at the last follow-up date. Two EBV-seronegative donor-recipient pairs were excluded from the analysis of EBV reactivation and EBV-LPD. GVHD was diagnosed and graded according to consensus criteria.[45] Chronic GVHD was evaluated among patients who survived at least 100 days after transplantation. TRM was defined according to standard criteria.[46] Time to EBV reactivation, EBV-LPD, acute and chronic GVHD, and TRM were estimated by the Kaplan-Meier method, and Kaplan-Meier curves were generated to illustrate differences between subgroups of patients.[47] The following

variables were included in the analysis of prognostic factors: sex, male patient and female donor, age, risk status, donor (sibling versus matched unrelated donor), source of stem cells (bone marrow versus peripheral blood), type of transplant (non-TCD versus TCD without ATG versus TCD with ATG) and graft characteristics (number of MNCs, number of $CD34^+$ cells, number of $CD3^+$ and CFU-GMs infused). Univariate survival analysis was performed using the log-rank test and Cox regression to see whether there was a difference between subgroups.[48,49] The variables that appeared significant in the univariate analysis were also included in a multivariate Cox regression. Moreover, Cox regression was performed using EBV reactivation within day 180 as a time-dependent covariate to assess whether EBV reactivation predicted EBV-LPD and TRM. All reported P-values are 2-sided and a significance level of α=0.05 was used.

3. Results

EBV reactivation

The probability of developing EBV reactivation was greater after TCD-allogeneic stem cell transplantation than after non-TCD stem cell transplantation (Figure 1, Table 2).

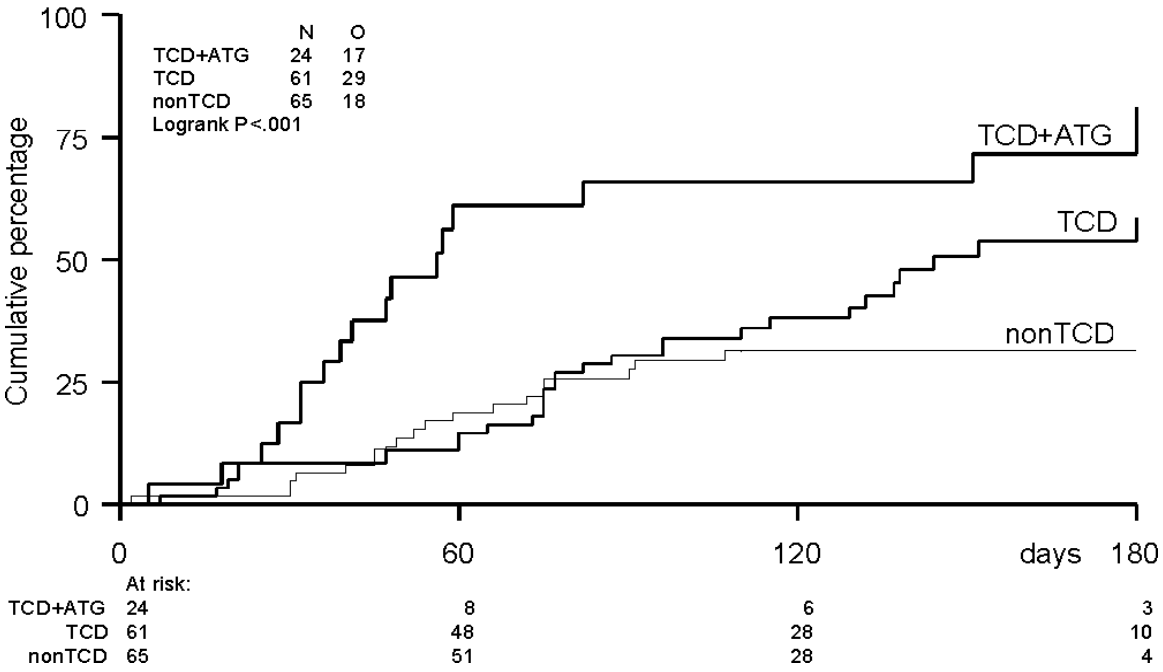

Figure 1. Incidence of EBV-reactivation. Incidence of EBV-reactivation after TCD-allogeneic hematopoietic stem cell transplantation with ATG (n=24), TCD stem cell transplantation without ATG (n=61), and non-TCD stem cell transplantation (n=65). Only TCD combined with ATG significantly increased the risk of EBV reactivation (P < 0.001).

Table 2. EBV reactivation and EBV-LPD

Parameter	T-cell-depleted allo-SCT (n=85)		Unmanipulated allo-SCT (n=65)	
No. of patients with EBV reactivation (%)	46	(54)	18	(28)
Time (d) to first EBV reactivation (median, range)	58	(5-180)	63	(2-107)
Maximum viral load (geq/ml) of first EBV reactivation (median, range)	535	(50-3,200,000)	808	(55-540,000)
No of patients (%) with recurrent EBV reactivation	14	(16)	2	(3)
No of patients (%) with EBV-LPD	10	(12)	-	
Time (d) from SCT to EBV-LPD (median, range)	87	(50-168)	-	
Time (d) from first EBV reactivation to EBV-LPD (median, range)	22	(13-120)	-	
EBV-LPD viral load (geq/ml, median, range)	110,0000	(1,800-790,000)	-	

geq/ml indicates genome equivalents EBV-DNA/ml. Other abbreviations are explained in Table 1.

That difference, however, could be largely attributed to the use of ATG in conjunction with TCD (Figure 1, Table 3). Probabilities of viral reactivation were not different between recipients of a non-TCD stem cell transplantation and recipients of TCD stem cell transplantation without concomitant ATG. Median time to first reactivation was 58 days (range, 5-180 days) in the TCD group and 63 days (range, 2-107 days) in the non-TCD

group (not significant). Plasma EBV-DNA levels measured at the peak of the first reactivation did not differ between the groups.
Recurrent reactivation was significantly more frequent after TCD (Table 2): 14 of 85 patients (16%) experienced multiple episodes of EBV reactivation after TCD stem cell transplantation, including 8 patients with 2 episodes, 5 patients with 3 episodes, and 1 patient showing 4 distinct periods of reactivation. This is exemplified for a recipient of a TCD donor graft who experienced 3 episodes of EBV reactivation without developing EBV-LPD (Figure 2).

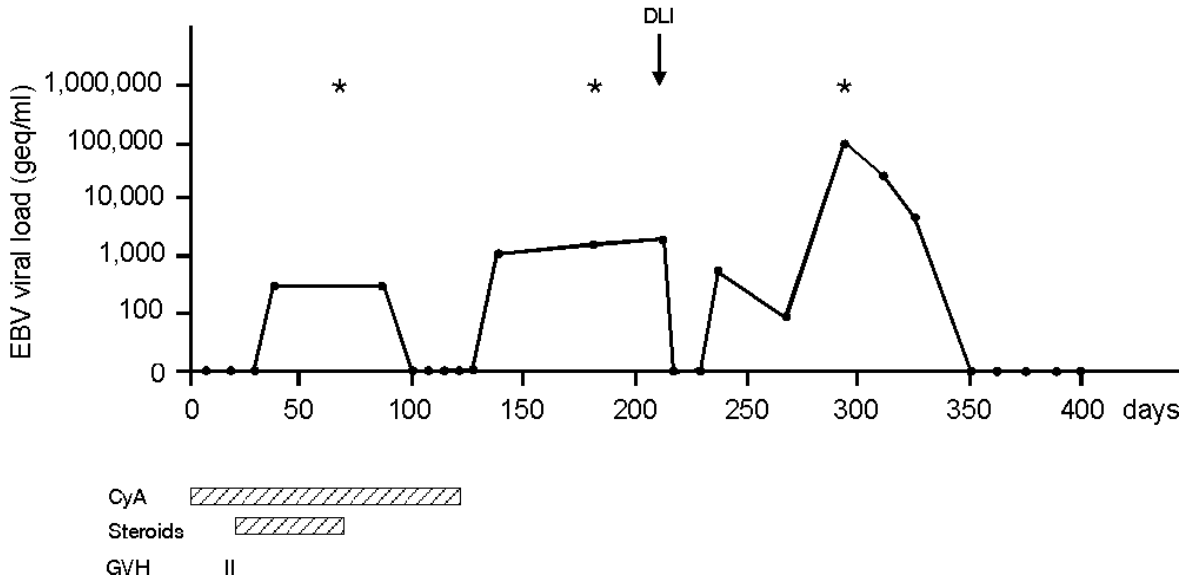

Figure 2. Monitoring EBV viral load after matched unrelated stem cell transplantation. A 16-year-old EBV-seropositive male with a philadelphia chromosome-positive (Ph^+) ALL in first complete remission received a TCD matched unrelated donor graft from an EBV-seropositive donor. Multiple EBV reactivations were observed; however, no EBV-LPD ensued. Frequent examination of bone marrow for the presence of monoclonal B cells and whole-body CT to detect lymphadenopathy were negative at various time points (*). At day 211, DLI (1.0 x 10^5 $CD3^+$ T-cells/kg) was administered because of molecular relapse of his Ph^+ ALL. Currently, the patient is free of disease and well at day 800 after SCT. CyA indicates cyclosporin A.

In contrast, only 2 of 65 patients (3%) receiving non-TCD grafts had a second period of reactivation. ATG appeared not to be associated with recurrent reactivation, as only 2 out of 14 patients with recurrent reactivation after TCD also received ATG as part of the conditioning regimen. Several risk factors predicted for first reactivation in univariate analysis (Table 3), including TCD ($P = 0.02$), use of ATG in the conditioning regimen ($P < 0.001$), transplantation of unrelated donor graft ($P = 0.02$), and a high $CD34^+$ cell number of the graft ($P = 0.001$) (Figure 3). Following multivariate analysis, only use of ATG and high $CD34^+$ cell count (> 1.35 x 10^6/kg) remained independently associated with EBV

reactivation (Table 3). Numbers of $CD34^+$ and $CD3^+$ cells were not associated with each other.

Table 3. Univariate and multivariate Cox regression analysis of risk factors for Epstein-Barr virus reactivation

Risk factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
T-cell depletion, no ATG	1.5	0.8-2.7	0.02	1.5	0.8-2.9	0.3
T-cell depletion, ATG	3.5	1.8-6.9	< 0.001	3.4	1.6-7.1	0.001
High-risk status	1.6	1.0-2.8	0.07	1.4	0.8-2.6	0.2
Unrelated donor	1.8	1.1-2.9	0.02	0.9	0.3-2.9	0.8
$CD34^+$ cell count of the graft (> 1.35 x 10^6/kg)	2.4	1.4-4.1	0.001	2.6	1.5-4.6	0.001

HR indicates hazard ratio; CI, confidence interval; ATG, antithymocyte globulin.

EBV-LPD

EBV-LPD was only observed following TCD stem cell transplantation (Table 2, Figure 4). Five patients developed EBV-LPD after HLA identical sibling SCT and 5 after unrelated donor stem cell transplantation (Table 4). Five of these patients had received ATG before unrelated donor stem cell transplantation, and 9 of them had been treated for high-risk disease. All EBV-LPD donor-recipient pairs were EBV seropositive. One donor had negative EBV serology before transplantation. Median time from first reactivation to EBV-LPD was 22 days (range, 13-120 days) (Table 2). Median EBV-DNA level at EBV-LPD diagnosis was 110,000 geq/ml (range 1,800-790,000). Histological proof of a diagnosis of EBV-LPD and classification according to the criteria of Knowles et al [44] were obtained in 8 patients. Patient 8 (Table 4), who received an HLA-identical sibling stem cell transplantation for multiple myeloma, was diagnosed with EBV-LPD by the presence of monoclonal B cells in his cerebrospinal fluid and an elevated plasma EBV-DNA level. Patient 9, who received an unrelated donor stem cell transplantation because of severe

aplastic anemia, was diagnosed with EBV-LPD because of massive lymphadenopathy on CT scanning and a highly elevated plasma EBV-DNA level.

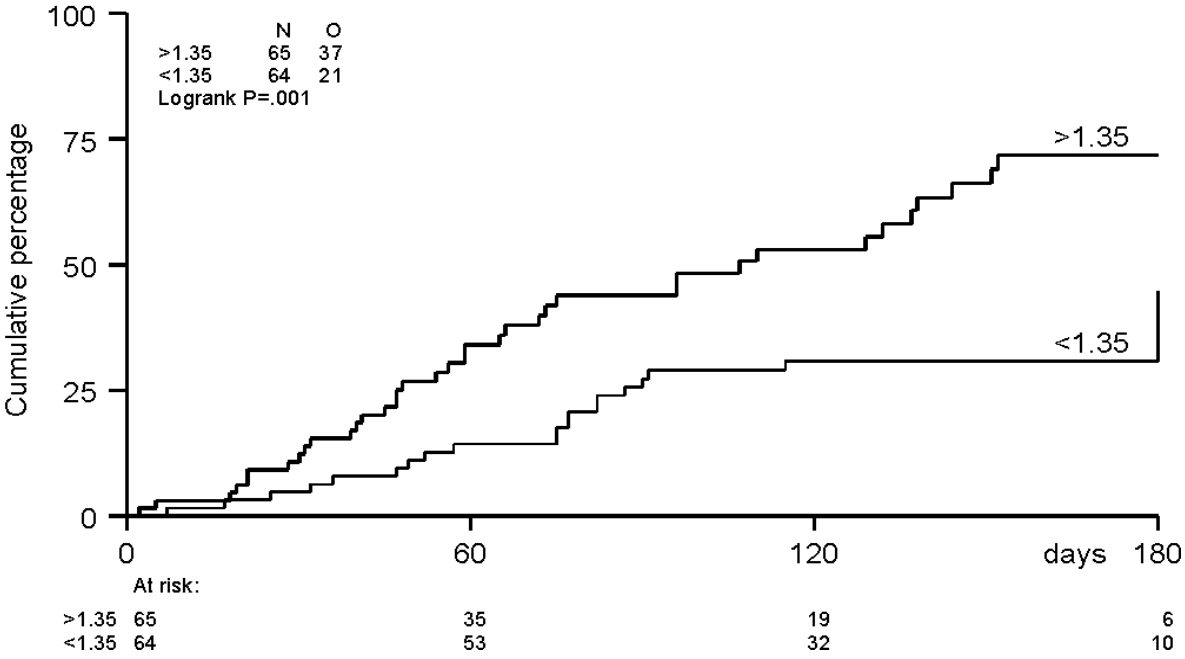

Figure 3. Incidence of EBV reactivation by number of CD34$^+$ cells in the graft. The median number of CD34$^+$ in the graft was 1.35 x 10^6/kg. Patients with grafts containing more than 1.35 x 10^6/kg were at higher risk (P = 0.001) of EBV reactivation.

Six patients received anti-B-cell monoclonal antibody therapy (rituximab), 5 patients received DLI, and immune suppression was discontinued in 8 patients (Table 4). Five patients obtained a complete remission and 5 other patients died of progressive EBV-LPD. Two responding patients are currently alive with a follow-up of 620 and 351 days. Three responding patients developed severe GVHD, 2 following DLI, and died due to GVHD-related complications.

Use of ATG, application of TCD, and high-risk status of underlying disease significantly predicted EBV-LPD in univariate analysis. Multivariate analysis was not performed because the latter 3 variables appeared strongly associated and the small number of events did not allow a reliable multivariate analysis. Several risk factors occurring after stem cell transplantation were evaluated for a possible association with EBV-LPD by time-dependent analysis. A lower lymphocyte count at first EBV reactivation appeared not predictive for developing EBV-LPD. In contrast, EBV load significantly predicted EBV-LPD in a quantitative manner. A stepwise increase of EBV DNA by 1 log (Table 5)

yielded a hazard ratio (HR) of 2.9 (95% confidence interval [CI], 1.7-4.8) for those patients receiving a TCD graft (P < 0.001).

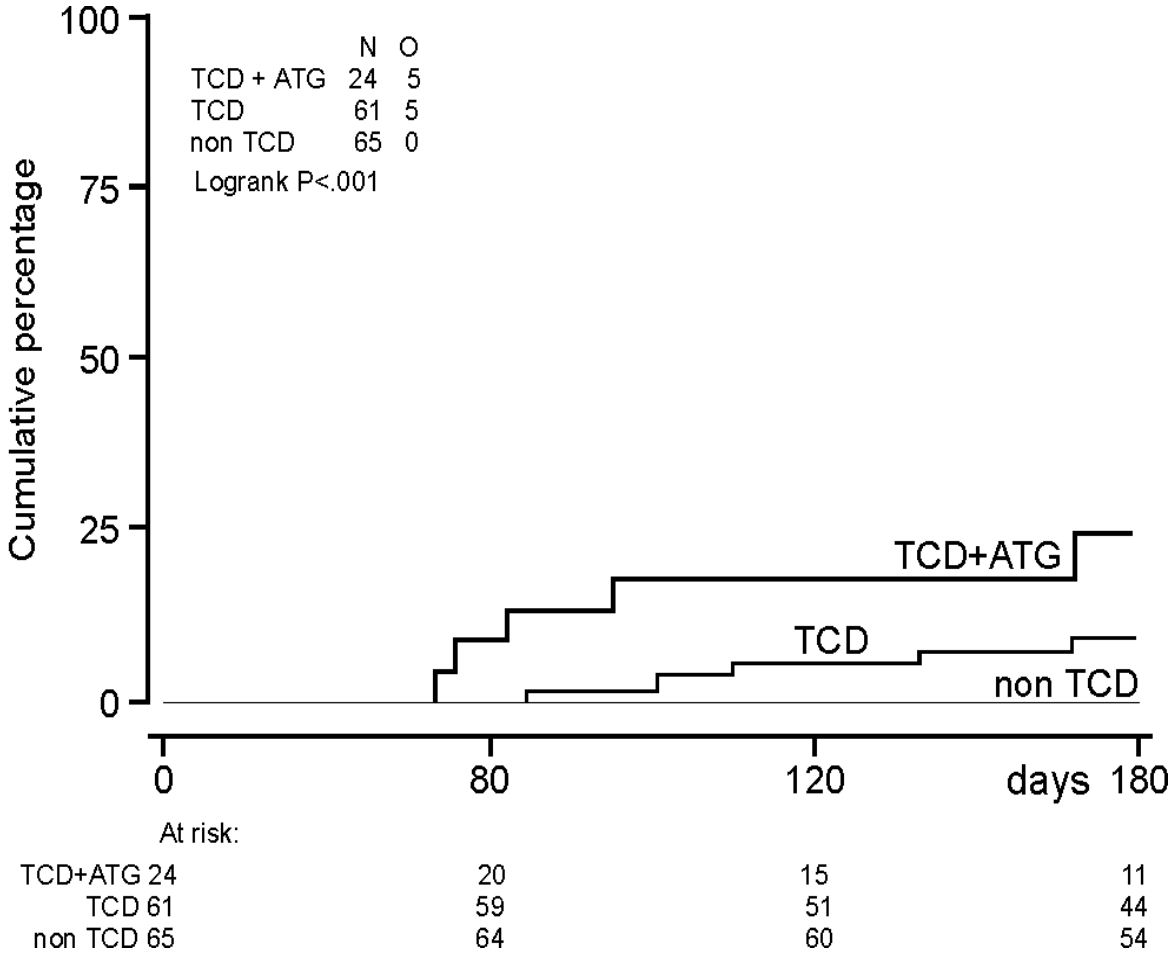

Figure 4. Incidence of EBV-LPD. Incidence of EBV-LPD (n=10) after TCD-allogeneic hematopoietic stem cell transplantation combined with ATG (n=24), TCD hematopoietic stem cell transplantation without ATG (n=61), and non-TCD hematopoietic stem cell transplantation (n=65).

Numbers of patients with a TCD stem cell transplantation with plasma levels of EBV-DNA exceeding a certain threshold value and the corresponding positive and negative predictive values for EBV-LPD for that subset of patients are shown in Table 5. Although the positive predictive value was 24% for patients with a copy number of 100 geq/ml or higher, it rose to 100% at the level of 500,000 geq/ml. However, only one patient with EBV-LPD reached that high number, and consequently the negative predictive value measured 89%.

Table 4. Epstein-Barr virus-lymphoproliferative disease following T-cell depleted allogeneic hematopoietic stem cell transplantation

Patient no	Donor Type	EBV-LPD diagnosis			Therapy			Outcome		
		Morphology	Clonality	Plasma EBV DNA (geq/ml)	SI	Anti-CD20	DLI	Response	Survival (d)	COD
1	Sib	III	Mono	1,800	+	-	+	PD	Dead	EBV-LPD
2	MUD	II	Poly	92,000	+	+	+	CR	Dead	GVHD
3	MUD	II	Poly	6,500	+	+	-	CR	Alive, 620^{+}	-
4	Sib	III	Mono	790,000	+	+	+	PD	Dead	EBV-LPD
5	Sib	III	Mono	128,000	+	+	-	CR	Alive, 351^{+}	-
6	Sib	II	Mono	74,000	+	+	-	CR	Dead	GVHD
7	MUD	III	Mono	133,000	+	+	+	PD	Dead	EBV-LPD
8	Sib	nd	Mono	7,900	+	-	-	PD	Dead	EBV-LPD
9	MUD	nd	nd	310,000	-	-	+	CR	Dead	GVHD
10	MUD	III	Mono	206,000	-	-	-	PD	Dead	EBV-LPD

SCT indicates stem cell transplantation; Sib, HLA identical family donor; MUD, matched unrelated donor; EBV-LPD, Epstein-Barr virus associated lymphoproliferative disease; I, plasmacell hyperplasia; II, polymorphic hyperplasia; III, Non-Hodgkin's lymphoma (criteria according to Knowles et al [44]); nd, not determined; Mono, monoclonal disease; Poly, polyclonal disease; SI, stop immunosuppression; anti-CD20, monoclonal anti B-cell therapy; DLI, donor lymhocyte infusion; PD, progressive disease; CR, complete remission; COD, cause of death; GVHD, graft-versus-host disease.

Table 5. Incidence of Epstein-Barr virus-lymphoproliferative disease by viral load following T-cell depleted allogeneic hematopoietic stem cell transplantation

EBV load (geq/ml)	No. of patients with specified reactivation	No. of patients with EBV-LPD	Predictive values	
			Positive (%)	Negative (%)
100	41	10	24%	100%
1,000	26	10	39%	100%
10,000	14	7	50%	96%
100,000	7	5	71%	94%
500,000	1	1	100%	89%

geq/ml indicates genome equivalents per ml. Other abbreviations are explained in Table 1.

Graft-versus-host disease

The actuarial probability of acute GVHD II-IV at day 100 was 57% ± 4% for the whole group and was not significantly different for patients receiving a TCD graft as compared with patients following unmanipulated allogeneic hematopoietic stem cell transplantation. An unrelated donor graft and a high $CD34^+$ cell count of the graft (independent from the number of $CD3^+$ T cells in the graft) were the only significant risk factors for developing acute GVHD following multivariate analysis. EBV reactivation was not associated with acute GVHD. Actuarial probabilities of chronic limited and extensive GVHD at 12 months post stem cell transplantation were significantly higher for non-TCD patients (83% ± 5%) than for TCD patients (38% ± 6%) ($P < 0.001$).

Treatment-related mortality

The actuarial probability of TRM was 29% ± 4% at 1 year for all patients and did not differ between TCD and unmanipulated allogeneic hematopoietic stem cell transplantation. Higher age and a higher $CD34^+$ cell count (> 1.35 x 10^6/kg) of the graft predicted higher TRM in multivariate analysis. Following time-dependent analysis, EBV reactivation (HR: 1.9, 95% CI: 1.0-3.3, P = 0.04) and acute GVHD grade I-IV (HR: 1.8, 95% CI: 1.0-3.3, P = 0.05) were associated with higher TRM. In addition, a higher lymphocyte count (> 0.6 x 10^9/l) at the time of first EBV reactivation significantly predicted less TRM (HR 0.3; 95% CI, 0.1-0.8; P = 0.02).

4. Discussion

This study demonstrates that EBV reactivation is a very frequent event after both TCD and unmanipulated allogeneic hematopoietic stem cell transplantation. In particular, recipients of stem cell grafts with high numbers of $CD34^+$ MNCs appeared to be at risk for EBV reactivation. However, patients receiving a TCD stem cell transplantation were at significantly higher risk for recurrent reactivation and only these patients developed EBV-LPD. The development of impending EBV-LPD in these patients could be predicted quantitatively by monitoring viral load in plasma at regular intervals during the first 6 months after SCT.

EBV reactivation was observed frequently after TCD stem cell transplantation and after unmanipulated allogeneic hematopoietic stem cell transplantation as well. The high incidence of first EBV reactivation after TCD stem cell transplantation could be largely attributed to the use of ATG and, as a result, TCD per se did not appear to be an independent risk factor for early EBV reactivation. However, patients receiving a TCD stem cell transplantation showed more recurrence of reactivation and EBV-LPD was observed only after TCD. Because the conditioning regimen has eradicated autologous EBV-specific immunity after both TCD and unmanipulated stem cell transplantation, early EBV reactivation may occur after both modes of allogeneic hematopoietic stem cell transplantation. [50,51] However, the significantly higher risks for recurrent EBV reactivation and EBV-LPD in TCD stem cell transplantation as compared with unmanipulated stem cell transplantation may be explained by the impaired capacity of patients receiving TCD grafts to mount an effective immune response to the reactivating virus. The strongly reduced numbers of EBV-specific memory T cells in TCD as compared with unmanipulated grafts may play a major role in this respect. [52,53]

Apart from the use of ATG as part of the conditioning regimen, we identified the number of $CD34^+$ cells in the graft as a novel independent risk factor for developing EBV reactivation (Table 3, Figure 3), and also for acute GVHD and TRM. Przepiorka et al [54] recently reported that recipients of peripheral blood stem cell grafts with high $CD34^+$ cell counts were at higher risk for acute GVHD, an effect that appeared independent of the number of $CD3^+$ T cells.[54] They suggested that GVHD at high $CD34^+$ cell doses may be exacerbated by cytokines released by the markedly expanding myeloid population at the time of engraftment. This explanation is supported by high levels of proinflammatory cytokines in patients with severe GVHD. [55-57] In the present study, acute GVHD significantly predicted TRM in a time-dependent analysis. Therefore, the association of $CD34^+$ cell dose and TRM might be explained by an increased incidence of GVHD. The association of $CD34^+$ cell dose and EBV reactivation is, however, less likely to be explained by more GVHD, as EBV reactivation preceded the onset of acute GVHD in a significant number of patients. Alternative explanations may include infusion of a higher number of EBV-infected B cells together with larger stem cell grafts, or stimulation of B-cell proliferation by cytokines produced by the higher number of rapidly maturing myeloid progenitors. The latter

explanation is supported by a number of preclinical as well as clinical studies showing that proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α and -β, and IL-6, may very effectively stimulate the growth of EBV-infected B cells. [58] In particular, IL-6 may play an important role as a growth factor, promoting the progression toward overt EBV-LPD. [59-62] Apart from monocyte-macrophages and endothelial cells as an established source of proinflammatory cytokines, the rapid proliferating myeloid population of grafts containing high $CD34^+$ cell doses may add to cytokine release and thus contribute to viral reactivation.

A number of studies have demonstrated a correlation between high levels of viral load and a diagnosis of EBV-LPD after both stem cell transplantation and solid-organ transplantation. [26-43] No study, however, has longitudinally followed allogeneic hematopoietic stem cell transplantation recipients with multiple risk factors from day 0 until day 180 and reported positive and negative predictive values. Lucas et al [41] evaluated the predictive value of a quantitative PCR using DNA extracted from peripheral blood MNCs in a cohort of 195 patients receiving a solid-organ transplantation. [41] Although the negative predictive value appeared very high (100%), the positive predictive value was 38%. Our results observed in recipients of an allogeneic hematopoietic stem cell transplantation are in line with these findings. Considering both TCD and non-TCD transplants, the negative and positive predictive values of a copy number of 1,000 geq/ml were, respectively, 100% and 28%. Higher predictive values were obtained when the analysis was restricted to patients receiving a TCD stem cell transplantation. The positive predictive value of a high EBV-DNA level of more than 1,000 geq/ml and more than 10,000 geq/ml for patients receiving a T-cell depleted stem cell transplantation were 39% and 50%, respectively (Table 5).

Although highly significant, these predictive values also indicate that most patients (even recipients of TCD grafts) were able to mount an effective immune response and clear their viral reactivation. Monitoring of the reconstitution of HLA-specific T lymphocytes may add to the predictive value of viral load quantification. For this purpose, rapid assays are now available, such as the enumeration of EBV-specific T lymphocytes by tetramer binding or the induction of intracellular interferon-γ in T cells after specific stimulation. [63] The accurate prediction of impending EBV-LPD in patients at risk is important because pre-emptive therapy might be more effective than therapy of established EBV-LPD. Despite the application of new treatment modalities such as DLI and anti-B-cell immunotherapy, the mortality of patients with established EBV-LPD is still high. Ten patients developed EBV-LPD in the present study: 5 died due to progressive EBV-LPD and 3 patients secondary to GVHD following DLI, resulting in a 80% (8 of 10) mortality. Pre-emptive infusion of EBV-specific cytotoxic T cells has been shown to reduce viral load and may prevent the evolution toward EBV-LPD. [20] However, the preparation and use of such EBV-specific T cells is expensive and difficult to implement on a wide scale. B-cell depletion of the donor graft has been shown to effectively reduce the incidence of EBV-LPD. [7,16] Therefore, anti-B-cell immunotherapy aimed at in vivo B-cell depletion after stem cell transplantation in patients at

high risk of EBV-LPD might be a promising new means of pre-emptive therapy. A prospective phase II study with that specific aim is currently being performed. [64] Because the depletion of B cells may add to the impaired immune status of these patients, one may argue to restrict pre-emptive therapy to those patients at highest risk. A threshold of 1,000 geq/ml, as observed in our patient population, may thereby serve as a critical level of viral load to start pre-emptive therapy. Thus, pre-emptive therapy may be administered selectively to high-risk patients to prevent EBV-LPD and to avoid treatment of patients who have recovered their EBV-specific immunity to protective levels. The frequent monitoring of EBV load after allogeneic hematopoietic stem cell transplantation may therefore be considered for patients with a high risk profile for EBV-LPD and may preferably be combined with close monitoring of the reconstitution of EBV-specific T lymphocytes.

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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.

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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 ($P = 0.001$).

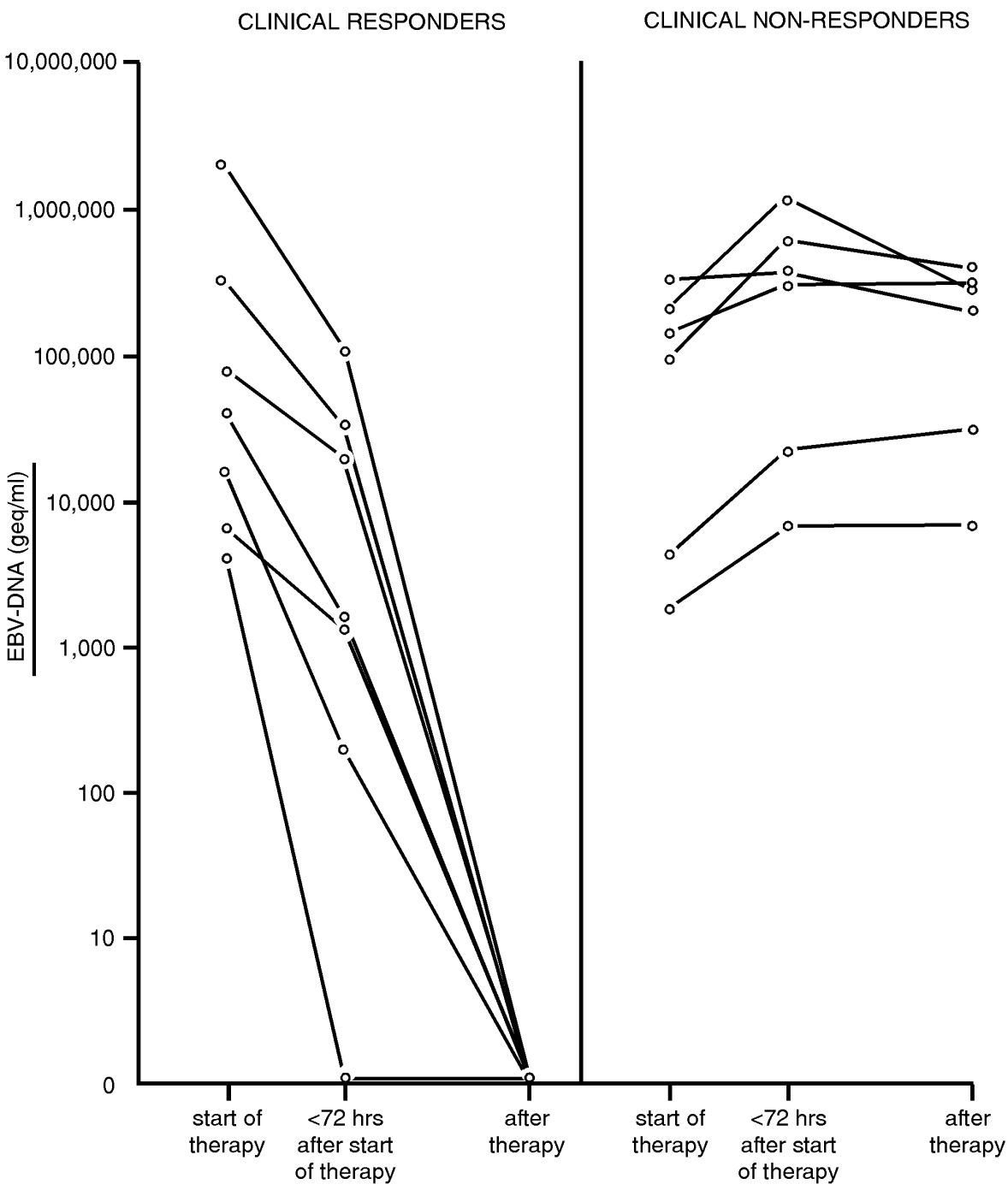

Figure 1. EBV load following therapy for EBV-LPD. Individual EBV-DNA levels for clinical responders (left) and clinical non-responders (right) at the start of therapy, after 72 hours and at clinical response evaluation. Responders were evaluated at a median of 17 days (range, 5-51 days) and non-responders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

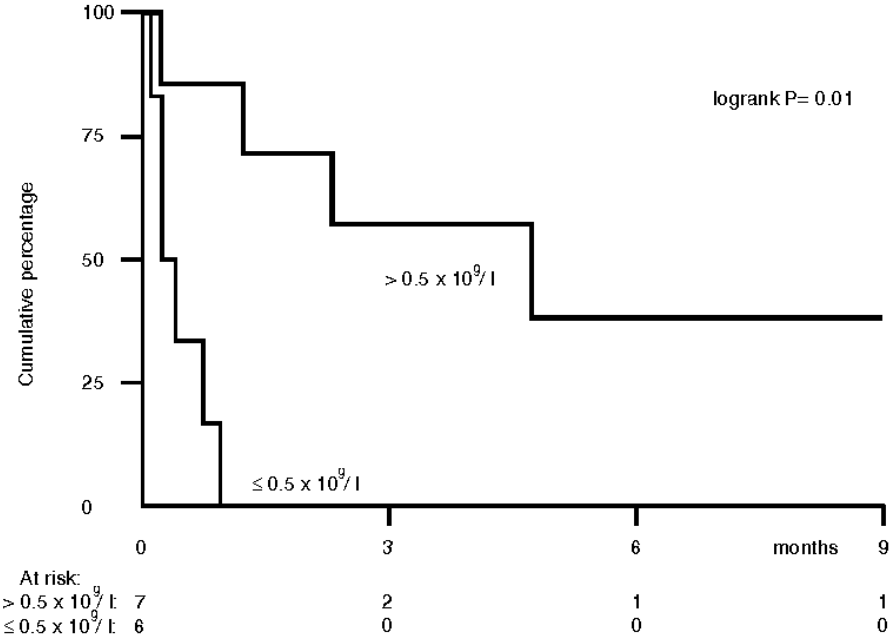

Figure 2. Lymphocyte count at diagnosis of EBV-LPD and overall survival. Overall survival (n=13) in relation to lymphocyte number (> 0.5 x 10^9/l versus ≤ 0.5 x 10^9/l) at diagnosis of EBV-LPD.

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).

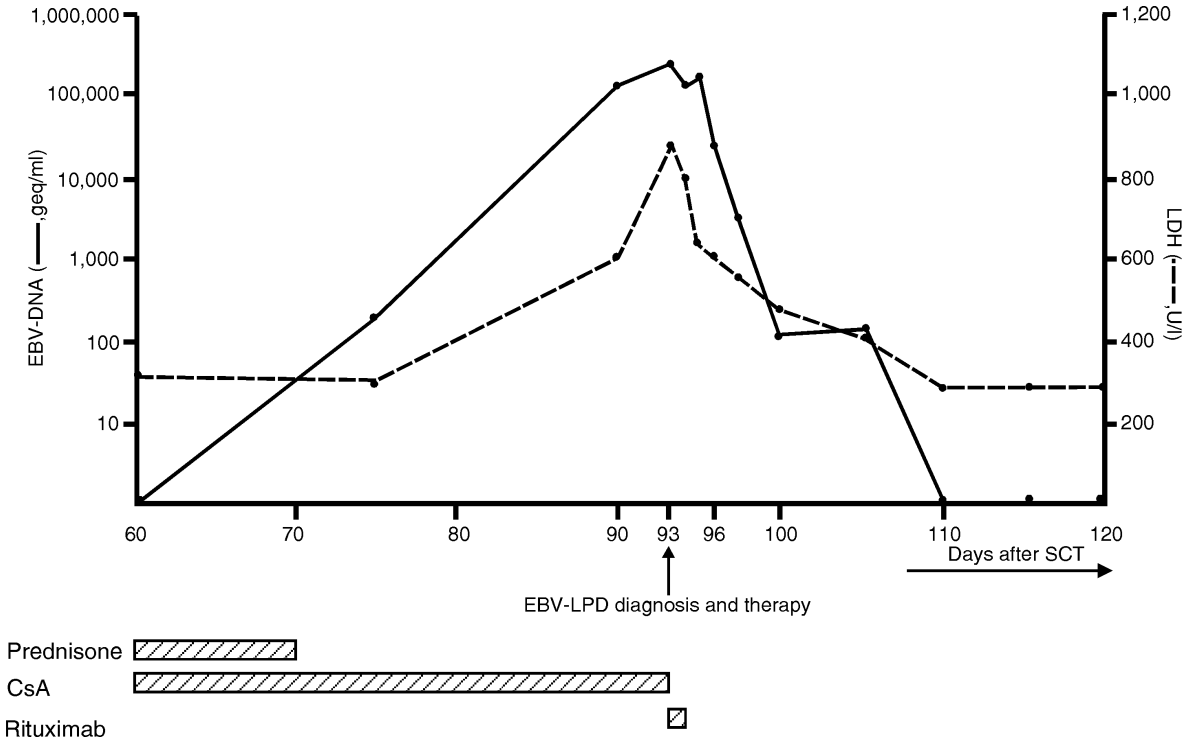

Figure 3. Therapy for EBV-LPD guided by EBV load. EBV-DNA levels and lactate dehydrogenase concentration (LDH) in patient no 14 who developed EBV-LPD at day 93 after allogeneic hematopoietic stem cell transplantation for multiple myeloma, and was treated using anti-CD20 immunotherapy and cessation of 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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5. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and pre-emptive rituximab in high-risk patients after allogeneic hematopoietic stem cell transplantation.

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Abstract

Recipients of a partially T-cell depleted (TCD) allogeneic hematopoietic stem cell transplantation (allo-SCT) developing reactivation of Epstein-Barr virus (EBV) with quantified viral DNA levels exceeding 1,000 genome equivalents/ml (geq/ml) are at high-risk for EBV-lymphoproliferative disease (EBV-LPD). We studied whether pre-emptive therapy with rituximab prevents EBV-LPD, EBV-LPD-mortality and abrogates viral reactivation in high-risk patients. We monitored 49 recipients of a TCD allogeneic hematopoietic stem cell transplantation weekly for EBV reactivation by quantitative real-time PCR. Pre-emptive therapy by a single infusion of rituximab was given to patients with viral reactivation $\geq$ 1,000 geq/ml. Results were compared with an historical control group of patients retrospectively monitored for EBV reactivation at similar intervals. There were 17 prospectively monitored patients who showed EBV reactivation $\geq$ 1,000 geq/ml and 15 received pre-emptive therapy. Median time to pre-emptive therapy was 113 days (range, 41-202 days) after stem cell transplantation. Fourteen patients showed complete response (CR) as characterized by prevention of EBV-LPD and complete clearance of EBV-DNA from plasma, which was achieved after a median number of 8 days (range, 1-46 days). One patient progressed to EBV-LPD despite pre-emptive therapy, but obtained complete remission after 2 infusions of rituximab and donor lymphocyte infusion. There were 2 patients who had already developed EBV-LPD prior to pre-emptive rituximab, but obtained complete remission following 2 rituximab infusions. Comparison of this prospectively followed series to our historical cohort with the same high-risk profile showed a reduction of EBV-LPD incidence (18 ± 9% versus 49 ± 11%, respectively) and a complete abrogation of EBV-LPD-mortality (0% versus 26 ± 10%, respectively) (P=0.04) at 6 months from EBV-DNA $\geq$ 1,000 geq/ml. Frequent quantitative monitoring of EBV reactivation and pre-emptive therapy by rituximab improves outcome in patients at high-risk of EBV-LPD.

1. Introduction

Herpes viruses including Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) continue to affect outcome of allogeneic hematopoietic stem cell transplantation (allo-SCT) and solid organ transplantation. Considerable progress has been made in the last decade in the ability to prevent CMV-infection and CMV-disease. [1] Key elements to that effect are the accurate identification of high-risk patients and the introduction of new effective antiviral agents. [2] Currently, a risk adapted strategy with pre-emptive or prophylactic ganciclovir in patients with a high-risk profile for CMV-disease, has become the preferred approach. [3]
In contrast to CMV, the precise identification of patients at high-risk for EBV-lymphoproliferative disease (EBV-LPD) has been hampered by lack of early and sensitive markers of EBV reactivation, which accurately predict impending EBV-LPD. The use of polymerase-chain reaction (PCR)-based assays, however, has enabled the early diagnosis of EBV-LPD and also the monitoring of EBV reactivation. [4-10] We recently showed a high incidence of EBV reactivation after allogeneic hematopoietic stem cell transplantation. [10] However, only recipients of a T-cell depleted (TCD) allogeneic hematopoietic stem cell transplantation appeared to be at risk for EBV-LPD. Furthermore, impending EBV-LPD could quantitatively be predicted by the frequent monitoring of viral load in plasma by quantitative real-time PCR. A viral load of 1,000 genome equivalents per ml (geq/ml) proved to be a level of EBV reactivation associated with a high predictive value. Clearly, the prevention of EBV-LPD in high-risk patients would be preferable, as outcome of established EBV-LPD is still not optimal. [9-14]
The recent introduction of rituximab has provided a relatively simple and safe treatment modality, [15,16] which has already been applied in LPD-treatment, [10,14,17,18] but might be preferred for prevention in a selected group of high-risk patients. We set out to study whether the pre-emptive use of rituximab in TCD allogeneic hematopoietic stem cell transplantation patients with viral reactivation of at least 1,000 geq/ml would prevent the development of EBV-LPD. By comparing results with an historical control group of patients with a similar risk-profile, it is shown that viral reactivation, progression to EBV-LPD and mortality from EBV-LPD can effectively be abrogated by pre-emptive therapy selectively given to patients at high-risk of developing EBV-LPD.

2. Patients and Methods

Prospective cohort

We prospectively monitored 49 consecutively treated patients receiving a partial TCD allogeneic hematopoietic stem cell transplantation either from an HLA antigen genotypically matched sibling donor (Sib; n=35) or a matched unrelated donor (MUD; n=14) following myeloablation were prospectively monitored at weekly intervals for EBV-DNA between January 1999 and March 2001 (Table 1).

Table 1. Patient Characteristics

Clinical Parameter	Study population (n=49)
Sex: male/female	27/22
Age (years, median, range)	38 (16-56)
Diagnosis	
AML CR1	11
AML > CR1	2
ALL CR1	4
ALL > CR1	2
CML CP1	7
CLL	2
Hodgkin	1
MDS	1
Multiple Myeloma	9
NHL	9
SAA	1
Risk Status SR/PR	21/28
Donor Type	
Sib	35
MUD	14
Conditioning Regimen	
Cy/TBI	33
Cy/TBI/ATG	15
Bu/Cy	1
Graft Characteristics	
MNC x 10^8/kg	0.09 (0.01-0.74)
CD34 x 10^6/kg	1.68 (0.53-11.1)
CD3 x 10^5/kg	2.0 (1.0-4.0)
CFU-GM x 10^4/kg	19.0 (3.0-128.0)
Stem Cell Source	
BM	37
PB	12

Table 1. Patient Characteristics (continued)

Clinical Parameter	Study population (n=49)
EBV-serology (D/R)	
D+/R+	44
D-/R-	1
D-/R+	1
D+/R-	3

AML CR1/>CR1 indicates acute myeloid leukemia first/subsequent complete remission; ALL CR1/>CR1, acute lymphoblastic leukemia first/subsequent CR; CML CP1, chronic myeloid leukemia in first chronic phase; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; SAA, severe aplastic anemia; SR/PR, standard-/poor-risk disease; Sib, genotypically matched sibling donor; MUD, matched unrelated donor; Cy, cyclophosphamide; TBI, total body irradiation; ATG, anti-thymocyte globulin; Bu, busulphan; MNC, mononuclear cells; CFU-GM, colony forming units-granulocytes macrophages; BM, bone marrow; PB, peripheral blood; EBV, Epstein-Barr virus; D+/-, donor EBV seropositive/-negative; R+/-, recipient EBV seropositive/-negative.

There were 21 patients who had standard-risk underlying disease and 28 patients who suffered from poor-risk disease. Standard risk was defined by acute lymphoblastic leukemia (ALL) in first complete remission (CR1), acute myeloid leukemia (AML) in CR1, chronic myeloid leukemia (CML) in first chronic phase (CP1), and untreated (very) severe aplastic anemia (SAA). All other diagnosis were considered poor risk. One donor/recipient pair had negative EBV-serology, 4 discordant EBV-serology and the remaining donor/recipient pairs had positive EBV-serology (anti-viral capsid antigen Ig G) prior to transplantation. Patients experiencing an EBV reactivation $\geq$ 1,000 geq/ml were admitted to receive pre-emptive B-cell immunotherapy by use of a single infusion of rituximab (MabThera®; Roche, Basel, Switzerland). Prior to infusion, patients were carefully examined for signs and symptoms of EBV-LPD. Patients with an established diagnosis of EBV-LPD were eligible for a therapeutic protocol including 2 infusions of rituximab followed by donor lymphocyte infusion (DLI) if the viral load had not been reduced to less than 50 percent by 72 hours after first rituximab infusion as previously described. [9] Transplant and rituximab protocols were approved by local institutional review boards and all patients provided informed consent.

Historical cohort

We included 85 consecutively treated patients receiving a TCD allogeneic hematopoietic stem cell transplantation as controls. The retrospective monitoring of EBV load was performed at weekly intervals in these patients; the results were reported recently. [10] Patients were treated at Utrecht Medical Center and at Erasmus Medical Center / Daniël den Hoed Cancer Center, Rotterdam, the Netherlands using the same protocols for transplantation and graft manipulation. Records of these 85 patients were updated for the present study.

EBV reactivation and EBV-lymphoproliferative disease
The real-time PCR assay for detection of EBV-DNA in plasma has been described before.[8] In short, primers were selected from the EBV-DNA genome encoding the BNRF1-p143 protein and results were related to a reference standard quantified by electron microscopy. The assay accurately detects viral DNA in plasma over a linear span between 50 and 10^7 geq/ml. Reactivation was defined by a plasma EBV-DNA exceeding 50 geq/ml in donor/recipient pairs with positive EBV-serology prior to transplantation. Viral load was prospectively monitored at weekly intervals starting at the time of stem cell transplantation until day 180 after stem cell transplantation and beyond day 180 in patients with chronic graft-versus-host disease (GVHD). A diagnosis of EBV-LPD was made on lymph node histology and/or cytology as described recently, [9,10] and was classified according to the criteria of Knowles. [19]

Transplantation
Most patients received cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (6 Gy on each of 2 successive days with partial shielding of the lungs for a total lung dose of 2 x 4.5 Gy) as a conditioning regimen (Table 1). Rabbit anti-thymocyte globulin (ATG) (Imtix Sangstat, Amstelveen, The Netherlands) was added (2 mg/kg from day –7 through day –3) for patients who received an unrelated donor graft for prevention of rejection (Table 1). Hematopoietic stem cells were obtained by bone marrow aspiration under general anesthesia or by peripheral blood stem cell collection. Grafts were partially depleted of T-cells using sheep erythrocyte rosetting or CD34 selection (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany). The T-cell number in the graft was adjusted to a fixed low number of 10^5 $CD3^+$ T-cells/kg, if the depletion procedure had resulted in less than 10^5 $CD3^+$ T-cells/kg (recipient body weight). [20] Supportive care protocols were as previously described. [9,10]

Pre-emptive therapy
Patients with an EBV reactivation ≥ 1,000 geq/ml within 180 days following allogeneic hematopoietic stem cell transplantation were admitted to receive pre-emptive rituximab. In order to verify for EBV-LPD at initiation of pre-emptive therapy, physical examination, computerized tomography (CT), bone marrow morphology and flow cytometry were

performed. Rituximab was given as a single infusion (375 mg/m^2 dissolved in 0.9% NaCl in a final concentration of 1 mg/ml) and immunosuppressive medication was continued. EBV load was monitored daily during the first 72 hours, then twice weekly until 2 negative test-results and thereafter at each outpatient visit. Absolute B-cell numbers were evaluated prior to and within 1 week and at 1, 3, 6, 9, and 12 months following rituximab infusion. Complete response to pre-emptive rituximab was defined as clearance of EBV-DNA (< 50 geq/ml) from plasma and absence of signs and symptoms of EBV-LPD.

Endpoints and statistical analysis

Data were analyzed as of May 2001. Endpoints of the study included: (1) incidence of viral reactivation $\geq$ 1,000 geq/ml, (2) the incidence of EBV-LPD, (3) EBV-LPD mortality, (4) EBV load and (5) B-cell recovery following pre-emptive rituximab infusion. Patient characteristics were compared with a group of previously described controls [10] using the Fisher exact test or Pearson chi-squared test, whichever appropriate in case of discrete variables, or the Wilcoxon rank-sum test in case of continuous variables. Time to EBV reactivation $\geq$ 1,000 geq/ml was determined from the date of stem cell transplantation until the date of last plasma sample. Time to EBV-LPD was measured from the date of viral reactivation $\geq$ 1,000 geq/ml. Patients without EBV-LPD were censored at the date of death or last follow-up. EBV-LPD-mortality was calculated from stem cell transplantation until death due to progressive EBV-LPD. Time to viral reactivation, time to EBV-LPD, EBV-LPD-mortality were estimated by the Kaplan-Meier method, [21] and the Kaplan-Meier curves of the 2 cohorts were compared using the logrank test. [22] All reported P-values are two-sided and a significance level $\alpha = 0.05$ was used.

3. Results

EBV reactivation and pre-emptive therapy

There were 27 of 49 (55%) prospectively studied patients showed EBV reactivation and 17 (35%) progressed to a viral load of $\geq$ 1,000 geq/ml. Median time to EBV reactivation $\geq$ 1,000 geq/ml was 112 days (range, 39-189 days) after stem cell transplantation. Median EBV-DNA level measured 2,100 geq/ml (range, 500-14,000) prior to admission and a median of 3 days (range, 1-14 days) elapsed between that day and initiation of pre-emptive therapy. Of these 17 patients, 2 appeared to present with active EBV-LPD upon examination (see below). The other 15 patients were eligible for pre-emptive therapy and received rituximab at a median time of 113 days after stem cell transplantation (range, 41-202 days). Of 15 treated patients, 14 had a complete and sustained response. EBV-DNA in plasma became undetectable after a median of 8 days (range, 1-46 days) (Table 2). Recurrent reactivations were not observed in any of these patients with a median follow up of 12 months (range, 1.0-24 months). One patient (case no. 5) did not respond as was evident by a continuing increase of viral load and progression to EBV-LPD.

Table 2. Pre-emptive rituximab after TCD allogeneic hematopoietic stem cell transplantation for prevention of EBV-LPD

Patient No.	Donor type	EBV ≥ 1000 geq/ml		Initiation of pre-emptive rituximab (d*)	Max EBV-DNA level (geq/ml)	EBV< 50 (geq/ml, d after start pre-emptive rituximab)	EBV-LPD
		Day of onset*)	EBV-load				
1	Sib	73	2,700	84	5,800	12	-
2	Sib	122	2,300	129	2,300	6	-
3	Sib	150	1,400	157	2,800	14	-
4	Sib	188	2,100	202	3,800	5	-
5	MUD	47	3,300	50	1,100,000	46	+
6	MUD	39	8,800	43	675,000	45	-
7	Sib	129	14,000	133	85,000	6	-
8	Sib	108	1,400	110	1,400	7	-
9	Sib	171	3,000	174	110,000	26	-
10	MUD	118	1,800	125	1,800	27	-
11	MUD	46	2,100	47	2,100	1	-
12	Sib	61	3,600	63	3,600	4	-
13	Sib	112	1,800	113	15,000	8	-
14	MUD	40	500	41	1,350	21	-
15	Sib	189	1,200	192	1,150	4	-

*Sib indicates HLA genotypically matched sibling donor; MUD, matched unrelated donor; EBV, Epstein-Barr virus; geq/ml, genome equivalents/ml; EBV-LPD, EBV-lymphoproliferative disease. *) days after stem cell transplantation.*

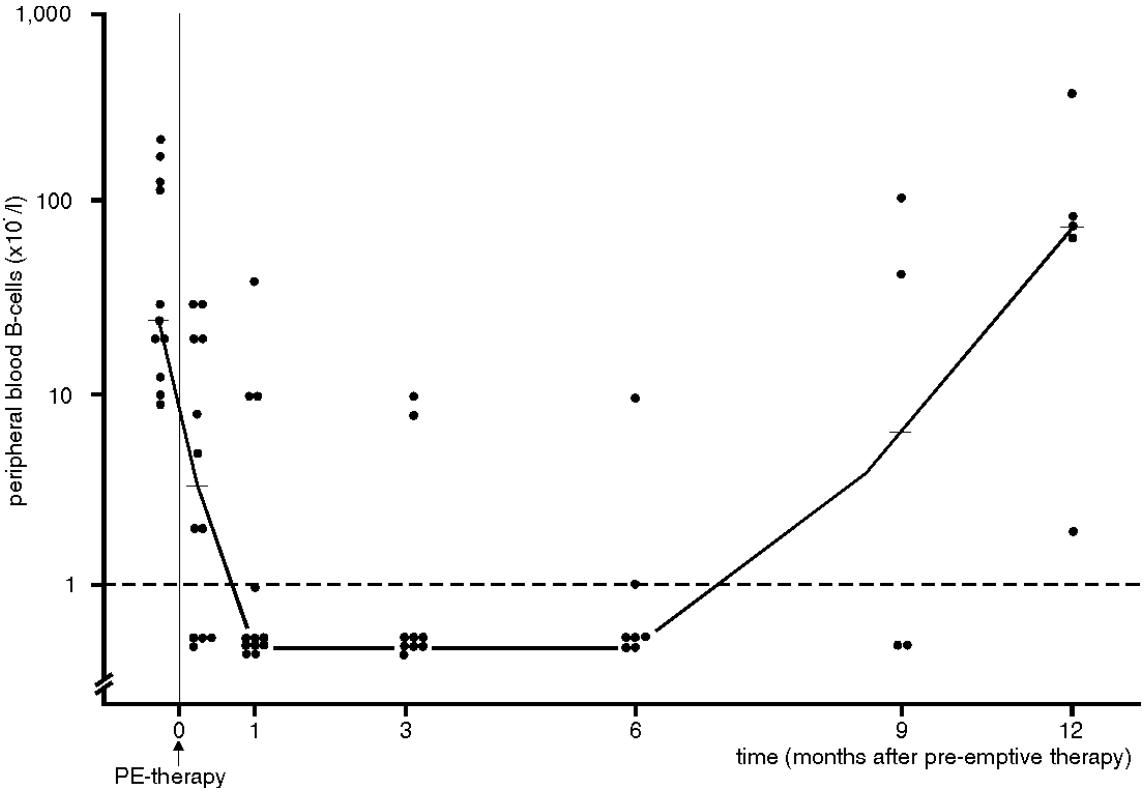

Figure 1. B-cell lymphopenia following rituximab treatment Median and individual peripheral blood B-cell numbers in recipients of a TCD allogeneic hematopoietic stem cell transplantation with EBV-DNA $\geq$ 1,000 geq/ml before and after pre-emptive rituximab (PE-therapy) given at day 0. (Dashed line denotes detection limit of assay, horizontal solid lines indicate median value).

This patient was treated with a second infusion of rituximab (375 mg/m^2) and donor lymphocyte infusion (1 x 10^6 CD3$^+$ T-cells/kg) (Table 2), and had a sustained response without subsequent EBV reactivation. B-cell numbers rapidly declined following rituximab infusion and became undetectable in 12 out of 15 patients. B-cell lymphopenia in these patients persisted for several months (Figure 1) and recovery started at approximately 6 months from pre-emptive therapy. Opportunistic infections (common toxicity criteria [CTC] grade 3 or 4) were observed in all 8 patients having extensive chronic GVHD. In contrast only 3 out of 7 patients without extensive chronic GVHD experienced CTC grade 3 or 4 infections following rituximab treatment (P=0.03). There were 4 B-cell lymphopenic patients who developed pneumonia, including 2 polymicrobial pneumonias (bacterial: 2, fungal: 4, viral: 1), all these 4 patients suffered at the time from extensive chronic GVHD necessitating intensive immunosuppressive therapy. Neutropenia (absolute neutrophil count < 0.5 x 10^9/l) within 4 weeks of infusion occurred in only 2 of the 15 patients; both had chronic extensive GVHD. The single patient (no. 5, Table 2),

who failed pre-emptive therapy and was treated using DLI, developed bronchiolitis obliterans organizing pneumonia, complicated by bacterial pneumonia.
Two patients presented with lymphadenopathy at the time of admission and were not eligible for pre-emptive therapy, because pathological and immunological examination of lymph node biopsies were consistent with a diagnosis of EBV-LPD. Both patients had received a TCD unrelated donor graft and had also received ATG as part of the conditioning regimen. They showed rapid progression of viral reactivation with 2 and 7 days, respectively, between the first signs of reactivation and the onset of lymphadenopathy (Figure 2). These 2 patients were enrolled in a therapeutical protocol, including 2 infusions of rituximab guided by viral load. Both patients obtained a complete response and they are alive at the date of last follow-up, day +338 and day +415 respectively. EBV-DNA levels were 8,750 geq/ml and 17,500 geq/ml at the time of clinical admission and complete and persistent clearance of EBV-DNA was achieved after 16 days and 34 days, respectively (Figure 2).

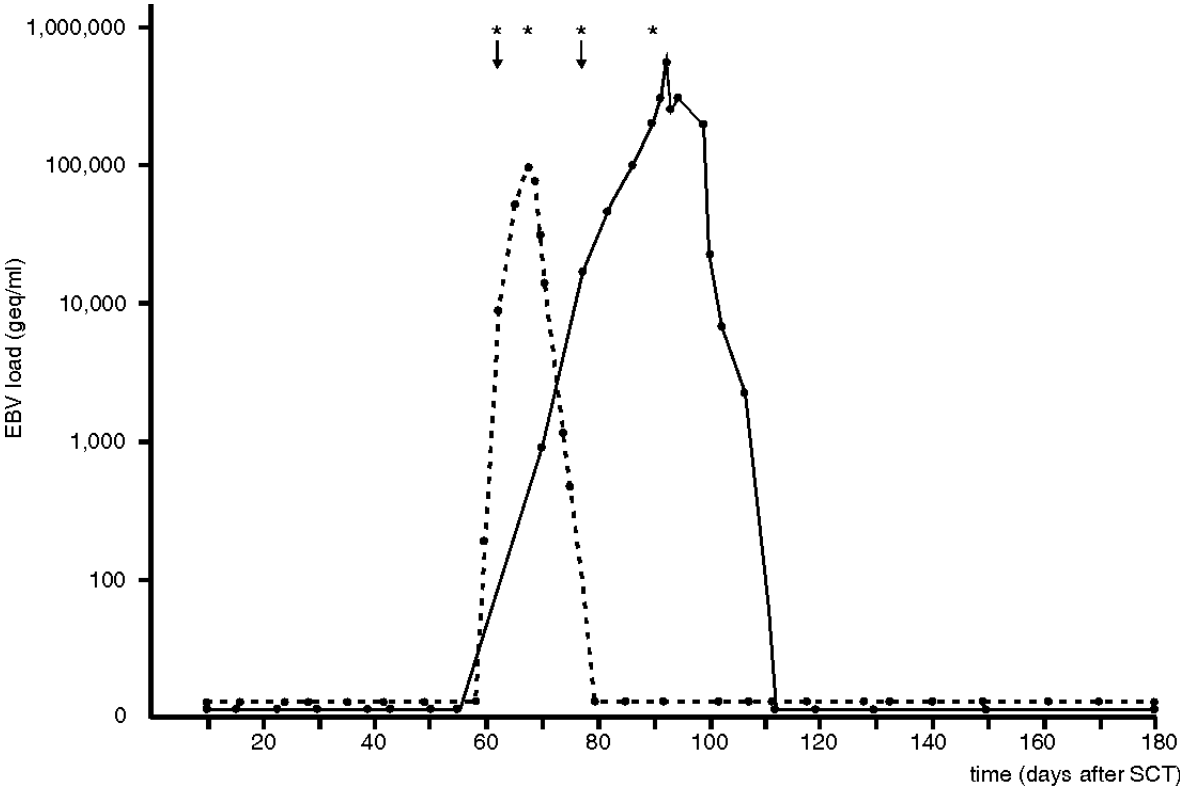

Figure 2. Viral load following therapy for established EBV-LPD. EBV load in 2 recipients of a TCD allogeneic hematopoietic stem cell transplantation with established EBV-LPD prior to planned pre-emptive therapy. EBV-LPD was diagnosed on lymph node biopsies in both patients at the day of first rituximab infusion. Both patients developed a sustained complete response after 2 successive infusions of rituximab combined with dose reduction of cyclosporin A.(↓ denotes diagnosis of EBV-LPD, * single infusion of 375 mg/m^2 rituximab).

Comparison with historical cohort

The positive and negative predictive values of a viral load of 1,000 geq/ml have been established in a group of 85 recipients of a TCD allogeneic hematopoietic stem cell transplantation. [10] Plasma samples were retrospectively examined for EBV reactivation at weekly intervals in these 85 patients. Considering a threshold level of 1,000 geq/ml, the negative predictive value was 100%. The cumulative probability of developing EBV-LPD was 38 ± 11% at 2 months and 49 ± 11% at 4 months from the date of EBV DNA $\geq$ 1,000 geq/ml (Figure 3A). Results of the current prospective study were compared with those historical controls with respect to EBV reactivation, incidence of EBV-LPD, and EBV-LPD-mortality. Patient characteristics of patients at high-risk of progression to EBV-LPD as defined by reactivation of $\geq$ 1,000 geq/ml after a TCD allogeneic hematopoietic stem cell transplantation, did not differ between prospectively followed patients (n=17) and controls (n=26, Table 3). In both cohorts, the majority of patients suffered from poor-risk underlying disease. In the historical cohort 42% of patients had received ATG and an unrelated donor graft, as compared to 41% in the prospective cohort. In addition, graft characteristics were similar (Table 3).

Table 3. Characteristics of high-risk patients *

Parameter	Historical cohort (n=26)	Prospective study (n=17)
Age	40 (18-55)	40 (19-51)
Underlying disease Risk status SR/PR	2/24	4/13
Donor Type		
Sib	15	10
MUD	11	7
ATG	11	7
Stem cell source		
Bone marrow	24	14
Peripheral blood	2	3
Graft Characteristics		
CD3 (10^5/kg)	2.0 (1-6.8)	2.0 (1.0-4.0)
CD34 (10^6/kg)	1.6 (0.26-4.57)	1.7 (0.53-8.10)

SR/PR indicates standard- / poor-risk disease; Sib, HLA-genotypically matched sibling donor; MUD, matched unrelated donor; ATG, anti-thymocyte globulin.
* *High-risk, as defined by 1. allogeneic hematopoietic stem cell transplantation by partial TCD, 2. EBV load $\geq$ 1,000 geq/ml.*

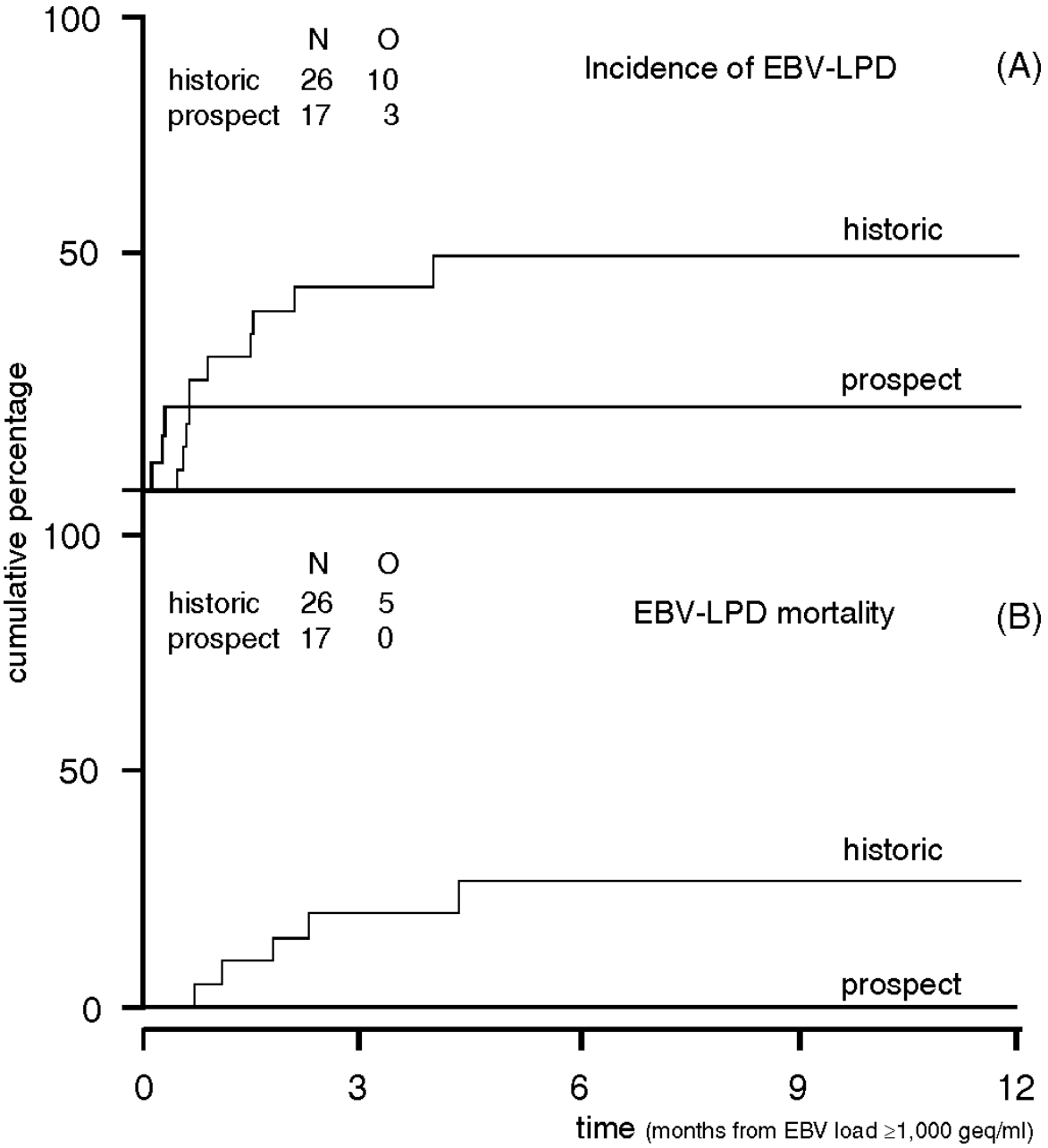

Figure 3. Incidence of EBV-LPD and EBV-LPD mortality as compared to historical controls. (A) Incidence of EBV-LPD. Cumulative incidence of EBV-LPD in historical control patients (n = 26) with EBV-DNA $\geq$ 1,000 geq/ml versus the incidence of EBV-LPD in the prospectively followed group after EBV-DNA $\geq$ 1,000 geq/ml (n=17) (P=0.13).
(B) EBV-LPD mortality. Cumulative incidence of EBV-LPD mortality in historical control patients (n=26) with EBV-DNA $\geq$ 1,000 geq/ml versus the incidence of EBV-LPD mortality in patients (n = 17) prospectively studied (P=0.04). N, indicates numbers of patients studied; O, indicates observations (endpoints) done in the study group.

GVHD prophylaxis was similar in both cohorts and consisted of TCD and cyclosporin A when a sibling donor was used, ATG was added in case of a MUD stem cell transplantation. Cumulative incidences of acute- and chronic GVHD did not differ between both cohorts. The cumulative incidence of acute GVHD grade II-IV was 51% at 100 days post stem cell transplantation versus 54% at 100 days for prospectively followed patients and historical controls, respectively. The cumulative incidence for chronic GVHD (limited and extensive) was 37% for prospectively followed patients versus 36% in the historical cohort. The probabilities of viral reactivation > 50 geq/ml and ≥ 1,000 geq/ml were similar in both cohorts. Probabilities of EBV reactivation (≥ 1,000 geq/ml) at 4 months from stem cell transplantation were 26 ± 5% versus 28 ± 7% for historical controls and prospectively monitored patients, respectively (Figure 4). Among the historical group, 10 of 26 patients developed EBV-LPD of whom 5 died due to progressive EBV-LPD despite the therapeutic use of rituximab and DLI, and 3 other patients died due to extensive chronic GVHD secondary to DLI. Among the prospectively monitored patients, 1 of 15 patients treated preemptively developed EBV-LPD and 2 other patients presented with EBV-LPD before initiation of pre-emptive therapy (Figure 3A, P=0.13). None of these 17 patients died from progressive EBV-LPD (Figure 3B, P=0.04). Viral reactivation was abrogated in all of these 17 patients without any recurrences.

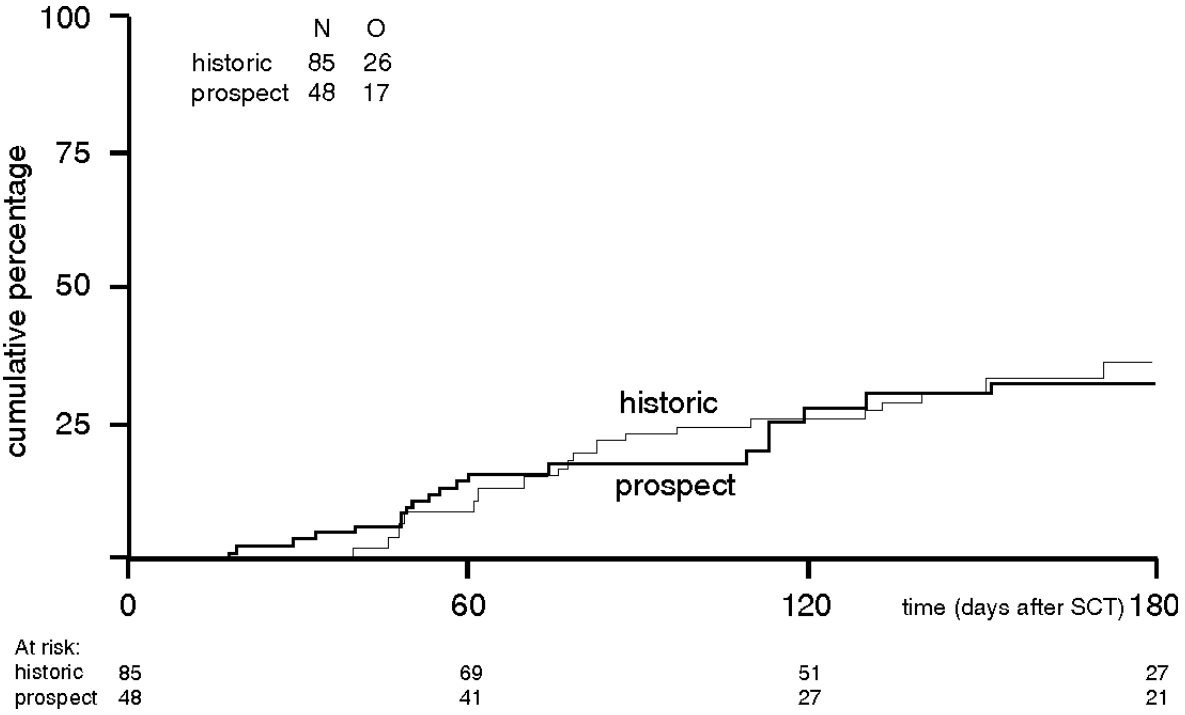

Figure 4. Cumulative incidence of EBV reactivation ≥ 1,000 geq/ml. Cumulative incidence of EBV reactivation ≥ 1,000 geq/ml in prospectively studied EBV-seropositive donor/recipient pairs (n=48) versus the incidence in the historical control group (n=85) (P=0.86).

4. Discussion

The present study shows that pre-emptive rituximab selectively administered to high-risk patients abrogates EBV reactivation and reduces the incidence of EBV-LPD. Furthermore, mortality due to EBV-LPD no longer contributed to treatment-related mortality in these prospectively monitored patients with EBV reactivation after TCD allogeneic hematopoietic stem cell transplantation.
Outcome of clinically established EBV-LPD is still not optimal, although new promising treatment modalities have been introduced, such as anti-CD20 immunotherapy [12,14,17,18] and adoptive transfer of T-cell immunity.[13,23-25] Failure of treatment may be due to rapidly progressive EBV-LPD,[9-11,14] development of resistance,[26] viral immune evasion, [27] and loss of CD20 antigen expression. [28] Studies focusing on the therapeutic value of rituximab have shown a mortality of 17-25% due to progressive LPD.[14,17] In addition, GVHD following donor lymphocyte infusion may also adversely affect outcome. [9,10,13,23] Therefore, effective preventive approaches may be preferred to reduce mortality associated with EBV-LPD. Such approaches, however, should specifically be developed for high-risk patients, because EBV-LPD is a rare complication after allogeneic hematopoietic stem cell transplantation and unnecessary treatment of patients with a low probability should be avoided. Retrospectively, we established a viral reactivation of 1,000 geq/ml as a threshold value with high positive and negative predictive values in recipients of a TCD allogeneic hematopoietic stem cell transplantation.[10] Using that threshold value, we were now able to selectively administer pre-emptive therapy to 15 out of 49 patients. EBV-LPD was effectively prevented by rituximab in those recipients. Only 1 out of 15 patients receiving pre-emptive therapy progressed to EBV-LPD, but the patient was rescued by a second infusion of rituximab and DLI as well. This patient had received ATG prior to TCD allogeneic hematopoietic stem cell transplantation, which may explain the more aggressive evolution of viral reactivation toward EBV-LPD. Retrospectively, ATG was strongly associated with a higher incidence of EBV reactivation and an earlier and more rapid evolution of reactivation and a higher incidence of EBV-LPD.[10,11,29] Two other patients, who had also received ATG, showed early viral reactivation, followed by rapid progression to EBV-LPD before pre-emptive therapy could be instituted. However, both patients developed a sustained complete response after a second infusion of rituximab given at a relatively early time point in the course of their disease, and EBV-LPD mortality was effectively prevented. Although these patients escaped the pre-emptive approach, the frequent monitoring allowed an early diagnosis and thereby an early therapeutic intervention.

Peripheral B-cell numbers rapidly declined following a single infusion of rituximab and became undetectable in 12 patients. In addition, B-cell lymphopenia persisted for several months (Figure 1). Only few relatively mild infections were observed in B-cell lymphopenic patients without chronic GVHD, which may be explained by unaffected plasma cell counts and thereby unaffected immunoglobulin production. [15] In contrast,

patients with B-cell lymphopenia and chronic extensive GVHD appeared to be at higher risk for opportunistic pneumonias, which may reflect the immunodeficiency associated with chronic GVHD rather than with B-cell lymphopenia as such. So far, patients treated with more intensive rituximab immunotherapy in other studies have not shown an increased risk of opportunistic infections despite effective and prolonged B-cell lymphopenia. [15,16]

The development of EBV-LPD is the result of uncontrolled B-cell proliferation due to failure of immunological control. Therefore, other investigators have focussed on a pre-emptive approach of improving EBV-specific immunity in patients at high-risk for EBV-LPD and reported on pre-emptive infusion of EBV-specific cytotoxic T-cells in patients with elevated EBV-DNA levels. [24,25] Effective prevention of EBV-LPD was strongly suggested by a decrease of viral DNA levels and a low incidence of EBV-LPD in the patient populations studied. The approach is, although attractive, hampered by the rather elaborate procedures needed to prepare EBV specific cytotoxic T-cells. These and other studies have focussed on detection of cellular EBV-DNA levels to identify patients at highest risk of EBV-LPD. [4-7,24,25] We have used a quantitative PCR of EBV-DNA in plasma, which appeared to accurately predict impending EBV-LPD as well as response to therapy in recipients of a TCD-allogeneic hematopoietic stem cell transplantation. [8-10] Especially in recipients of stem cell transplants, quantification of viral load in plasma may be advantageous, as the technique is relatively fast and simple and patients with lymphopenia may have insufficient cell numbers for reliable quantification of peripheral blood mononuclear cell viral load. Furthermore, response to therapy of EBV-LPD may be followed more accurately by plasma PCR, as suggested by our findings and those of Yang et al. [9,30] Our assay may monitor lytic EBV infection and/or release of viral DNA from latently infected B-cells. If viral DNA is mainly derived from lytic replication, our results may suggest that active lytic infection contributes to the development of EBV-LPD, which results would be in line with a number of previous studies showing that active lytic infection participates in the development of EBV-LPD. [31-35] The latter studies have raised the question whether the prophylactic or pre-emptive administration of antiviral agents such as aciclovir or ganciclovir would prevent EBV-LPD following transplantation of allogeneic stem cells or allogeneic solid organs. However, preventive approaches with these antiviral drugs have generally been disappointing, (reviewed by Davis [31]), which may be explained by their inability to inhibit proliferating B-cells, once these have acquired an autonomous growth pattern.

In conclusion, we developed a risk-adapted strategy to abrogate viral reactivation and prevent EBV-LPD. It is shown that a single infusion of rituximab is effective as pre-emptive therapy for EBV-LPD and prevention of EBV-LPD mortality in a selected group of allogeneic hematopoietic stem cell transplantation recipients at high-risk for EBV-LPD. Considering the possible rapid and aggressive evolution of viral reactivation towards EBV-LPD in recipients having received ATG, the frequent monitoring of reactivation and early institution of pre-emptive therapy should be advocated in recipients treated with ATG.

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6. Impaired recovery of Epstein-Barr virus (EBV)-specific CD8+ T-cells after allogeneic hematopoietic stem cell transplantation identifies patients at high risk for progressive EBV reactivation and lymphoproliferative disease

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(Submitted)

Abstract

The cytotoxic T-cell immune response towards Epstein-Barr virus (EBV) is considered pivotal for prevention of lymphoproliferative disease (LPD) in recipients of an allogeneic hematopoietic stem cell transplantation (allo-SCT). The aim of this study was to evaluate the recovery of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation and to study the relation between EBV-specific $CD8^+$ T-cells, EBV reactivation and EBV-LPD. EBV-specific immunity was studied using a panel of 11 HLA class I tetramers presenting peptides derived from 7 EBV proteins. Blood samples were taken at regular intervals after allogeneic hematopoietic stem cell transplantation in 61 patients and EBV-DNA levels were assessed by real-time polymerase chain reaction (PCR). Forty-five patients showed EBV-reactivation, including 25 with high-level reactivation (≥ 1,000 geq/ml). Nine of these patients progressed to EBV-LPD. $CD8^+$ T-cells specific for latent and lytic EBV epitopes repopulated the peripheral blood at similar rates. Absolute numbers of EBV-specific $CD8^+$ T-cells increased after allogeneic hematopoietic stem cell transplantation to normal levels within 6 months in the majority of patients. Concurrently, the incidence of EBV reactivation strongly decreased. Patients with insufficient recovery were at higher risk for EBV reactivation in the first 6 months after stem cell transplantation. Absence of EBV-specific $CD8^+$ T-cells in patients with high-level reactivation was significantly associated with the subsequent development of EBV-LPD (P=0.048). Thus, the earlier defined positive predictive value of approximately 39%, based on high-level viral reactivation only, increased to 100% in patients without EBV-specific T-cells. These results show that the absence of EBV-specific $CD8^+$ T-cells in patients with high-level viral reactivation may identify a subgroup of patients at extremely high risk for EBV-LPD, and support that EBV-specific $CD8^+$ T-cells may protect allogeneic hematopoietic stem cell transplantation recipients from progressive EBV-reactivation and EBV-LPD.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous γ-herpesvirus that infects more than 90% of the world population. Following primary infection in the oropharynx, EBV remains latently present in B-cells. [1] Latent EBV infection is normally controlled by a cell-mediated immune response and $CD8^+$ T lymphocytes directed against the immunodominant latent proteins EBNA3A, -3B, 3C and the lytic proteins BZLF1 and BMLF1 can be detected in the circulation in the majority of healthy EBV seropositive individuals. [2-4] EBV-infected B-cells may evolve to lymphoproliferative disease (EBV-LPD) in immuno-suppressed transplant recipients, due to inhibition of immunological control of latently infected B-cells. EBV-LPD is a serious complication following allogeneic hematopoietic T-cell depleted allogeneic hematopoietic stem cell transplantation (allo-SCT) and solid-organ transplantation and may be associated with considerable mortality.[5-8] Quantifying EBV-DNA in plasma, currently allows for the monitoring of EBV reactivation in transplant recipients.[9-11] We recently reported a longitudinal study that revealed EBV reactivation as a frequent event after allogeneic hematopoietic stem cell transplantation.[11] In addition, EBV-LPD could be quantitatively predicted by the frequent monitoring of viral load in plasma. A viral load of 1,000 genome equivalents per millilitre (geq/ml) proved to be a level of EBV reactivation associated with a positive predictive value of 39 % and a negative predictive value of 100%.[11]

The cytotoxic T-cell immune response towards EBV is considered important for controlling EBV in allogeneic hematopoietic stem cell transplantation recipients and the prevention of EBV-LPD.[1,3] Especially, the clinical success of adoptive cellular immunotherapy of EBV-LPD using EBV-specific cytotoxic T lymphocytes (CTL) has indicated the pivotal role of these CTL in controlling EBV in allogeneic hematopoietic stem cell transplant recipients.[12-14] While the overall recovery of $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation has been studied extensively, little is known as regards the recovery of EBV-specific $CD8^+$ T-cells in allogeneic hematopoietic stem cell transplantation patients.[15-18] Antigen-specific $CD8^+$ T-cells can be enumerated by staining with tetrameric MHC-class I-peptide complexes. It has revealed a much higher frequency of antigen-specific circulating T cells than estimated before by limiting dilution assays. [2,19-21] The aim of this study was to evaluate the recovery of EBV-specific $CD8^+$ T-cells using tetramer technology and to evaluate the relationship between regeneration of EBV-specific T-cells and viral reactivation and progression to EBV-LPD.

2. Patients and methods

Patients

The study population included 61 consecutive patients, who received a T-cell depleted allogeneic hematopoietic stem cell transplantation between January 1998 and December 2000. Patient, donor and graft characteristics are presented in Table 1.

Table 1. Patient characteristics

Parameter	Study population (n=61)
Sex male/female (n)	32/29
Median age (y, range)	38 (16-55)
Diagnosis (n):	
AML	17
ALL	10
MDS	1
CML	8
SAA	1
MM	8
Lymphoma	16
Donor type (n)	
Sib	39
MUD	22
Conditioning regimen (n)	
Cy/TBI	35
Cy/TBI/ATG	23
Bu/Cy	3
Graft characteristics: (median, range)	
CD3 x 10^5/kg	2.0 (1.0-7.5)
CD34 x 10^6/kg	1.6 (0.5-8.8)
EBV-serology (n):	
D+R	3
D+R+	55
D-R+	3
Stem cell source (n):	
BM	51
PB	10

Legend to table 1.
AML indicates acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome; CML , chronic myeloid leukaemia; SAA, severe aplastic anaemia; MM, multiple myeloma; Sib, HLA identical family donor; MUD, matched unrelated donor; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; ATG, anti-thymocyte globulin; D+/- , EBV-seropositive/seronegative donor; R+/-, EBV seropositive/seronegative recipient; BM, bone marrow; PB, peripheral blood.

Patients were positive for at least one of the following HLA-alleles: A*0201, A*1101, B*0702, B*0801 and B*3501. The distribution of these HLA-alleles, corresponding to the EBV-tetramers, is shown in Table 2. The HLA-A*0201 allele was present in 35 patients, HLA-A*1101 in 7 patients, HLA-B*0702 in 17 patients, HLA-B*0801 in 17 patients and HLA-B*3501 in 12 patients. All patients were conditioned with cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (12 Gy in 2 fractions with partial lung shielding). Rabbit ATG (Imtix Sangstat, Amstelveen, The Netherlands) was given for prevention of rejection in recipients with an unrelated donor at days –7 to –4 preceding transplantation (total dose: 8 mg/kg). If patients had previously been treated with locoregional irradiation, the conditioning regimen consisted of oral busulfan (4 mg/kg on each of 4 successive days) and cyclophosphamide (120 mg/kg). Partial T-cell depletion was performed using sheep erythrocyte rosetting (n=31) or CD34 selection (Miltenyi, Bergisch Gladbach, Germany) (n=30). Ten patients received peripheral blood-derived stem cells and 51 patients received bone marrow-derived stem cells. Cyclosporin A (3 mg/kg) was given as graft-versus-host prophylaxis from day –3 until day + 100 after allogeneic hematopoietic stem cell transplantation. All patients received ciprofloxacin and fluconazole for prevention of infection during neutropenia, and cotrimoxazole was given after neutrophil recovery until day 180 after allogeneic hematopoietic stem cell transplantation. Erythrocyte and platelet products for transfusion were filtered to remove leukocytes and subsequently irradiated (25 Gy).

Quantitative EBV-specific PCR

EBV-DNA levels were measured as described previously. [22] Briefly, 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. A standard with a fixed EBV copy number (ABI Advanced Biotechnologies, Columbia, MD, USA) was used for standardisation of the assay. Serial dilutions ranging from 10 to 10^7 EBV-DNA genome equivalents per millilitre (geq/ml) were made to characterise linearity, precision, specificity, and sensitivity. The Taqman assay detects viral DNA in plasma over a linear range between 50 and 10^7 geq/ml. Test results below 50 geq/ml were considered negative. Plasma samples for quantification were obtained at weekly intervals in patients without EBV-LPD and daily in patients with established EBV-LPD. EBV reactivation was defined as EBV DNA levels in plasma of > 50 geq/ml, EBV DNA levels of <1,000 geq/ml were

defined as low-level reactivation and EBV DNA levels of ≥ 1,000 geq/ml were defined as high-level reactivation in this particular group of patients.[11] As from January 1999, patients with high-level reactivation were treated pre-emptively with rituximab (375mg/m^2) (Roche, Basel, Switzerland) as recently described. [23]

Table 2. HLA-allele distribution

HLA-allele	n
A*0201	17
A*0201 A*1101	1
A*0201 B*0702	4
A*0201 B*0702 B*3501	2
A*0201 B*0702 B*0801	2
A*0201 B*0801	8
A*0201 B*3501	1
A*1101	5
A*1101 B*3501	1
B*0702	6
B*0702 B*0801	1
B*0702 B*3501	2
B*0801	5
B*0801 B*3501	1
B*3501	5

EBV-LPD diagnosis

EBV-LPD was diagnosed using histology and/or cytology and was classified according to the criteria of Knowles et al. [24] Immunohistology included staining with monoclonal antibodies specific for CD19 (BD Biosciences, San Jose, CA), EBV-encoded latent membrane protein 1 (LMP1) and kappa and lambda light chains (all from Dako, Glostrup, Denmark). In situ hybridisation was performed to detect the expression of EBV encoded RNA's (EBER) using an EBV-EBER probe (Dako). PCR was performed for detection of EBV-DNA for the BamHI fragment. Furthermore, EBV-LPD staging included physical examination, whole-body computed tomography (CT) scanning and detection of monoclonal B-lymphocytes in blood and bone marrow derived mononuclear cell suspensions using flow cytometry. Patients with a diagnosis of EBV-LPD were treated

with interruption of immunosuppressive drugs and rituximab guided by viral load as described. [25] Patients were treated with donor leukocyte infusions, if no response ensued following rituximab. [25]

Enumeration of $CD8^+$ T lymphocytes specific for class I HLA-restricted EBV-encoded epitopes

Heparinised blood samples were obtained from transplant recipients at 2, 3, 6, 9, 12, 18 and 24 months after allogeneic hematopoietic stem cell transplantation and from healthy controls, i.e. 37 laboratory workers and 39 allogeneic hematopoietic stem cell transplantation donors prior to mobilisation of bone marrow donation. Peripheral blood mononuclear cells (MNC) were isolated using Ficoll-Isopaque density grade centrifugation and cryo-preserved until required. In parallel, absolute numbers of $CD8^+$ T lymphocytes were assessed in whole blood samples within 6 hours after venipuncture. $CD8^+$ T-cells were enumerated using a 3-color, single platform whole blood immunostaining technique. [26,27] The following monoclonal antibodies were used: CD45 (clone 2D1, conjugated with fluorescein isothiocyanate (FITC); BD Biosciences (BD), San Jose, CA), CD8 (clone SK1 conjugated with phycoerythrin (PE); BD) and TCR PAN α/β (clone BMA031 conjugated with PE-Cy5; Immunotech, Marseille, France). Of each sample 50,000 leukocytes were acquired using a FACSCalibur flow cytometer (BD). During data analysis, $CD8^+$ T-lymphocytes were defined as events with low to medium forward light scatter, low sideward light scatter, $CD45^+$, $TCR\alpha/\beta^+$ and $CD8^+$. The proportion of EBV-specific $CD8^+$ T-cells was assessed using tetramer technology on cryo-preserved and thawed MNC. EBV tetramers used in this study have been characterized as described before and are summarised in Table 3. [28-31] Mononuclear cell suspensions were incubated with EBV tetramers and CD8 (clone SK1 conjugated with allophycocyanin) for 30 min on melting ice. After 1 wash, cells were resuspended in PBS containing 1µg/ml 7-aminoactinomycin D (7-AAD; Sigma, St. Louis, MO). Following acquisition of 20,000 living $CD8^+$ T-lymphocytes (defined as having low to intermediate forward light scatter, low sideward light scatter as well as being $7\text{-}AAD^-$ and $CD8^+$), the proportions of living $CD8^+$ T-cells binding the tetramer(s) was assessed. The absolute number of circulating tetramer-binding $CD8^+$ T-cells was calculated from the proportion of $CD8^+$ T-cells binding the tetramer and the simultaneously obtained absolute $CD8^+$ T-cell count. [27] The lower limit of detection was 0.1 EBV-specific $CD8^+$ T-cells/µl.

Statistical analysis

Regeneration kinetics as a function of time were described for the individual EBV-specific $CD8^+$ T-cell subsets. As the regeneration kinetics of $CD8^+$ T-cells specific for latent and lytic EBV epitopes did not differ significantly (see results), these data were pooled for the subsequent analyses addressing the interaction between recovery of EBV-specific $CD8^+$ T-

cells, EBV reactivation and development of EBV-LPD. In these analyses, each patient was entered at each time point with a single EBV-specific $CD8^+$ T-cell count. For patients studied with multiple tetramers, the result of the EBV-specific $CD8^+$ T-cell subset with the highest absolute count was used for these analyses. EBV-specific $CD8^+$ T-cell recovery was considered effective when any EBV-specific $CD8^+$ T-cell subset had reached the threshold level of ≥ 0.5 cells/μl. Fisher's exact test was used to analyse 2-by-2 tables. Statistical evaluation of the differences between two groups was performed using the non-parametric Mann-Whitney U ranking test. P values < 0.05 were considered significant.

3. Results

Clinical outcome of EBV reactivation

EBV serology was positive in both donor and recipient in 55 out of 61 patients. In 3 out of 61 patients, EBV serology was positive in the donor and negative in the recipient. EBV serology was negative in the donor and positive in the recipient in the 3 remaining patients. Forty-five patients showed EBV reactivation (> 50 geq/ml). The median time to first reactivation was 65 days (range, 4 - 447). High-level EBV reactivation (≥ 1,000 geq/ml) was apparent in 25 of the latter 45 patients. Ten of these 25 patients were pre-emptively treated with anti-B-cell monoclonal antibody therapy (rituximab). [23] None of these patients progressed to overt EBV-LPD. Among the 15 patients not treated pre-emptively, 9 progressed to EBV-LPD. Seven LPD-patients responded to therapy, the 2 non-responders died from progressive EBV-LPD (Table 4). The median follow-up for EBV-specific $CD8^+$ T-cell recovery and EBV reactivation was 10 months (range 2 to 38 months following stem cell transplantation).

Repopulation of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation

Recovery of EBV-specific $CD8^+$ T-cells was evaluated by measuring these cells at 2, 3, 6, 9, 12, 18 and 24 months after allogeneic hematopoietic stem cell transplantation using a panel of 11 EBV-specific tetramers (Table 2, Table 3). Figure 1 shows the recovery patterns of $CD8^+$ T-cell subsets with different EBV specificities. As only few datapoints for the epitopes AVF and IVT were available, data for these epitopes are not shown. Normal values were defined for each epitope as the 5 to 95 percentile values assessed in 20-25 healthy EBV-seropositive donors (Table 3). The recovery directed against both lytic and latent epitopes appeared to be relatively fast. Most EBV-specific $CD8^+$ T-cell subsets returned to their respective normal ranges within 3 – 6 months after allogeneic hematopoietic stem cell transplantation in the majority of patients (Figure 1). With respect to lytic antigen derived epitopes, high median numbers were observed for GLC and RAK specific T-cells at 3 months post- stem cell transplantation.

Table 3. EBV tetramers used in this study

EBV antigen (co-ordinates)	HLA-restriction	Sequence	Reference	Healthy donors* Median (5^{th}-95^{th} percentile) [n]
Latent				
EBNA1 (407-417)	B*3501	HPVGEADYFEY	30	2.0 (0.2 - 5.2) [20]
EBNA3A (379-387)	B*0702	RPPIFIRRL	29	1.8 (0.3 - 4.7) [20]
EBNA3A (502-510)	B*0702	VPAPAGPIV	29	0.6 (<0.5-3) [20]
EBNA3A (325-333)	B*0801	FLRGRAYGL	28,29	1.4 (0.4 - 3.9) [21]
EBNA3A (458-466)	B*3501	YPLHEQHGM	30	1.3 (0.1 – 50) [20]
EBNA3B (399-408)	A*1101	AVFDRKSDAK	ur	1.2 (0.9 – 4.0) [11]
EBNA3B (416-424)	A*1101	IVTDSVIK	ur	0.6 (0.4 – 1.5) [11]
EBNA3C (284-293)	A*0201	LLDFVRMGV	29	0.9 (0.5 - 5.1) [20]
LMP2 (426-434)	A*0201	CLGGLLTMV	29	0.5 (0.2 - 5.7) [22]
Lytic				
BZLF1 (190-197)	B*0801	RAKFKQLL	28	2.9 (0.2 – 32) [20]
BZLF1 (54-64)	B*3501	EPLPQGQLTAY	31	5.0 (0.2 – 43) [20]
BMLF1 (280-288)	A*0201	GLCTLVAML	28	1.3 (0.4 – 10) [25]

** Median and 5^{th} and 95^{th} percentile of absolute numbers of EBV-specific $CD8^+$ T-cells determined in healthy EBV-seropositive donors. Between brackets the number of healthy donors analysed; ur indicates unpublished results D. van Baarle, F. Miedema.*

Table 4. Patient outcome

Parameter	No. of patients (n=61)
No EBV reactivation	16
EBV reactivation	45
50 – 1000 geq/ml	20
≥ 1000 geq/ml	25
EBV-LPD	9
EBV-LPD-mortality	2

As regards the latent epitopes, the $CD8^+$ T-cells directed against most of these epitopes also repopulated to normal levels within 3-6 months after allogeneic hematopoietic stem cell transplantation, but T-cells with specificity for the LLD-epitope, derived from EBNA-3C, recovered by 9 months after stem cell transplantation. Of note, GLC-, FLR- and HPV-specific T-cells recovered to supranormal levels within 3-6 months in 5 patients and also remained at those levels. Only 1 out of these 5 patients developed EBV reactivation, but the EBV viral load in that particular patient was rapidly cleared and did not exceed the low level of 80 geq/ml. The recovery of EBV-specific $CD8^+$ T-cells was delayed in patients who received ATG as part of the conditioning regimen. The effect of ATG on the recovery of RAK-specific T-cells is illustrated in Figure 2. It shows that patients, who didn't receive ATG as part of the conditioning regimen, had recovered RAK-specific T-cells to normal levels by 3 months after stem cell transplantation, while these levels had not been reached in patients pretreated with ATG until 9 months after stem cell transplantation. A similarly delayed recovery of EBV-specific $CD8^+$ T-cells following ATG was also observed for the lytic epitope EPL and the latent epitopes CLG, RPP, FLR, HPV and YPL (results not shown).

EBV-specific $CD8^+$ T-cells and EBV reactivation

The relation between the recovery of EBV-specific $CD8^+$ T-cells and EBV reactivation was studied by evaluating patients at risk in time intervals, starting from a particular time-point of T-cell monitoring until the next time-point. Results are presented in Table 5. At each time-point, only those patients were included of whom EBV-specific $CD8^+$ T-cells had been enumerated at least once and for whom follow-up for EBV reactivation was effectively monitored until the next time-point of evaluation of T-cell recovery. It is shown that the number of patients who recovered EBV-specific $CD8^+$ T-cells rapidly increased in time.

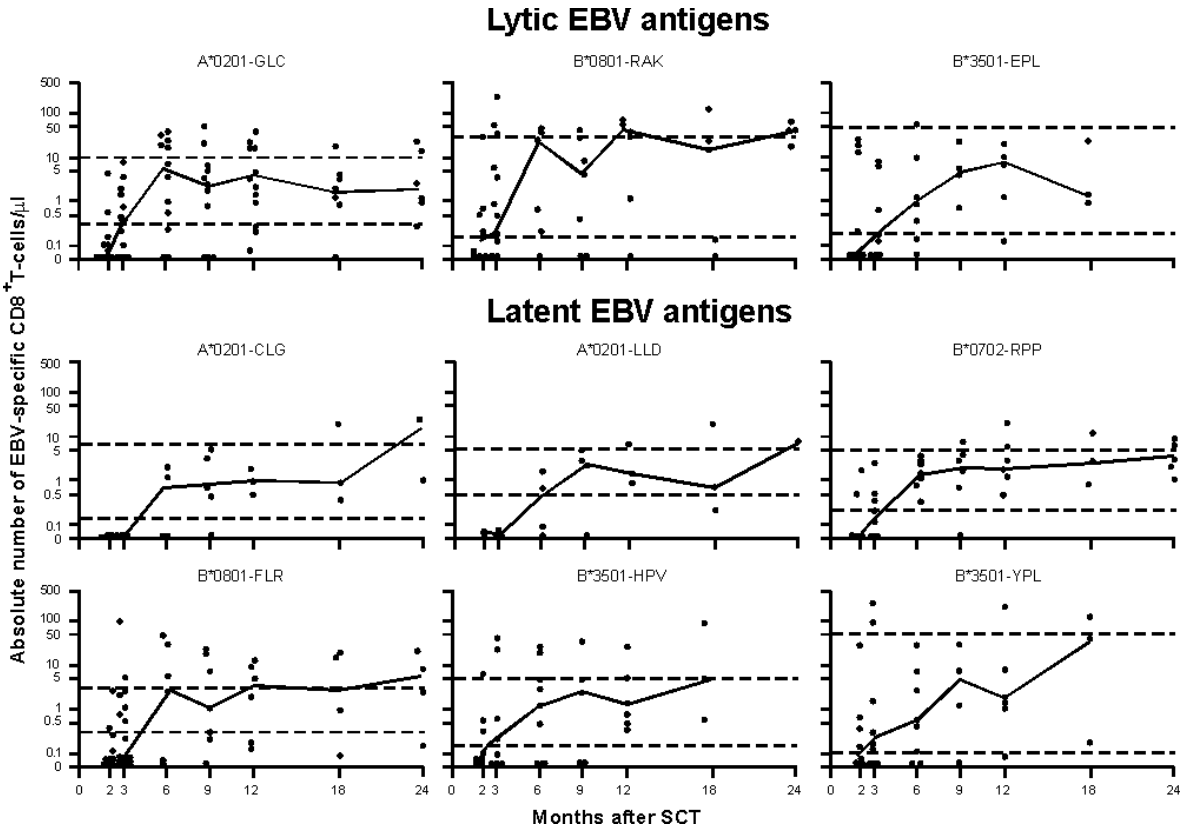

Figure 1. Repopulation of EBV-specific CD8$^+$ T-cells after allogeneic T-cell depleted hematopoietic stem cell transplantation. Each panel represents the recovery of CD8$^+$ T-cells directed against a single EBV-specific epitope as measured by tetramer technology. IVT and AVF are not included, since only few datapoints were available. For each EBV-encoded epitope, the median values per time point are connected with a line. Horizontal lines indicate the normal ranges as defined by the 5th and the 95th percentiles of EBV-specific CD8$^+$ T-cells as measured in healthy EBV-seropositive donors.

Seven out of 35 patients (20%) had recovered EBV-specific CD8$^+$ T-cells by 2 months and 73% (22/30) of patients had done so by 6 month following SCT. This increase was accompanied by an increase in the median level of EBV-specific T-cells during the first 6 months after allogeneic hematopoietic stem cell transplantation, which remained at a stable level thereafter (Table 5, Figure 1). Concurrently, the incidence of EBV reactivation decreased from 30-40% at 2-3 months to less than 20% of patients after 6 months post-stem cell transplantation. These data show that most EBV reactivations occurred during the period of insufficient EBV-specific CD8$^+$ T-cell recovery. A trend towards a correlation between insufficient recovery and EBV reactivation was observed for those patients, who had been monitored for T-cell recovery at 2 and/or 3 months post- stem cell transplantation and monitored for EBV reactivation up to the 6 month time-point.

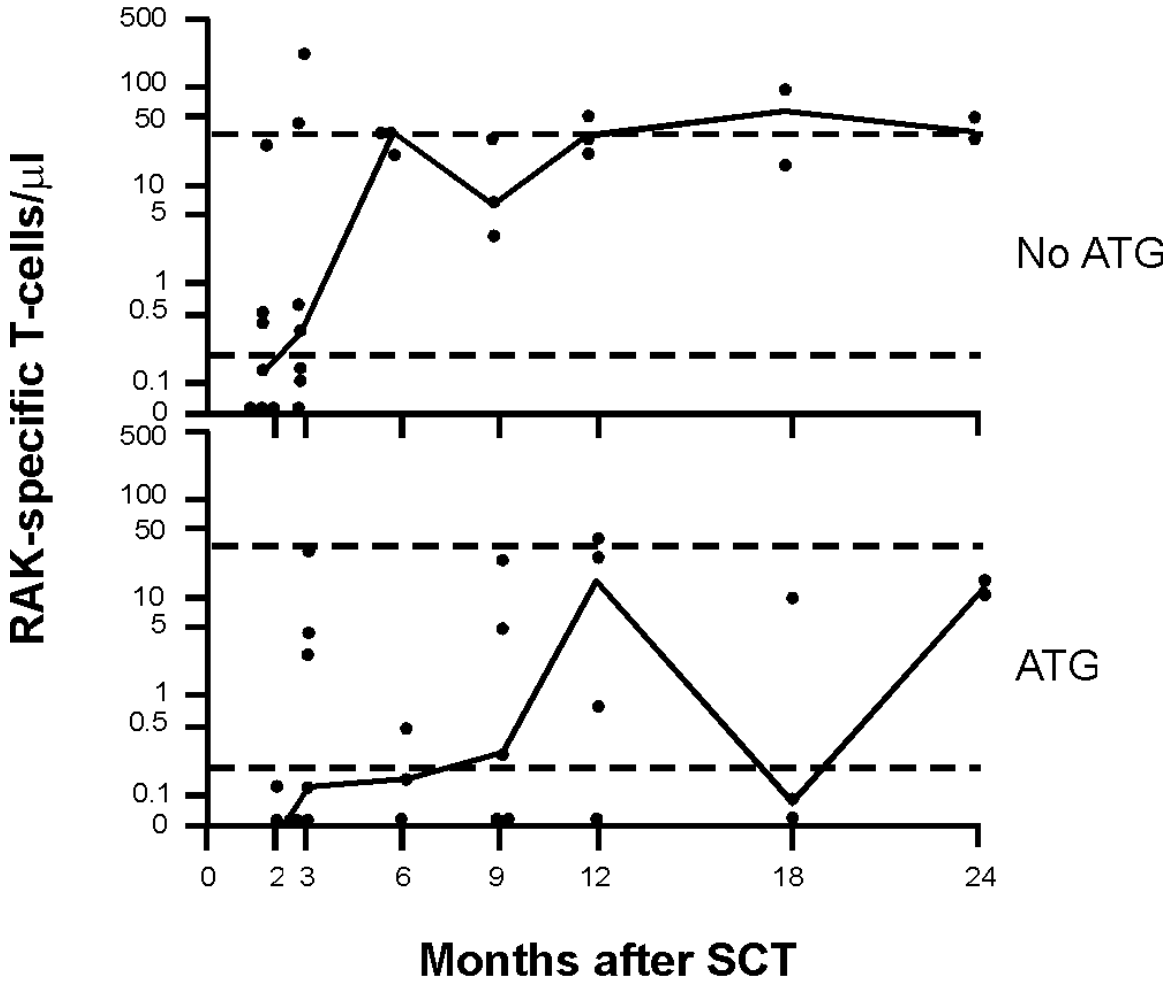

Figure 2. ATG delays the recovery of RAK-specific CD8$^+$ T-cells after allogeneic hematopoietic stem cell transplantation. RAK-specific T-cells were measured following allogeneic hematopoietic stem cell transplantation in patients who did not receive ATG (upper panel) and patients who received ATG as a part of the conditioning regimen (lower panel). See further legend to Figure 1.

Sixteen out of a total of 43 patients, studied at 2 and 3 months, effectively recovered an EBV-specific CD8$^+$ T-cell response before 6 months and only 3 of them (19%) developed EBV reactivation. No detectable EBV-specific CD8$^+$ T-cells were found in 27 of 43 patients (63%), and 13 of these 27 patients developed EBV reactivation (48% versus 63%, P = 0.053). Thus, patients with recovery of EBV-specific CD8$^+$ T-cells at 2 –3 months after allogeneic hematopoietic stem cell transplantation are at lower risk to of EBV reactivation, as compared to patients with an impaired EBV-specific CD8$^+$ T-cell response.

Table 5. EBV-specific $CD8^+$ T-cell recovery and EBV reactivation

Parameter	Time-point after transplantation (months)						
	2	**3**	**6**	**9**	**12**	**18**	**24**
EBV-specific T-cell recovery							
• No. of patients recovered / No. of patients at risk**	7/35	13/37	22/30	16/22	15/17	12/14	3/3
• Level of recovery (T-cell/µl) Median (range)	< 0.1 *(0.1-28.9)*	0.16 *(0.1-253)*	3.6 *(0.1-54.9)*	4.52 *(0.1-166)*	6.3 *(0.1-224)*	3.44 *(0.1-151)*	12 *(3.5-23.6)*
EBV reactivation*							
• No. of patients reactivating / No. of patients at risk**	11/35	15/37	6/30	4/22	2/17	1/14	0/3

**) patients were evaluated in time intervals, starting from the time-point indicated until the next time-point,*

***) numbers of patients at risk pertime-point were determined by (1) availability of tetramer tests and (2) follow-up for EBV reactivation until next time-point*

EBV-specific CD8$^+$ T-cells in patients with high-level EBV reactivation

We subsequently evaluated whether the absence of EBV-specific CD8$^+$ T-cells would correlate with a high quantitative level of EBV reactivation (≥ 1,000 geq/ml), since these patients have been shown to be at high risk to develop EBV-LPD. [11] Patients with high-level EBV reactivation (≥ 1,000 geq/ml) and patients with low-level EBV reactivation (< 1,000 geq/ml) were compared. Twenty-four patients, in whom EBV-specific T-cells had been assessed prior to the onset of EBV reactivation, were studied. As shown in Table 6, only 2 of 15 patients (13%) developing high-level EBV reactivation had recovered EBV-specific T-cells preceding EBV reactivation, while 6 of 9 patients (67%) with low-level EBV reactivation had done so (13% versus 67%, P=0.02). These differences in absolute numbers of EBV-specific T-cells did not relate to a difference in absolute numbers of CD8$^+$ T-cells. Absolute numbers of CD8$^+$ T-cells preceding EBV reactivation were similar for both groups (< 1000 geq/ml and ≥ 1000 geq/ml) (data not shown).

Table 6. EBV low- and high-level reactivation and EBV-specific CD8$^+$ T-cells

EBV reactivation (geq/ml)	No. of patients with EBV-specific CD8$^+$ T-cells		P-value
	< 0.5/µ versus	≥ 0.5/µ	
50 -1000	3	6	0.02
≥ 1000	13	2	

High-level reactivation was defined as an EBV copy number ≥ 1,000 geq/ml

EBV-specific CD8$^+$ T-cells and EBV-LPD

Next we addressed the question whether the presence or absence of EBV-specific CD8$^+$ T-cells could improve the positive predictive value as earlier defined by quantitative viral load. [11] The presence of EBV-specific CD8$^+$ T-cells was analysed in 9 patients with high viral load (≥ 1000 geq/ml), who had not been treated pre-emptively with rituximab. Table 7 shows that EBV-LPD developed in all 5 patients with high viral load reactivation with no effective recovery of EBV-specific CD8$^+$ T-cells. In contrast, only 1 of 4 patients, who had effectively repopulated EBV-specific T-cells in the face of high viral load, developed EBV-LPD (P=0.048). Thus, the positive predictive value increased from 39% to 100% in patients with high-level reactivation without detectable EBV-specific T-cells. Conversely, the positive predictive value was reduced to 25% in patients with high-level reactivation, who actually did recover EBV-specific T-cells up to a level of ≥ 0.5 T-cells/µl. Of note, the earlier defined negative predictive value of a viral load of < 1,000 geq/ml was 100% and

therefore remained unchanged. [11] Next we studied the recovery of EBV-specific $CD8^+$ T-cells prior and after a diagnosis of EBV-LPD in further detail. Only results of the subset of EBV-specific T-cells with the highest absolute counts per patient and per time interval preceding and following a diagnosis of EBV-LPD are depicted (Figure 3). Nine EBV-LPD-patients were studied after LPD-diagnosis and 6 patients of them could also be studied before diagnosis (Table 7).

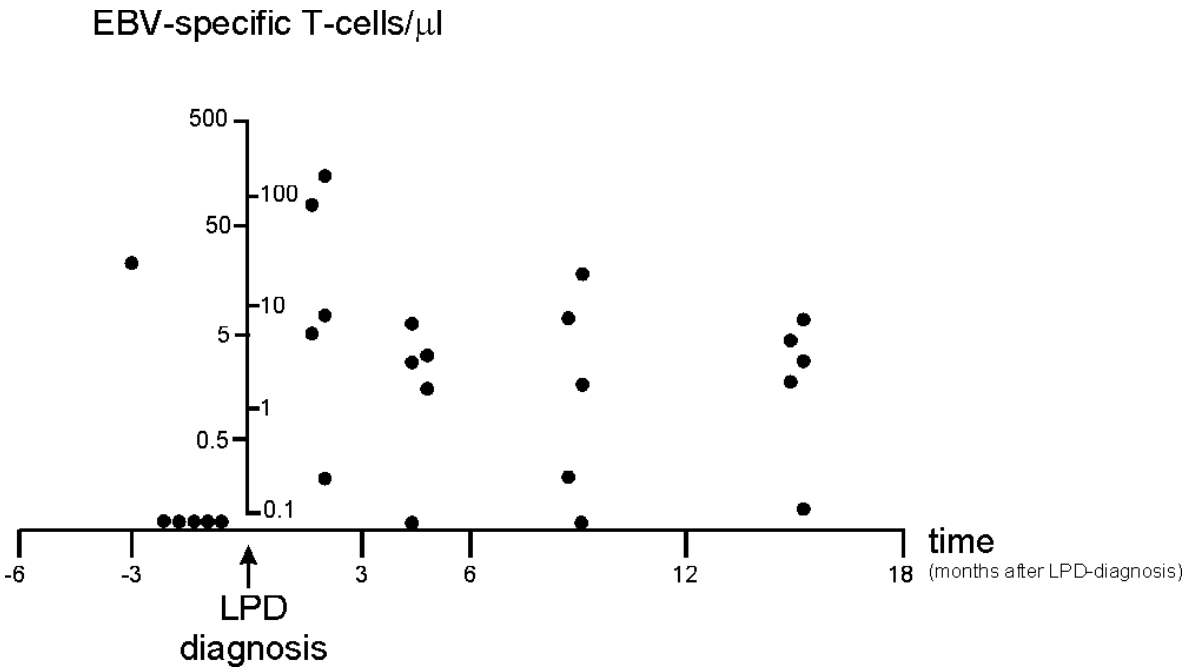

Figure 3. Recovery of EBV-specific $CD8^+$ T-cells prior and after a diagnosis of EBV-LPD. Absolute numbers of EBV-specific T-cells preceding and following EBV-LPD diagnosis in 9 patients. Six patients were evaluated prior to EBV-LPD diagnosis. Only the single highest value of the various tetramer-binding T-cells per patient are depicted.

Table 7. EBV-LPD and EBV-specific $CD8^+$ T-cells

Presence of EBV-specific T-cells (≥ 0.5/µl)	No. of patients with EBV-DNA ≥ 1000 geq/ml**		P-value
	Without EBV-LPD	Progressing to EBV-LPD	
Absent	0	5	0.048
Present	3	1	

*Absolute numbers of EBV-specific $CD8^+$ T-cells were monitored from allogeneic hematopoietic stem cell transplantation until day 180 or until diagnosis and treatment of EBV-LPD. **Patients with high-level reactivation receiving pre-emptive rituximab were excluded.*

EBV-specific T-cells were present in 1 of 6 patients preceding EBV-LPD. All patients were treated with a combination of rituximab and interruption of immunosuppression and 3 patients received donor lymphocytes as well. Seven out of 9 patients responded to therapy and EBV-load gradually decreased to become undetectable after a median of 17 days (range 5 – 59 days). All 7 responding patients had detectable EBV-specific $CD8^+$ T-cells post EBV-LPD, which recovery could already be detected within 4 weeks from treatment in 5 out of 7 patients. Two patients rapidly died due to progressive disease at respectively 10 and 41 days from EBV-LPD diagnosis. One of these 2 patients was investigated 5 days post EBV-LPD and had no detectable EBV-specific $CD8^+$ T-cells at that time.

4. Discussion

In the present study we evaluated the recovery of EBV-specific $CD8^+$ T-cells directed against lytic or latent antigens in recipients of T-cell depleted allogeneic hematopoietic stem cell grafts. We were particularly interested in the relation between the EBV-specific immune response, the incidence of EBV reactivation, and the development of EBV-LPD. It is shown that most EBV reactivations occur during the time lag before recovery of EBV-specific $CD8^+$ T-cells to normal levels. Furthermore, we also show a lack of EBV-specific $CD8^+$ T-cells in the majority of patients developing high-level reactivation and EBV-LPD. The absence of EBV-specific $CD8^+$ T-cells in patients with high-level EBV reactivation was significantly associated with EBV-LPD, indicating that the recovery of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation protects against uncontrolled reactivation and the development of EBV-LPD. Thus, the absence of EBV-specific $CD8^+$ T-cells strongly improved the positive predictive value of a viral load > 1,000 geq/ml. The earlier defined positive predictive value of a viral load > 1,000 geq/ml increased from 39% to 100% in patients without detectable EBV-specific $CD8^+$ T-cells. [11] Conversely, the positive predictive value was reduced to 25% in patients, who effectively recovered EBV-specific $CD8^+$ T-cells up to a level of at least 0.5 T-cells/µl. These data compare well to those of Smets et al, who monitored EBV-specific T-cells in recipients of liver allografts using interferon-γ ELISPOT stimulating peripheral blood lymphocytes with autologous EBV-derived lymphoblastoid cell lines. [32] Similarly, the combination of an elevated EBV viral load and the absence of EBV-specific $CD8^+$ T-cells resulted in a positive predictive value of 100% for the development of EBV-LPD in their patients. [32]

We did not evaluate the functional characteristics of the tetramer binding T-cells *in vitro*, nor did we evaluate the recovery of EBV-specific $CD4^+$ T-cells. However, the strong association between the level of EBV-specific $CD8^+$ T-cells and the observed protection against uncontrolled reactivation suggests that the EBV-specific T-cells detected are fully functional *in vivo* in the majority of our patients. Comparable results were obtained in

recipients of an allogeneic hematopoietic stem cell transplantation monitored for the recovery of CMV-specific $CD8^+$ T-cells by use of tetramers. [27] The mere presence of CMV-specific T-cells already proved to be strongly associated with in-vivo function, i.e. protection against CMV-disease. In contrast, van Baarle *et al.* evaluated numbers and function of EBV-specific $CD8^+$ T-cells in AIDS-related non-Hodgkin's lymphoma patients and showed that EBV-specific $CD8^+$ T-cells were not physically lost but rather lost their functionality. [28] This loss of function correlated with lower $CD4^+$ T-cell numbers and increasing viral load. Such a discrepancy between presence and function could possibly explain the high incidence of viral reactivation in recipients of unmanipulated allogeneic stem cell grafts, which patients were shown to experience as much reactivation as recipients of T-cell depleted grafts. [11] Despite higher peripheral T-cell numbers, recipients of unmanipulated stem cell grafts may experience viral reactivation during the time period in which they are treated with cyclosporin, which drug effectively inhibits the function of $CD4^+$ T-cells and thereby abrogates the indispensable help to $CD8^+$ T-cells. [33]

The EBV-specific $CD8^+$ T-cell response recovered within 3 – 6 months after allogeneic hematopoietic stem cell transplantation. Early recovery was already noted at 1 month after allogeneic hematopoietic stem cell transplantation in some of the patients. The rapid recovery of the EBV-specific T-cells corresponds well to the pattern of viral reactivation as monitored by our plasma real-time PCR. The median time to first reactivation was 2 months and early reactivation was already noted in the first month after allogeneic hematopoietic stem cell transplantation in 11 patients. These findings correspond well with those of Marshall et al, who described rapid repopulation of $CD8^+$ T-cells for latent and lytic antigens after allogeneic hematopoietic stem cell transplantation. [29] Early recovery of T-cells may be explained by oropharyngeal EBV-excretion, which has been demonstrated to occur already during the first month after allogeneic hematopoietic stem cell transplantation, thereby providing an antigenic stimulus immediately following transplantation. [34,35] From a pathophysiological point of view, early oropharyngeal EBV-excretion may represent a critical phase in which B-cells of the donor graft may become infected and transformed. [36] The T-cell response directed to lytic antigens may play a critical role in that early phase by limiting oropharyngeal lytic infection and thus subsequent B-cell infection.

Recovery of EBV-specific $CD8^+$ T-cells was delayed in patients, who had been treated with ATG. As shown in Figure 2, a delayed recovery lasting > 3 months was apparent for RAK-specific $CD8^+$ T-cells. A similar delay was observed for the other EBV-epitope-specific T-cells as well. ATG has been recognized as a particularly strong risk factor for the development of EBV-LPD. Several studies have reported increased hazard ratio's ranging from 5 to >10. [7,8,37] We have recently reported that ATG was associated with early reactivation of EBV and a higher probability of progression to EBV-LPD. [11] Based on the results of the present study, we postulate the probable elimination of recipient and graft derived EBV-specific T-cells by ATG which may lead to reduced levels of EBV-specific

$CD8^+$ T-cells in the early time-period post transplant. It further suggests that an early recovery of EBV-specific T-cell immunity is critical for prevention of EBV-LPD.
In conclusion, monitoring the recovery of EBV-specific $CD8^+$ T-cells by use of tetramers was shown to significantly enhance the predictive value of an increased viral load after allogeneic hematopoietic stem cell transplantation. Thereby, a more accurate identification of patients at high risk for EBV-LPD has become possible by combining the 2 assays. It may enable us to further narrow pre-emptive treatment and avoid over-treatment of recipients, who are able to mount an immune response that controls the proliferation of EBV-infected B-cells.

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7. General discussion

General discussion

The lack of early and accurate markers of EBV reactivation and disease has long hampered a timely diagnosis of post-transplant EBV lymphoproliferative disease. The introduction of polymerase chain reaction (PCR)-based assays, however, has allowed for sensitive and quantitative monitoring of viral DNA in peripheral blood samples. This thesis has addressed the question whether molecular monitoring of EBV-DNA would accurately predict for EBV-LPD and whether preventive and therapeutic strategies could be developed based on viral load monitoring. High positive and negative predictive values of viral load were retrospectively established in 152 recipients of an allogeneic hematopoietic stem cell transplant. Subsequently a preventive strategy using pre-emptive anti-CD20 monoclonal antibody therapy was developed, which strategy resulted in a reduction of mortality due to EBV-LPD in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplant. Hence, the molecular monitoring of EBV load has great clinical relevance as it offers a convenient predictive assay of EBV reactivation and EBV-LPD in our group of patients. Such monitoring now seems indispensable for prevention of mortality due to EBV-LPD. However, several new questions as regards molecular monitoring emerge which will be discussed in this final chapter.

1. Diagnosis of EBV-LPD

Histology of a pathological lymph node is still considered the gold standard for a diagnosis of EBV-LPD. However, should this standard change with the introduction of PCR-based assays? Are such assays sufficient, necessary or only additive for diagnosing EBV-LPD? In our retrospective study (chapter 3) the positive predictive value reached 100% at a level of 500,000 genome equivalents per ml plasma (geq/ml) in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplant. However, such high viral plasma levels were also observed in recipients of a T-cell replete hematopoietic stem cell transplantation without EBV-LPD, indicating that EBV reactivation as such may be associated with a high viral load without EBV-LPD. Moreover the highest viral load (3×10^6 geq/ml) was observed in a recipient of a T-cell replete allogeneic hematopoietic stem cell transplantation without EBV-LPD. These results compare well to earlier findings by Lucas et al., who measured highly elevated EBV-DNA levels following unmanipulated allogeneic hematopoietic stem cell transplantation in patients not developing EBV-LPD. [1] Therefore, the quantitative result of a PCR test is not sufficient for diagnosing EBV-LPD. Molecular monitoring of viral load, however, does seem necessary, as lymph node histology is not always possible. EBV-LPD following allogeneic hematopoietic stem cell transplantation may present as disseminated disease without overt lymphadenopathy. But lymphoproliferation may already be present as evidenced by the detection of monoclonal B-cells in the peripheral blood or bone marrow. Such patients, who present with aspecific symptoms of malaise and fever without lymphadenopathy, but with high viral load and monoclonal B-cells may be diagnosed as EBV-LPD. Preferably, the detection of EBV within the monoclonal B-cells, for example by anti-LMP antibodies, should then definitely prove a diagnosis of EBV-LPD.

2. Molecular monitoring of EBV-DNA

We defined EBV reactivation as the presence of at least 50 genome equivalents of EBV-BNRF1-DNA per ml plasma. The mere detection of that part of the viral genome does not indicate its origin. It may originate from fully assembled viral particles produced by lytic infection, but it may also come from B-cells latently infected by EBV, but transformed to autonomously proliferating and dying lymphocytes. Earlier studies have suggested that active lytic infection does participate in the development of EBV-LPD. [2-7] Experimentally, Rowe et al. showed that the development of human EBV-LPD lesions in severe combined immune deficiency mice was accompanied by the expression of lytic antigens in all tumors evaluated. [7] Furthermore, expression of lytic genes has also been shown in B-cells of a considerable proportion of patients with established EBV-LPD. [5,8] Expression of lytic genes may be followed by the induction of a specific cellular immune response. [9,10] As described in chapter 6, we observed a strong cytotoxic $CD8^+$ T-cell response to several epitopes from both lytic and latent proteins in patients with EBV-LPD and in patients with

EBV reactivation. These findings suggest that the theoretical sharp distinction between lytic infection and latently infected, autonomously proliferating, B-cells may not apply to the development of EBV-LPD in patients after allogeneic hematopoietic stem cell transplantation. Future studies should address the question, which genes are involved in EBV reactivation and the progression towards EBV-LPD, and to what extent lytic infection may drive the development of lymphoproliferative disease.

Several PCR based techniques have been used to determine viral load, including semiquantitative PCR, quantitative competitive PCR and quantitative real-time PCR (reviewed by Stevens et al. [11]). A disadvantage of semiquantitative PCR assays is the inability for adequate standardisation. [12] These problems were circumvented with the introduction of quantitative competitive PCR assays, which are based on competitive co-amplification of EBV-DNA with a fixed amount of an internal calibration standard added to the reaction. [13] Competitive PCR proved to be reproducible and accurate. But competitive PCR also proved very time-consuming and it requires intensive sample handling and calculation. Recently, real-time PCR has been introduced, based on direct detection of fluorescent PCR products in a closed-tube system. It is associated with a low risk of contamination due to few handling procedures, thereby allowing high-throughput screening. [14] Real-time PCR also appeared reproducible, sensitive, and standardisation among different laboratories can effectively be accomplished (chapter 2).

Several specimens have been used to determine viral load, including peripheral blood mononuclear cells (MNC), serum, plasma, and whole blood. So far, all studies have shown that an elevated EBV load, irrespective of the source of the specimen, after allogeneic hematopoietic stem cell transplantation and solid organ transplantation increases the probability of developing EBV-LPD. [11] Although several studies have shown a correlation between EBV-DNA assessed in MNCs and plasma or serum, differences in sensitivity and specificity have been reported. [15-18] To date, only few comparative studies have addressed this issue in detail. Two studies compared plasma and MNC as the source of EBV-DNA assessed by real-time PCR in recipients of solid organ transplantation. [15,17] Both studies revealed a higher sensitivity of real-time PCR for EBV-DNA in MNC as compared to plasma, but they also showed a higher specificity if plasma was used as the source of EBV-DNA. As a result, the positive predictive value was greater using plasma samples.

In contrast, Stevens et al. compared whole blood samples versus plasma or serum samples in 4 patients with EBV-LPD following solid organ transplantation using a quantitative competitive PCR. [19] They found no correlation between viral load measured in whole blood as compared to plasma or serum. Furthermore, the EBV burden seemed restricted to the cellular blood compartment in most patients as several serum or plasma samples yielded negative results, despite a high viral load in corresponding whole blood samples. The authors concluded that whole blood samples are to be preferred as they may better reflect the total virus load by combining different blood compartments. However, as long

as the latter findings have not been validated in a larger longitudinal study in both patients with definite EBV-LPD and patients at risk for EBV-LPD, it remains uncertain whether whole blood is to be preferred.

We have longitudinally assessed positive and negative predictive values in a group of 152 recipients of an allogeneic hematopoietic stem cell transplant (chapter 3) using a quantitative real-time PCR. Viral reactivation as defined by $\geq$ 50 geq/ml preceded the development of EBV-LPD in all patients by a median number of 22 days (range, 13-120 days). The positive predictive value of a viral load $\geq$ 1,000 geq/ml was 39% at 2 months and 50% at 4 months, while the corresponding negative predictive values were 100%. These results indicate that the plasma viral load in recipients of an allogeneic hematopoietic stem cell transplant timely and reliably predicts for EBV-LPD (chapter 3). The specificity of the assay was further demonstrated in patients with established EBV-LPD who did or did not respond to therapy (chapter 4). While all responding patients showed rapid clearance of plasma viral load, all non-responders showed a progressive increase of EBV-DNA. These results are in contrast with several studies evaluating cellular viral load during and after therapy for EBV-LPD. High copy numbers were found to persist in a substantial number of responding patients, which did not differ from those in non-responding patients. [20]

Thus, while the cellular viral load may more sensitively reflect an early increase of EBV-DNA, the plasma viral load may be associated with a higher specificity and a higher positive predictive value. From a clinical point of view, decisions as regards pre-emptive treatment or adaptation of therapeutic regimens for established EBV-LPD will especially need to rely on assays with a high specificity and high positive predictive value. Quantifying the viral load in plasma by real-time PCR currently seems to meet these requirements best, in recipients of a solid organ or allogeneic hematopoietic stem cell transplantation. Further improvement of the positive predictive value can be achieved by combining real-time PCR with techniques to assess the EBV-specific cellular immune response. As shown in chapter 6, absence of EBV-specific $CD8^+$ T-cells, as quantified by the tetramer technique in patients with a high viral load ($\geq$ 1,000 geq/ml), was strongly associated with progression to EBV-LPD. Thus, the combination of these assays may permit a further improvement in accurately identifying patients at high risk for EBV-LPD.

3. Prevention of EBV-LPD

Outcome of clinically established EBV-LPD is still not optimal, although new promising treatment modalities have been introduced, such as monoclonal anti B-cell antibody therapy (rituximab) and adoptive T-cell immunotherapy. [21-24] Therefore, preventive strategies are to be preferred. Prevention may be applied as prophylaxis in patients at risk before the onset of EBV reactivation or, prevention may be performed by pre-emptive treatment in patients with established reactivation at high risk of progressing to EBV-LPD.

The latter approach critically depends on a timely and accurate identification of such patients. As described in this thesis, abrogation of EBV-LPD-mortality can effectively be achieved by molecular monitoring and pre-emptive rituximab in accurately identified high-risk patients. However, patients at risk may also be identified, albeit less accurately, by pre-transplant risk factors, such as the application of T-cell depletion, the use of anti-thymocyte-globulin, and alternative donor stem cell transplantation (reviewed in chapter 1, table 3). Instead of monitoring viral load in these patients, an alternative approach would be the administration of prophylaxis in all patients in order to prevent reactivation and the progression towards EBV-LPD. Such prophylaxis would result in significant over-treatment, because EBV-LPD is still a rare complication of allogeneic hematopoietic stem cell transplantation. On the other hand prophylaxis would be attractive if an effective agent is available with few side effects and at low cost. Are such modalities available? To date, prophylaxis with antiviral drugs such as aciclovir and ganciclovir have not been shown to prevent EBV reactivation and EBV-LPD. [25-27] In our retrospective study (chapter 3), 75 out of 152 patients were treated prophylactically with aciclovir, but the incidence of EBV reactivation did not differ between patients with or without prophylaxis.

Another way of prophylaxis is the depletion of B-cells from the donor stem cell graft, which has already been shown a highly effective approach. [28,29] Several groups using the monoclonal antibody alemtuzumab for both T-cell and B-cell depletion have reported a low incidence of EBV-LPD. [28,29] More recently, Liu et al. reported favourable but preliminary results of the in-vivo application of rituximab for B-cell depletion shortly after stem cell transplantation. [30] Although B-cell depletion of the donor graft (either performed in-vivo or in-vitro) may be very effective, the approach may be associated with a delayed immune recovery in general and a delay in EBV-specific T-cell immunity. [30] Furthermore, the approach would imply significant over-treatment. Therefore, weighing the pros and cons of prophylaxis versus pre-emptive treatment, the balance may turn in favour of pre-emptive treatment if one prefers to avoid unnecessary (expensive) treatment of patients with a low probability. Lastly, 67 recipients of unmanipulated hematopoietic stem cell grafts described in chapter 3 did not develop EBV-LPD, although they experienced no less frequent reactivations than did recipients of T-cell depleted grafts. Clearly, the T-cells infused with the donor graft were able to mount an immune response to prevent recurrent reactivation and the progression to EBV-LPD. Therefore, these patients do not require prophylactic treatment or intensive molecular monitoring and pre-emptive treatment for prevention of EBV-LPD. The question then arises whether the benefits of T-cell depletion still outweigh the disadvantages such as the risk of EBV-LPD.

Retrospectively, treatment related mortality did not differ between recipients of T-cell depleted stem cell grafts versus recipients of unmanipulated grafts (chapter 3). Furthermore, the incidence of acute graft-versus-host disease did not differ either. However, the probability of developing chronic limited and extensive graft-versus-host disease was significantly less following T-cell depletion (38 % ± 6% versus 83% ± 5%). These results compare well to a number of earlier studies evaluating the incidence of acute

and chronic graft-versus-host disease following partial T-cell depletion. [31,32] An even better prevention of acute and chronic graft-versus-host disease may be achieved by a more stringent, near complete depletion of T-cells. However, such reduction is achieved at the expense of an even slower immune recovery and a higher relapse rate of the primary malignancy (reviewed by Ho,[33]).
Therefore, partial T-cell depletion was developed in order to prevent acute and chronic graft-versus-host disease, while retaining some graft-versus-leukemia activity. [34] Although survival differences have not been demonstrated between recipients of T-cell depleted versus unmanipulated stem cell grafts, the reduction of acute and especially chronic graft-versus-host disease may be of significant benefit in terms of prevention of long lasting treatment related morbidity, necessitating prolonged use of immunosuppressive drugs. Disadvantages of T-cell depleted allogeneic hematopoietic stem cell transplantation such as viral reactivation and the need for cautious monitoring should therefore be weighed against the treatment related morbidity associated with a higher incidence of chronic graft-versus-host disease following unmanipulated allogeneic hematopoietic stem cell transplantation. So far, the approach pursued by hematopoietic stem cell transplant centers in the Netherlands has focussed on the prevention of acute and chronic graft-versus-host disease and the concurrent prevention of opportunistic infections and secondary malignancies such as EBV-LPD.

4. Treatment of EBV-LPD

Before the introduction of molecular monitoring of viral load, a diagnosis of EBV-LPD was often made relatively late following the onset of LPD. Patients usually presented with a critical illness and outcome was very poor despite the application of multiple treatment modalities. Surrogate markers for response were lacking, precluding a careful evaluation of different treatment modalities. The picture has significantly changed during the last 5 years following the introduction of molecular monitoring. The merits of molecular monitoring are several fold. First, EBV-LPD presents itself at diagnosis no longer as a medical emergency. Impending EBV-LPD alerts the clinician to institute pre-emptive treatment or to begin therapy at a relatively early time-point in the course of EBV-LPD. Secondly, different treatment modalities can now be evaluated by a highly specific surrogate marker of response. As described in chapter 4, the molecular quantification of viral load in patients with established EBV-LPD allows for a very early (< 72 hours) and accurate prediction of response, which now enables us to carefully select and adjust successive treatment modalities.

Which therapeutic approach should be pursued in patients with established EBV-LPD following allogeneic hematopoietic stem cell transplantation? Improving host defence and eliminating EBV-infected autonomously proliferating B-cells remain the current cornerstones of therapeutic management of EBV-LPD. But molecular monitoring now allows for a stepwise approach. Malignant B-cells can effectively be eliminated by

rituximab infusion guided by viral load. High response rates were observed in recipients of stem cell grafts and in recipients of solid organ grafts following multiple infusions of rituximab (reviewed in chapter 1). While complete peripheral blood B-cell depletion may already be achieved by a single infusion of rituximab, some patients may require multiple infusions. We observed incomplete peripheral blood B-cell depletion in 5 out of 17 patients (chapter 5) after a single rituximab infusion. Molecular monitoring in 2 out of these 5 patients revealed a progressive increase of viral load following the first infusion concurrent with the development of overt EBV-LPD. Subsequently, a decline of EBV-DNA accompanied with complete peripheral blood B-cell depletion was observed in both patients following a second infusion of rituximab. Future studies should address the question whether and how the scheme of rituximab can be optimized.

Host defence may be improved by interruption of immune suppressive drugs and/or the adoptive transfer of donor T-cells. As described in chapter 6, patients with established EBV-LPD may rapidly recover EBV-specific cytotoxic T-cells up to a protective level already within the first weeks following interruption of immunosuppressive agents. In order to allow for sufficient endogenous T-cell recovery, the adoptive transfer of donor T-cells may be postponed in patients with EBV-LPD for at least 1-2 weeks. T-cell immunotherapy may then very selectively be applied in patients, who do not recover EBV-specific immunity and who show a progressive increase of viral load despite cessation of immunosupression and rituximab infusion. Adoptive immunotherapy can be performed with unselected donor leucocytes or with donor-derived EBV-specific cytotoxic T-cell lines as developed and pioneered by Rooney and Heslop. [23,24] While unselected donor T-cell infusion may be complicated by graft-versus-host disease, they do provide immunity not only towards EBV but also to a number of other potential lethal opportunistic infections, including CMV, adenovirus, Aspergillus, etc. As recently reported by Einsele et al., patients lacking CMV-specific immunity may be treated with CMV-specific cytotoxic T-cells, but the lack of immunity towards other pathogens may still be associated with the development of lethal infections. [35] In addition, although effective, the laborious technical procedures needed to prepare cytotoxic T-cells may preclude their application on a wider scale.

Is there still a role for chemotherapy? Elimination of malignant B-cells may effectively be performed by rituximab, but relapse of EBV-LPD has been reported in 20-30% of patients treated for EBV-LPD following solid organ grafting (chapter 1). Failure of treatment may be due to development of resistance, viral immune evasion, rapidly progressive disease, and loss of CD-20 antigen expression. [22,36-38] Earlier studies evaluating response following chemotherapy showed high response rates in recipients of solid organ grafts with acceptable toxicity, if intensified dosages were avoided. [39-42] In contrast, the side effects of chemotherapy applied for EBV-LPD in recipients of allogeneic hematopoietic stem cell grafts appeared excessive, which may be explained by cumulative toxicity added to the preceding high dose chemo-radiotherapy. Therefore, cytotoxic chemotherapy may selectively be applied in the treatment of EBV-LPD following solid organ transplantation.

Preferably, a combination of rituximab and chemotherapy, such as has been studied by Coiffer et al in patients with Non-Hodgkin's lymphoma [43], would need to be studied in recipients of solid organ grafts to address the question whether the response rate can be improved and relapse can be prevented by combining rituximab with chemotherapy.

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8. Summary / samenvatting

English Summary

Epstein-Barr virus is a γ-herpesvirus that infects more than 90% of the world population. Following primary infection in the oropharynx, EBV remains latently present in B-cells. Latent EBV infection is normally controlled by a cell-mediated immune response. EBV-infected B-cells may evolve to EBV-lymphoproliferative disease (LPD) in severely immunocompromised patients, due to inhibition of immunological control of latently infected B-cells. Recipients of allogeneic hematopoietic stem cell grafts are at risk for EBV-LPD during the time-period of insufficient T-cell recovery after transplantation. EBV-LPD is a rare but serious complication following allogeneic hematopoietic stem cell transplantation and is associated with considerable mortality. The lack of early and accurate markers of EBV reactivation has long hampered a timely diagnosis of EBV-LPD. The introduction of polymerase chain reaction (PCR)-based assays has allowed for sensitive and quantitative monitoring of EBV-DNA in peripheral blood samples. This thesis has addressed the question whether molecular monitoring of EBV-DNA would predict for EBV-LPD and whether preventive and therapeutic strategies could be developed based on viral load monitoring.

Chapter 2 describes the development of a real-time quantitative assay for detection of EBV-DNA in plasma. EBV-DNA encoding for the non-glycosylated membrane protein BNRF1-p143 was used as the target gene in this assay. EBV-DNA was assessed in plasma samples from healthy EBV-seropositive individuals, patients with infectious mononucleosis, asymptomatic immunosuppressed solid organ transplant recipients, and patients with a histologically confirmed diagnosis of EBV-LPD. EBV-DNA could not be detected in plasma of healthy EBV-seropositive individuals whereas it could be detected in plasma of 19 % (21/109) of solid organ transplant recipients, in 73 % (16/22) of patients with infectious mononucleosis, and in 100% (10/10) of patients with EBV-LPD. Quantitative values of patients with infectious mononucleosis and patients with EBV-LPD differed significantly from solid organ transplant recipients. Furthermore, the assay appeared to be rapid, sensitive, specific and highly reproducible over a range between 100 and 10^7 genome equivalents/ml (geq/ml).

We then wished to examine the clinical utility of the EBV-DNA test as an assay for diagnosing EBV reactivation and EBV-LPD following allogeneic hematopoietic stem cell transplantation. For this purpose, in chapter 3 we retrospectively evaluated 152 recipients of an allogeneic hematopoietic stem cell transplantation at weekly intervals for the presence of EBV-DNA in plasma during the first 180 days following stem cell infusion. Endpoints of this longitudinal study were: incidence of EBV reactivation, EBV-LPD, graft-versus-host disease and treatment-related mortality. The incidence of EBV reactivation did not differ between recipients of unmanipulated versus T-cell depleted grafts and measured approximately 50 % for the whole group. In multivariate analysis

patients pretreated with anti-thymocyte globulin in the conditioning regimen and patients receiving higher $CD34^+$ cell numbers in the graft were found to be at greater risk for EBV reactivation. EBV-LPD was not observed after unmanipulated stem cell transplantation but occurred only in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplantation. Plasma EBV-DNA quantitatively predicted for EBV-LPD. The positive and negative predictive values of a viral load of 1,000 geq/ml were, respectively, 39% and 100% after T-cell depleted allogeneic hematopoietic stem cell transplantation. Thus, EBV reactivation appeared a frequent event after allogeneic hematopoietic stem cell transplantation and plasma viral load quantitatively predicted for EBV-LPD in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplantation.

In chapter 4 we set out to assess the value of serial monitoring of EBV-DNA plasma levels for predicting response to treatment and subsequent survival in recipients of a T-cell depleted hematopoietic stem cell with established EBV-LPD. Fourteen patients were monitored frequently from the time of EBV-LPD diagnosis until clinical response or death. Seven patients obtained a complete response and 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 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 strongly predicted for clinical response. In addition, lymphopenia at the time of EBV-LPD diagnosis was associated with non-responsiveness and poor outcome. It was concluded that the quantitative monitoring of viral load accurately predicts for response to therapy in patients with established EBV-LPD.

In chapter 5 we studied whether pre-emptive therapy with anti-CD20 B-cell antibody (rituximab) would prevent EBV-LPD, and EBV-LPD mortality. We monitored 49 recipients of a T-cell depleted allogeneic hematopoietic stem cell transplantation weekly for EBV reactivation by quantitative real-time PCR. Pre-emptive therapy by a single infusion of rituximab was given to patients as soon as EBV-DNA value exceeded 1,000 geq/ml as these values identify patients at high risk for EBV-LPD (chapter 3). Results were compared with a control group of patients retrospectively monitored for EBV reactivation at similar intervals. Seventeen prospectively monitored patients showed EBV reactivation $\geq$ 1,000 geq/ml and 15 of them received pre-emptive therapy. Fourteen patients had complete responses to therapy. One patient progressed to EBV-LPD despite pre-emptive therapy, but obtained complete remission after 2 infusions of rituximab and donor lymphocyte infusion. Two patients had already developed EBV-LPD prior to pre-emptive rituximab, but obtained complete remission following 2 rituximab infusions. Comparison of this prospective cohort of patients followed series to a historical cohort with a similar high-risk profile showed a reduction of EBV-LPD incidence and a complete abrogation of EBV-LPD mortality. These results indicated that frequent monitoring of EBV reactivation

and pre-emptive therapy by rituximab improves outcome in patients at high-risk for EBV-LPD.

It is assumed that cytotoxic T-cells play a critical role in the control of evolving EBV reactivation and the prevention of progression towards EBV-LPD. The aim of the study described in chapter 6 was to evaluate the recovery of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation and to study the relation between EBV-specific $CD8^+$ T-cells, EBV reactivation and EBV-LPD. EBV-specific immunity was studied using a panel of 11 HLA class I tetramers presenting peptides derived from 7 EBV proteins. Forty-five patients showed EBV reactivation, including 25 with high-level reactivation (≥ 1,000 geq/ml). Nine of these patients progressed to EBV-LPD. Repopulation of $CD8^+$ T-cells specific for latent and lytic EBV epitopes in the peripheral blood was similar. Absolute numbers of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation recovered to normal levels within 6 months in the majority of patients. Concurrently, the incidence of EBV reactivation strongly decreased. Patients with insufficient recovery were at higher risk for EBV reactivation in the first 6 months after stem cell transplantation. A profile of absent EBV-specific $CD8^+$ T-cells coupled to high-level EBV-DNA plasma levels was significantly associated with the subsequent development of EBV-LPD (P=0.048). Thus, the earlier defined positive predictive value of approximately 40%, based on high-level viral reactivation only, increased to 100% in patients without EBV-specific T-cells. These results show that the absence of EBV-specific $CD8^+$ T-cells in patients with high-level viral reactivation may identify a subgroup of patients at extremely high risk for EBV-LPD. This observation is consistent with the idea that EBV-specific $CD8^+$ T-cells protect recipients of an allogeneic hematopoietic stem cell graft against progressive EBV reactivation and EBV-LPD.

Finally, the results of the studies presented in chapters 2-6 are discussed in chapter 7 (General Discussion). Emerging questions with respect to diagnosis, prevention, and treatment of EBV-LPD based on molecular monitoring are addressed.

Nederlandse Samenvatting

Inleiding

Het Epstein-Barr virus (EBV) behoort tot de groep van herpesvirussen waartoe o.a. ook het waterpokken-virus en het koortslip-virus behoort. Ongeveer 90% van de wereldbevolking wordt geïnfecteerd met EBV. Infectie komt tot stand door speekseloverdracht (vandaar de naam "kissing disease") en verloopt in het merendeel van de gevallen symptoomloos. Soms treden er symptomen op zoals, koorts, keelpijn, gezwollen keelamandelen, en een opgezette lever of milt. Dit beeld staat bekend als de ziekte van Pfeiffer. Na de primaire infectie in de mond-keel holte nestelt het Epstein-Barr virus zich levenslang in lymfocyten en wel de zogenaamde B-lymfocyten. Nadat het virus door middel van een specifieke receptor de B-cel is binnengedrongen, verhuist DNA van het virus naar de celkern en ontregelt het groeiprogramma van deze cel, waardoor deze in een potentiële kankercel verandert. Echter, uitgroei van dergelijke B-cellen tot een kwaadaardige woekering wordt voorkomen door afweercellen (T cellen), die zich specifiek richten tegen het EBV. Hiermee ontstaat een balans tussen EBV geïnfecteerde B-cellen en de EBV specifieke T-cellen resulterend in een levenslange vorm van samenleven tussen gastheer en virus. Wel kan er periodieke reactivatie en uitscheiding van virus in het speeksel optreden waardoor het afweersysteem geprikkeld wordt en andere personen door speekseloverdracht kunnen worden geïnfecteerd. In perioden van (sterk) verminderde T-cel afweer kan de balans tussen T en B cellen worden verstoord. Een voorbeeld hiervan is de situatie die ontstaat na een orgaan- (b.v. hart of nier) of beenmerg transplantatie.

Patiënten met bepaalde bloedziekten kunnen in bepaalde omstandigheden in aanmerking komen voor een transplantatie van stamcellen van een gezonde donor. Na intensieve chemotherapie en radiotherapie, waarbij het eigen (zieke) beenmerg wordt opgeruimd, kunnen de donor stamcellen worden toegediend (allogene stamcel transplantatie) die vervolgens de beenmergfunctie overnemen. De bloedvormende stamcellen van het transplantaat vestigen zich in de beenmergholte en groeien na verloop van tijd uit tot rode bloedcellen (zuurstof transport), witte bloedcellen (afweer) en bloedplaatjes (stolling). Ook moeten er opnieuw T-cellen ontstaan, die EBV-geïnfecteerde B-cellen kunnen remmen. In de eerste maanden na een allogene stamcel transplantatie ontbreekt de immunologische afweer en schiet ook de EBV specifieke afweer tekort. Het is dan ook in deze periode dat EBV geïnfecteerde B-cellen kunnen uitgroeien tot een kwaadaardige ziekte. De autonoom groeiende B-cellen kunnen zich nestelen in bloed, beenmerg, en vooral lymfeklieren. Deze ziekte wordt post-transplantatie lymfoom genoemd (EBV-lymphoproliferative disease, EBV-LPD).

Doel van het onderzoek

Het doel van ons onderzoek was om te bestuderen of met behulp van een nieuwe gevoelige moleculaire techniek DNA van het EBV in het bloed gemeten zou kunnen worden en of de aanwezigheid van EBV-DNA het optreden van EBV-LPD zou kunnen voorspellen na donor stamcel transplantatie. Op basis van een betrouwbare voorspelling zou dan een preventieve therapeutische benadering ontwikkeld kunnen worden.

Het onderzoek

In hoofdstuk 2 wordt de bepaling onderzocht die gebruikt is om bovenstaande vraagstelling te beantwoorden. Het gaat hierbij om een polymerase kettingreactie ofwel PCR die kwantitatief de aanwezigheid van stukjes EBV-DNA kan meten in het plasma. Verschillende patiëntengroepen werden onderzocht. Het bleek dat er geen EBV-DNA aantoonbaar was bij gezonde personen die de ziekte van Pfeiffer in het verleden hadden doorgemaakt. Bij patiënten met een actieve ziekte van Pfeiffer werd in 73% van de gevallen EBV-DNA in plasma gevonden. Patiënten met een post-transplantatie lymfoom hadden in alle gevallen EBV-DNA in het plasma. Verder bleek dat de hoeveelheid EBV-DNA per milliliter plasma voor de patiënten met een post-transplantatie lymfoom vele malen hoger was dan bij de andere patiëntengroepen met aantoonbaar EBV-DNA. De bepaling leek derhalve bruikbaar om bij patiënten EBV gerelateerde ziektebeelden te vervolgen.

In hoofdstuk 3 hebben we bij een grote groep patiënten die een stamcel transplantatie ondergingen wekelijks de hoeveelheid EBV-DNA in het plasma gemeten. Het bleek dat EBV-DNA frequent kon worden aangetoond in het plasma van patiënten die een stamcel transplantatie hadden ondergaan en dat naarmate de hoeveelheid EBV-DNA in het plasma toenam, de kans op het ontstaan van een post-transplantatie lymfoom steeg. Post-transplantatie lymfomen werden alleen gezien na stamcel transplantaties waarbij een groot deel van de T-cellen uit het transplantaat was verwijderd en niet bij ontvangers van een volledig stamcel transplantaat, dat wil zeggen met het normale aantal T-cellen. Daarnaast was het mogelijk om op basis van de hoeveelheid EBV-DNA in plasma een inschatting te maken van de kans op een post-transplantatie lymfoom.

In hoofdstuk 4 worden de resultaten beschreven van een retrospectieve studie bij 14 transplantatie patiënten, die behandeld werden voor een post-transplantatie lymfoom. Bij de patiënten die goed op therapie reageerden halveerde de hoeveelheid EBV DNA in het plasma binnen 3 dagen na start van de therapie. De hoeveelheid DNA in plasma steeg daarentegen bij patiënten zonder reactie op therapie. Patiënten uit deze laatste groep overleden ten gevolge van de ziekte. Verder bleek dat van de patiënten die wel ziektevrij werden door de behandeling er uiteindelijk een groot aantal overleden aan de complicaties van de behandeling. De conclusie van dit hoofdstuk is dat het verloop van de hoeveelheid

EBV-DNA in plasma een nauwkeurige weerspiegeling geeft van de reactie van een post-transplantatie lymfoom op de ingestelde therapie.
Daar de prognose van patiënten met een post-transplantatie lymfoom in het algemeen slecht is, werd in hoofdstuk 5 onderzocht of het mogelijk was om het optreden van een post-transplantatie lymfoom te voorkomen in een goed gedefinieerde risicogroep door middel van vroegtijdige herkenning en ingrijpen. Uit het onderzoek beschreven in hoofdstuk 3 was duidelijk geworden dat patiënten met een EBV-DNA $\geq$ 1000 geq/ml in het plasma een grote kans hebben om een post-transplantatie lymfoom te krijgen. Patiënten met een EBV-DNA boven deze waarde kregen in dit onderzoek een éénmalige toediening van een medicament, dat B-cellen effectief vernietigt (anti B-cel therapie). Bij de patiënten die op deze wijze werden behandeld werd geen sterfte ten gevolge van het post-transplantatie lymfoom waargenomen en bovendien bleek het aantal patiënten dat uiteindelijk een post-transplantatie lymfoom ontwikkelde eveneens beduidend lager dan in het verleden het geval was. Anti B-cel therapie toegepast in een vroege fase van EBV reactivatie na allogene stamcel transplantatie verbetert de overleving van patiënten met een grote kans op een post-transplantatie lymfoom.

In hoofdstuk 6 wordt beschreven hoe de T-cel afweer tegen het EBV zich ontwikkelt na een stamcel transplantatie. Hierbij werd gebruik gemaakt van een nieuwe techniek (tetrameren test), die het mogelijk maakt om individuele EBV-specifieke T-cellen te detecteren, door deze T-cellen te laten reageren met stukjes EBV-eiwit, waaraan een merkstof gekoppeld is. In dit onderzoek hebben we bij een groot aantal stamcel transplantatie patiënten met behulp van deze techniek de EBV-specifieke afweer onderzocht. De resultaten hiervan werden gecorreleerd aan de metingen van EBV-DNA in plasma. Bij het merendeel van de patiënten ontwikkelt de EBV specifieke afweer zich geleidelijk binnen de eerste 6 maanden na stamcel transplantatie. Patiënten met onvoldoende EBV-specifieke T-cellen vertoonden frequenter een EBV reactivatie, waarbij ook meer EBV-DNA in het plasma gemeten kon worden. Patiënten gekenmerkt door de combinatie van een grote hoeveelheid EBV-DNA en afwezigheid van EBV-specifieke T-cellen bleken het hoogste risico te hebben op een post-transplantatie lymfoom. Het meten van de EBV-specifieke afweer levert een duidelijke bijdrage aan de identificate van hoogrisico patiënten.

Tenslotte hebben wij in hoofdstuk 7 de resultaten van de verschillende studies bediscussieerd en ons daarbij vooral gericht op diagnosestelling, voorkomen en behandeling van het post-transplantatie lymfoom in de huidige tijd, waarin we met moleculaire technieken gevoelig en vroeg reactivatie van het Epstein-Barr virus kunnen aantonen. Al met al kan geconcludeerd worden dat de gebruikte polymerase kettingreactie techniek een belangrijke bijdrage heeft opgeleverd aan de identificatie van patiënten met een hoog risico op een post-transplantatie lymfoom en dat meting van EBV-DNA een leidraad kan vormen voor toe te passen therapieën bij patiënten met een post-transplantatie lymfoom.

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Curriculum vitae

De auteur van dit proefschrift werd op 24 augustus 1964 in Roermond geboren. In 1983 behaalde hij het VWO-diploma aan het Bisschoppelijk College Schöndeln te Roermond. In hetzelfde jaar werd gestart met de studie geneeskunde aan de Rijksuniversiteit Limburg, te Maastricht, alwaar in 1989 het artsexamen werd behaald. Van oktober 1989 tot en met januari 1991 vervulde hij zijn militaire dienstplicht bij de Koninklijke Marine ondermeer als arts bij de Centrale Ziekenboeg, te Den Helder (hoofd: Kapitein Luitenant-ter-zee arts H.J. Hofkamp) en aan boord van de Hr. Ms. Witte de With en Hr. Ms. Alkmaar. Hierna trad hij in februari 1991 in dienst bij de maatschap interne geneeskunde van het Maasland ziekenhuis te Sittard, alwaar hij in april 1991 met de opleiding interne geneeskunde begon (opleider: Dr. Th.W.M. van de Wiel en Dr. A.M.J. Moers). Vanaf april 1993 tot en met december 1996 werd de opleiding voortgezet in het Academisch Ziekenhuis Maastricht (opleider: Prof. dr. J.A. Flendrig †, Prof. dr. A.C. Nieuwenhuijzen Kruseman, Prof. dr. H.F.P. Hillen). Hierna kwam hij in dienst bij de afdeling hematologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt (hoofd: Prof. dr. B. Löwenberg) alwaar in april 1997 zijn registratie als internist plaatsvond gevolgd door zijn registratie als internist-hematoloog in september 1997. Vanaf januari 1998 tot en met december 2001 was hij als internist-hematoloog werkzaam op de afdeling hematologie van de Dr. Daniël den Hoed Kliniek (hoofd: Prof. dr. B. Löwenberg) gedurende welke periode ook dit proefschrift werd bewerkt. Sinds januari 2002 is hij bezig met het aandachtsgebied medische oncologie in voornoemde instelling (Opleider: Prof. dr. G. Stoter). Hij is gehuwd met Hanneke de Bruijn en zij hebben 2 zonen, Job en Dirk.