ORIGINAL ARTICLE

The Epstein–Barr virus DNA load in the peripheral blood of transplant recipients does not accurately reflect the burden of infected cells

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SUMMARY

Transplant recipients frequently exhibit an increased Epstein–Barr virus (EBV) load in the peripheral blood. Here, we quantitated the EBV-infected cells in the peripheral blood of these patients and defined the mode of viral infection, latent or lytic. These data indicated that there is no strong correlation between the number of infected cells and the EBV load (EBVL). This can be explained by a highly variable number of EBV copies per infected cell and by lytic replication in some cells. The plasma of these patients did not contain any free infectious viruses, but contained nevertheless EBV DNA, sometimes in large amounts, that probably originates from cell debris and contributed to the total EBVL. Some of the investigated samples carried a highly variable number of infected cells in active latency, characterized by an expression of the Epstein–Barr nuclear antigens (EBNA2) protein. However, a third of the samples expressed neither EBNA2 nor lytic proteins. Patients with an increased EBVL represent a heterogeneous group of patients whose infection cannot be characterized by this method alone. Precise characterization of the origin of an increased EBVL, in particular, in terms of the number of EBV-infected cells, requires additional investigations including the number of EBV-encoded small RNA-positive cells.

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Introduction

Patients with an immunodeficiency, and in particular transplant recipients, have a reduced ability to control infectious agents [1]. This applies to viruses that establish a lifelong chronic infection such as herpesviruses that infect a large proportion of the general population [2]. In the presence of a diminished functional T-cell response, a substantial proportion of individuals who were previously infected with the Epstein–Barr virus (EBV) cannot prevent infected B cells

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from resuming proliferation [3]. Transplant recipients, in particular children, who undergo primary infection while already under immunosuppressive treatment carry an even higher risk of EBV-associated diseases [4,5].

In vitro, the EBV infection leads to B-cell infection and proliferation within a few days [6]. This process can also lead to the development of a lymphoproliferation in immunosuppressed patients that can be rapidly lethal in the absence of an appropriate therapy [7]. EBV transforms B cells by expressing a restricted set of latent proteins that belong to the Epstein–Barr nuclear antigens (EBNA) or to the latent membrane protein families, together with the BHRF1 microRNAs [8,9].

The identification of post-transplant lymphoproliferative disorders (PTLD) at an early, subclinical, stage would allow an early or a pre-emptive treatment that is more likely to be efficient. This is the rationale behind the quantification of the EBV load (EBVL) in the peripheral blood that measures the concentration of EBV DNA in the peripheral blood or in plasma or serum by quantitative PCR (qPCR) [10]. However, regular monitoring of transplant recipients with this assay was shown to have a poor predictive value for the development of PTLD [11]. Nevertheless, this test has an excellent negative predictive value, close to 100%, showing that the large majority of patients with EBV-positive PTLD have an excess of EBV DNA in the peripheral blood [12]. Despite these limitations, quantitative monitoring of the peripheral blood EBVL is considered as the gold standard to identify stem cell recipients at risk of PTLD and remains the cornerstone of many clinical trials targeting PTLD in a larger population of transplant recipients [13,14]. There are several potential explanations for the poor positive predictive value of the EBVL. An increase in viral DNA in the peripheral blood can be generated by an increase in the number of EBV-driven proliferative cells, as seen in patients with PTLD [15,16]. Alternatively, viral DNA can also be generated when cells undergo lytic replication, a complex process that leads to virus progeny [17]. In this study, we collected a series of blood samples from kidney and bone marrow transplant recipients and precisely investigated the infected cells to delineate the mechanisms that led to the increase in viral DNA.

Material and methods

Ethics statement and study design

Blood samples were obtained from immunosuppressed patients who underwent renal or stem cell transplantation at the University of Heidelberg. The EBVL was quantified routinely following transplantation. Twenty-three patients with informed consent had an EBVL $\geq$ 1000 cop/ml and were enrolled in the study. This study was a prospective nonrandomized, nonblinded leave nonblinded observatory study. It lasted 18 months and was approved by the Ethics Committee of the University of Heidelberg (S-005/2014).

B cell, serum, and plasma preparation

Plasma samples were prepared from 18 ml of EDTA whole blood (WB) (S-Monovette^{R} EDTA, Sarstedt, Germany). We used coagulated WB to obtain serum samples (S-Monovette^{R} Z-Gel). The blood samples were centrifuged at 313 *g* and aliquots stored at −80 °C. Primary human B cells were isolated from 18 ml of EDTA-treated WB by positive selection with CD19 Dynabeads (Invitrogen Waltham, Massachusetts, USA) after density gradient centrifugation on a Ficoll cushion.

DNA extraction

DNA was extracted from WB samples (200 µl), purified B cells (1–3 × 10E5 cells), and from plasma or serum samples (200 µl) using a QIAmp DNA Blood Mini Kit (QIAGEN, Venlo, Niederlande). DNA was stored in TE buffer at −20 °C.

qPCR

DNA extracted from WB, purified B cells, and serum samples was used for qPCR analyses to determine the EBV viral load. We performed TaqMan qPCR analyses using primers and probes designed to amplify the EBV polymerase (BALF5, forward primer 5′-CTTTGGCGCG GATCCTC-3′, reverse primer 5′-AGTCCTTCTTGGCTA GTCTGTTGAC-3′, probe 5′-Fam-CATCAAGAAGCTGC TGGCGGCC-Tamra-3′) or the BGLF5 gene (forward primer 5′-CCTCTTTTCCAAGTCAGAATTGAC-3′, reverse primer 5′-TGACCTCTTGCATGGCCTCT-3′, probe 5′-Fam-CCATCTACCCATCCTACACTGCTTTACA-Tamra-3′). Amplification reactions were performed in a total volume of 25 µl, including 12.5 µl of TaqMan universal PCR master mix (Applied Biosystems, Waltham, Massachusetts, USA), 2.5 µl each of forward and reverse primers (2 µM), 1 µl of 5 µM FAM-labeled Pol-probe, 1.5 µl of water, and 5 µl of buffer containing the purified DNA samples. After initial activation of the DNA polymerase for 10 min at 95 °C, samples were amplified for 40 cycles (15 s at 95 °C and 60 s at 60 °C). The ABI 7300 real-time PCR system (Applied Biosystems)

was used for detection of the fluorescent signals. A serial dilution of an EBV bacterial artificial chromosome (BAC) preparation (p2089) was included to calculate the viral DNA content of the different supernatants.

Cell lines and cell culture

B 95-8 is an EBV-positive cell line established from a patient suffering from infectious mononucleosis. M81 is a virus isolated from a cell line established from nasopharyngeal carcinoma (NPC) tissue of a Chinese patient [18]. Elijah is a Burkitt's lymphoma cell line, and we used an EBV-negative clone thereof for this study. All cell lines were kept in RPMI 1640 medium (Gibco, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS, St. Louis, Missouri, USA) (Sigma).

EBV-encoded small RNA (EBER) staining

Isolated B cells from patients with detectable EBVL were applied onto glass slides and fixed for 30 min in 4% paraformaldehyde supplemented with 5% glacial acetic acid. A peptide nucleic acid (PNA) probe specific for the EBER noncoding RNAs was purchased from Dako and used as instructed by the provider (Dako PNA ISH Detection Kit, Hamburg, Germany).

Immunostaining

Detection of the immediate early antigen BZLF1, late lytic protein gp350 or latent protein EBNA2 was performed using monoclonal antibodies specific to these proteins (EBNA2 clone PE2, gp350 clone 72A1, BZLF1 clone BZ.1). Cells were fixed with 4% paraformaldehyde, permeabilized in PBS 0.5% Triton X-100 except for samples stained for gp350 that were fixed in pure acetone. Cells were incubated with the first antibody for 30 min, washed in PBS three times, and incubated with a secondary antibody conjugated to the Cy-3 fluorochrome for 30 min.

FISH

For fluorescence *in situ* hybridization (FISH), B cells were treated with an hypotonic solution containing 0.0075 M KCl, repeatedly fixed in ice cold methanol : acetic acid (vol:vol 75:25) and applied onto glass slides. We generated an EBV-specific probe by nick translation of the complete B 95-8 BAC as described before [19]. Denaturation of cells was performed in 2 × SSC 70% deionized formamide for 2 min at 75 °C and followed by dehydration in an increasingly concentrated alcohol series. The probe was denatured at 75 °C for 5 min, applied onto dehydrated cells, and incubated overnight at 37 °C. Slides were then washed in 4 × SSC. The probe was detected by streptavidin-conjugated fluorescein Alexa 488 (Life Technologies, Carlsbad, California, USA). Slides were analyzed using a Leica epifluorescence microscope Wetzlar, Germany.

Binding assay

4 × 10E4 EBV-negative Elijah cells were incubated with 200 µl of serum for 4 h at 4 °C, washed in PBS, and applied onto glass slides. The treated cells were fixed in pure acetone for 20 min. Immunostaining was performed as described above using an antibody against gp350. Nuclei were counterstained with DAPI. Incubation of EBV-negative Elijah cells with either pure FBS or infectious supernatants containing M81 served as negative and positive controls, respectively.

Transformation assay

1 × 10E5 B cells from healthy donors were incubated with 200 µl of plasma for 4 h at 4 °C. The treated B cells were transferred onto a layer of irradiated feeder cells in one well of a 96-well plate. Cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Sigma). Cells were regularly observed for transformation over a period of 3 months. B cells infected with M81 or B 95-8 virus served as positive controls.

Statistics

Statistical analyses were performed using the Graphpad prism 6 software, La Jolla, CA, USA. Some results are given as arithmetic mean with standard error (SEM). We performed correlation studies by calculating the Pearson's correlation coefficient using prism 6.

Results

Clinical characteristics

We investigated peripheral blood samples from 23 transplant recipients who presented with an increased EBVL (mean 8583 cop/ml WB, range 1000–24 900 cop/ml) (Table 1). Nine patients had undergone stem cell transplantation and 14 kidney transplantation. The clinical data of the patients, including age, sex, time from transplantation to the detection of the increased EBVL,

Table 1. Characteristics of the studied patients (Pat-1 to 23) with an elevated EBV load.

Patient number	Age	Sex	Transplanted organ	Time from transplantation to increased EBVL (in days)	EBV cop/ml WB	Immunosuppressive regimen	Rituximab treatment	Time from rituximab treatment to negative EBVL (in days)	FU (in months)	Viral load in cop/ml WB at last FU (in months)	Status at last FU
Pat-1	66	m	SCT	26	3150	CSA, MTX	+	4	12	0 (5)	Alive
Pat-2	66	f	KT	1821	18 600	CSA, MMF, ST	–		15	15 700 (15)	Alive
Pat-3	64	m	SCT	29	1000	FK, MMF	–		21	0 (9)	Dead (relapsed ALL)
Pat-4	66	f	KT	3024	1730	ST	–		3	-	Alive
Pat-5	63	m	SCT	273	16 200	FK	+	7	14	0 (14)	Alive
Pat-6	60	f	KT	2853	1920	BELA, ST	–		10	1210 (10)	Alive
Pat-7	28	m	KT	5704	4570	FK, ST, SIR	–		9	12 500 (3)	Alive
Pat-8	56	f	KT	18	8450	CSA, MMF, ST	–		16	1470 (16)	Alive
Pat-9	37	m	KT	810	16 400	FK, MMF, ST	–		7	-	Alive
Pat-10	48	m	KT	5121	12 060	CSA, ST	–		10	10 500 (3)	Alive
Pat-11	54	f	SCT	25	4450	CSA	–		17	7840 (3)	Alive
Pat-12	53	f	KT	6199	3450	ST	–		15	-	Alive
Pat-13	52	m	SCT	32	16 000	CSA, MMF	+	10	7	0 (4)	Alive
Pat-14	61	f	KT	2703	12 200	CSA, MMF, ST	–		6	1690 (6)	Alive
Pat-15	39	m	SCT	30	3780	FK, MMF	+	13	11	0 (2)	Alive
Pat-16	65	m	KT	1556	10 600	CSA, MMF, ST	–		5	34 300 (3)	Alive
Pat-17	56	f	KT	1187	1900	CSA, AZA, ST	–		5	1050 (5)	Alive
Pat-18	32	f	SCT	43	1000	ST, FK	+	17	5	0 (5)	Alive
Pat-19	68	m	KT	8371	16 000	FK, MMF, ST	–		7	-	Dead (sepsis)
Pat-20	69	m	KT	613	1000	BELA, ST	–		6	0 (6)	Alive
Pat-21	29	f	KT	10 144	1040	CSA, MMF, ST	–		5	1930 (5)	Alive
Pat-22	72	f	SCT	519	24 900	*	+	21	5	0 (5)	Alive
Pat-23	49	m	SCT	60	17 000	CSA	+	14	3	0 (3)	Alive

EBVL, EBV load; CSA, cyclosporin A; MMF, mycofenolate mofetil; FK, tacrolimus; SIR, sirolimus; AZA, azathioprine; MTX, methothrexate; BELA, belatacept; KT, kidney transplantation; SCT, stem cell transplantation; ST, steroid; m, male; f, female; cop, copies; WB, whole blood; FU, follow-up; ALL, acute lymphoblastic leukemia.

*Patient with paused immunosuppression.

immunosuppression regimens, and rituximab treatment, are given in Table 1. None of the kidney recipients had received an intensified immunosuppressive treatment or antilymphocyte therapy in the 6 months that preceded the development of the increased EBVL. None of the patients showed clinical or radiological evidence of a neoplastic process, in particular of PTLD, neither at diagnosis nor during follow-up.

The EBV load measured by qPCR in whole blood samples does not accurately reflect the number of EBV-infected B cells they contain

We started our investigations by determining the percentage of infected cells within the purified blood B cells of the investigated patients (Fig. 1). To this aim, we performed FISH on purified B cells using an RNA probe specific for the noncoding EBER RNAs that are expressed at very high levels in infected cells [20]. Knowing the B-cell counts per ml of WB, we calculated the number of infected B cells per ml of WB (number of B cells per ml WB × percentage of infected B cells). This analysis showed that the number of infected B cells ranged widely, between 13 and 2750 per ml WB (median 258 per ml). For comparison, immunocompetent individuals usually carry between 2.3 and 62 infected cells per million of B cells, that is, 0.6–15.7 infected cells per ml of WB [21–23]. Thus, a few patients diagnosed with an increased EBVL will have only a marginal increase in infected B cells relative to healthy controls. We then assessed the correlation between the qPCR-measured EBVL and the number of infected cells in the same blood samples by calculating the Pearson's correlation coefficient. To this end, we first calculated the natural logarithm of the values to obtain a normal distribution. We found that there was only a weak correlation between these two parameters [$r = 0.5586$, $P = 0.056$, 95% confidence interval (0.1901–0.7891)] (Fig. 2). Moreover, exclusion of the highest EBVL led to an absence of correlation. Thus, the EBVL does not accurately predict the number of infected B cells in the blood.

Patients with an increased EBV load carry transformed infected B cells at a highly variable rate

We then stained the purified B cells from 20 of the samples with an antibody specific to the EBNA2 protein (Fig. 3). This viral product is the most important factor in the classical EBV-mediated transformation pathway (also known as latency 3) that is operative in the majority of EBV-positive PTLD cases [24]. Immunostains showed that 50% of the patients carried EBNA2-positive B cells, but also that the percentage of EBNA2-positive cells ranged between 1.79 and 80% of EBER-positive B cells. The remaining patients had no EBNA2-positive

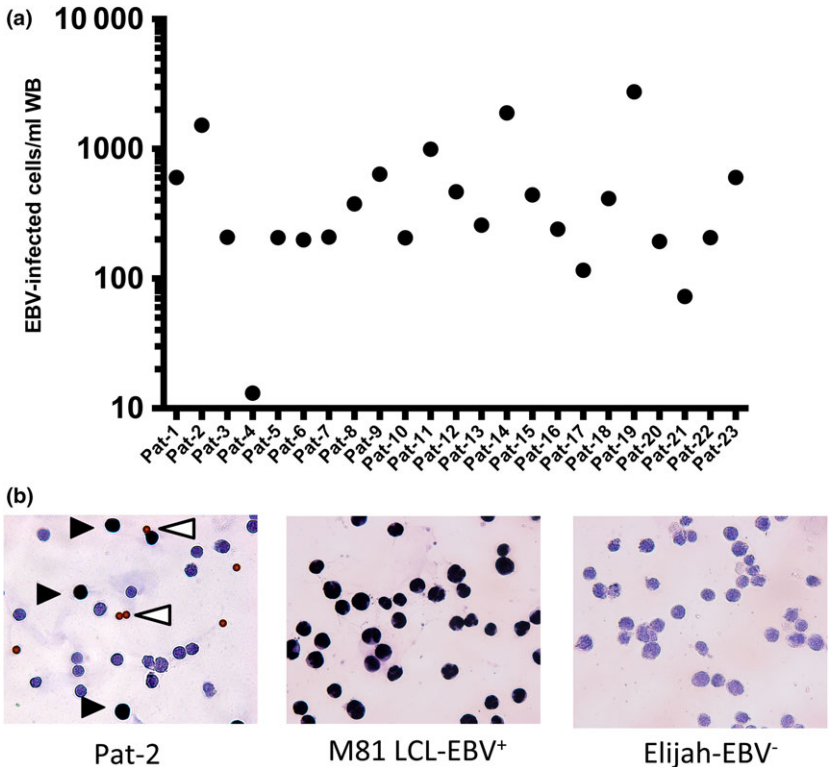

Figure 1 The absolute number of EBV-infected cells in whole blood (WB) of patients with an elevated EBV load (EBVL) is highly variable. (a) CD19-positive B cells from patients with increased EBVL were stained by EBER *in situ* hybridization to identify EBV-infected cells. The percentage of EBER-positive cells and the absolute number of isolated B cells per ml WB gave the absolute number of EBV-infected cells per ml WB that is given in the dot plot. (b) The pictures show an example of EBER staining. The positive cells display a nuclear black signal (indicated by black arrowheads). Some anti-CD19 Dynabeads that were used for B cell purification were visible and indicated by white arrowheads. An EBV-positive lymphoblastoid cell line (LCL) transformed by M81 served as positive control, the EBV-negative cell line Elijah as a negative control.

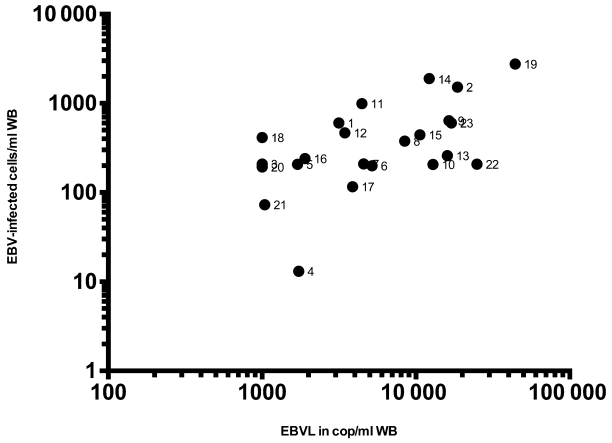

Figure 2 There is only a weak relationship between the EBV load (EBVL) in the whole blood (WB) and the number of EBV-infected cells per ml of WB. The EBVL was quantified using qPCR, and the number of EBV-infected cells per ml WB was determined by EBER *in situ* hybridization (also see Fig. 1).

cells, suggesting that these cells were not actively transformed.

Infected B cells carry variable numbers of EBV copies

We wished to understand the reasons that underlie the discrepancies between the actual B-cell infection rate in the peripheral blood and the EBVL. Two factors can *a priori* explain the absence of correlation. The number of EBV DNA copies per cell could influence the results. If the B cells in a given sample contain twice as many EBV copies than the B cells in another sample, the EBVL will be twice higher, even if the number of infected cells is identical in both samples. Thus, we performed qPCR on the same 20 B-cell samples to determine the average number of EBV copies per B cell. Knowing the percentage of infected B cells per sample, we could calculate the average EBV copy number per infected B cell (Fig. 4a). The investigated samples carried between 2 and 60 viral genome copies per cell. Thus, the variable number of viral copies per B cell will influence the total viral load, even if the number of infected B cells is identical. We had enough material to complete our analysis with a FISH assay, using a DNA probe specific for the EBV genome in five of the investigated samples. This method allows direct visualization of the viral genomes in the infected cells and their quantification. We first determined the percentage of B cells that exhibited signals to determine the rate of B-cell infection. We compared these numbers to those obtained after EBER staining and found a good correlation between both methods (Fig. 4b). In two clinical samples, we could investigate more than 30 infected cells per sample and determine the number of EBV copies per infected B cell (Fig. 4c). These values spread largely, showing that the number of EBV genomes per infected cell differs from cell to cell, even in the same clinical sample. We compared the mean of the values obtained by FISH in these two cases with the mean copy number calculated by qPCR and could find a good correlation (Fig. 4c). Although there was a large variation in the number of EBV genomes per cell, these low numbers were consistent with a predominantly latent infection in these two particular samples. We show examples of FISH in cells undergoing latent and lytic infection in Fig. 4d. Cells undergoing lytic replication generate several thousands of viral DNA copies per cell, giving rise to a very strong signal that completely covers the nucleus.

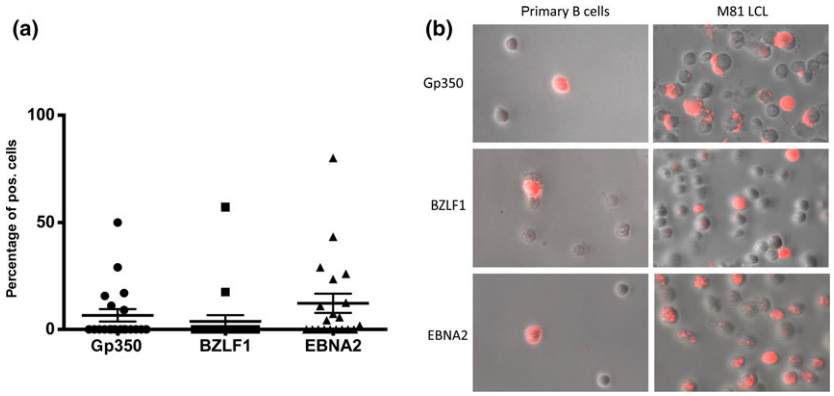

Figure 3 The latent and lytic protein expression in B cells of patients with increased EBV load (EBVL) widely varies between patients. (a) B cells of patients with an elevated EBVL were stained for the expression of the latent EBNA2 protein, for the immediate early lytic protein BZLF1, and for late lytic gp350 protein expression by immunofluorescence. The dot plot shows the mean expression levels of the different markers, normalized to the number of EBER-positive cells. We also show the mean and standard error of the mean of these values. (b) The pictures show examples of B cells that were positive for latent or lytic proteins (red). The M81 lymphoblastoid cell line (LCL) that expresses latent and lytic proteins served as a positive control.

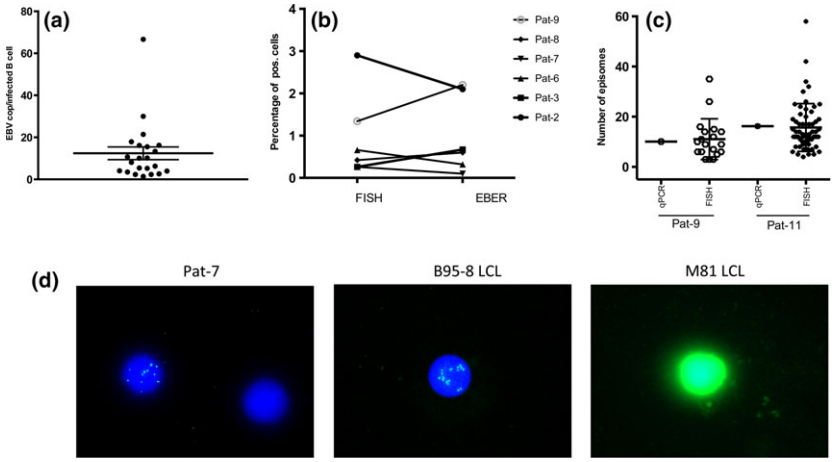

Figure 4 The EBV copy number in infected B cells varies widely between patient samples and within the same sample. (a) The dot plot shows the mean EBV copy number per infected B cell as determined by the results of the qPCR and of the EBER staining. (b) This graph shows the percentage of infected B cells in six samples, using either FISH or EBER staining of the same sample. (c) The figure shows the spreading of EBV copies per cell as determined by fluorescence *in situ* hybridization (FISH) in two blood samples. We also show the average EBV copy number per infected B cell calculated by qPCR. (d) The picture shows a B cell from Pat-7 stained by FISH that harbors 14 copies of the EBV genome. Cells from a cell line infected with EBV B 95-8 served as a positive control for latent EBV infection. We also show a cell transformed by M81 undergoing replication and carrying a very high number of copies that cannot be distinguished and lead to a homogenous staining of the nucleus.

Some patients with an increased EBV load carry B cells that undergo lytic replication

Thus, in cells that undergo lytic replication, the number of EBV copies will be several orders of magnitude higher than in latently infected cells and the presence of replicating cells within a blood sample will markedly enhance viral titers [25]. Approximately one-third of samples contained cells that express BZLF1 or gp350, two specific markers of lytic replication. Here again, the percentage of cells positive for one or the other marker varied widely between samples (Fig. 3a). Nevertheless, this indicates that the virus EBVL in some patients will be influenced by the presence of replicating cells.

The EBV load in the plasma does not reflect the presence of cell-free viruses

We continued our investigations by a characterization of the plasma of all 23 blood samples. We measured the EBVL and found that all samples contained EBV DNA, generally at a fraction of the concentration measured in the WB, although in some cases these two values were comparable (Table 1, Fig. 5a). The detection of EBV DNA in the plasma or serum has been taken as an indication of the possible presence of free infectious virus particles in some cases [26]. Therefore, we assessed the ability of the serum to transform resting primary B cells. The rationale behind this experiment was that if infectious viruses were present in the serum, they should be able to transform resting B cells. After serum exposure, B cells were placed on feeder cells and we monitored them to detect B-cell outgrowth. Although the addition of infectious viruses in the positive control readily gave rise to B-cell colonies, we could not detect any B-cell growth in any of the tested samples (Fig. 5b). This assay was completed by a binding assay in which viruses are detected at the surface of a B-cell line that is known to bind EBV with high efficiency. The presence of the virus bound to the surface is visualized by an immunostain against gp350 that is abundantly present in viruses (Fig. 5c). Here again, while the positive controls showed strong signals, we could not identify any bound particles after incubation with the sera of the patients. Thus, the plasma of patients with an increased EBVL contains DNA in variable amounts, but this DNA does not originate from infectious particles.

Rituximab treatment of patients with an increased EBV load

While stem cell transplant recipients were frequently treated with rituximab, the kidney transplant recipients were simply kept under intensive clinical surveillance [13,27]. None of the patients were managed by reduction or changes in the maintenance immunosuppressive regimen. The treatment with rituximab was very effective in reducing the DNA load and preventing clinical

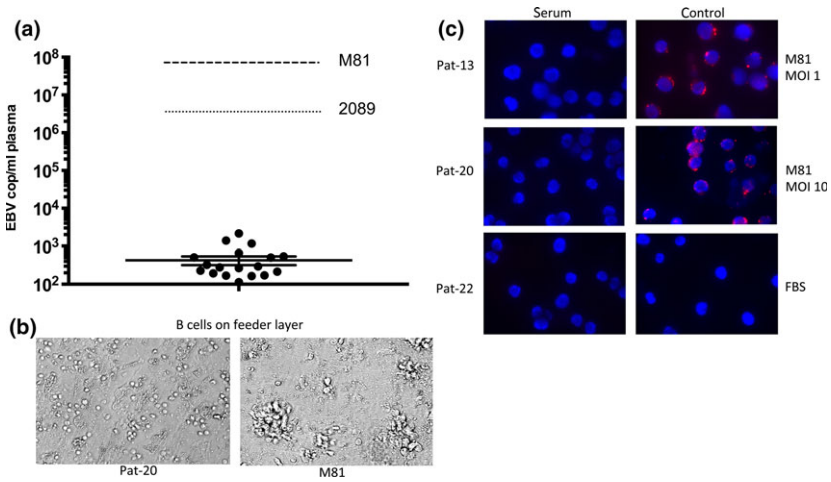

Figure 5 The plasma and serum of patients with increased EBV load (EBVL) does not contain infectious virions. (a) The plot shows the EBVL (EBV cop/ml supernatant or plasma) in plasma samples from patients with increased EBVL as determined by qPCR. We included supernatants from a latently infected lymphoblastoid cell line (LCL) transformed with B 95-8 and from a replicating cell line transformed with M81 as positive controls (dotted lines). (b) We exposed resting peripheral blood B cells to plasma from patients with increased EBVL or to M81 infectious viruses and monitored B-cell outgrowth on plates coated with fibroblastic feeder cells. While exposure to the controls led to the outgrowth of colonies made of large transformed B cells, cells exposed to plasma did not show any signs of outgrowth (Pat-20). (c) We performed binding assays with EBV-negative Elijah cells that were incubated with serum of patients with increased EBVL. We show here three examples (Pat-13, -20, -22) as well as a positive control in which the cells were infected with M81 viruses at a multiplicity of infection (MOI) of one or 10 viruses per cell. We used cells treated with fetal bovine serum (FBS) as a negative control. The cells were stained for the EBV lytic protein gp350 and counterstained with DAPI (blue nuclear signal). The staining generates the surface red signals in the positive controls.

PTLD in stem cell recipients (Table 1). Untreated kidney recipients showed no signs of lymphoproliferation during the follow-up period (Table 1).

Discussion

An increased EBVL in the peripheral blood of transplant recipients has been suggested as a predictive factor for the development of PTLD [28]. As PTLD consists of EBV-infected B lymphoctes, it makes indeed sense to have an assay that can quantify them in the peripheral blood and several investigators have found an increase in the number of EBV-infected B lymphocytes in the peripheral blood of patients with EBV-positive PTLD [15,16,29]. Although there is no formal proof that development of PTLD is always accompanied by an increased number of infected B lymphocytes, their detection in the peripheral blood is certainly suggestive of this lymphoproliferation. However, the present study demonstrates that quantification of the EBVL does not accurately reflect the number of infected cells in the blood. Two patients with the same EBVL could have more than a 100-fold difference in their B-cell numbers (Fig. 1). Such a discrepancy can be explained by multiple factors, some of which we could identify in the present study. The results of the FISH analysis indicated that the number of EBV copies varied substantially across different samples and across different cells in the same sample. Our results on the EBV genome copy numbers are concordant with previous studies of EBV-infected cells [30,31]. Calattini *et al.* [30] reported a median EBV copy number of 13.9 per cell in transplant recipients and patients with infectious mononucleosis. Other studies performed on a pediatric population found that patients with a low EBVL contained B cells with a low EBV genome copy number (59% of cells with one copy, 17% with 3–9 copies), while patients with higher EBVLs carried cells that displayed a higher EBV genome count [31]. These numbers even increased in patients with PTLD where half of infected cells carried more than 10 EBV genomes per infected B cell [31]. We could also detect evidence for lytic replication in a significant minority of samples that is likely to increase the amount of viral DNA produced by an infected cell. Finally, we found that a variable proportion of viral DNA is present in the plasma. Previous reports have suggested that the quantification of the EBVL in plasma or serum can detect the presence of DNAse-resistant cell-free infectious viruses in patients with NPC or more rarely in transplant recipients with PTLD [26,32]. We tested this hypothesis by exposing resting primary B cells to the

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plasma or serum of our patient series but could not detect any bound viruses or any signs of infection. These results fit with the observation that the plasma or sera of EBV-infected humanized mice do not contain infectious viral particles [18]. The strong avidity of EBV for B cells renders it unlikely that free virus can be found in the blood, as the virus progeny will be captured by the neighboring B cells [18]. Thus, a plasma EBVL is unlikely to originate from infectious viruses but rather from decayed cells and consists of viral DNA that can be DNAse-resistant but is nevertheless noninfectious. In some samples, the plasma virus load represented half of the total blood virus load, suggesting that the dead cells contributed significantly to the total EBVL.

Preemptive rituximab treatment of stem cell recipients with an increased EBVL has been shown to reduce the PTLD incidence, although some patients with increased load do not respond to this treatment [33–35]. Therefore, some authors have suggested treating stem cell recipients with an increased EBVL that exceeds 1000 cop/10E5 PBMCs or 1000 cop/ml WB, respectively [35,36]. Our results show that some of these patients only have a mild increase in infected cells, close to values found in normal individuals. For these patients, the benefits of a treatment with cytotoxic antibodies might be outweighed by the costs of the therapy and its toxicity, in particular cytopenia [37,38]. Our results suggest that it is difficult to appreciate the burden of infected B cells with the EBVL. Thus, the number of EBV-infected cells might be a better marker of lymphoproliferation than the EBVL but its value to guide clinical decisions needs to be evaluated in future studies.

EBNA2 or other members of the EBNA gene family that are expressed during latency 3 such as EBNA3C have been suggested as surrogate markers of an EBV-induced lymph proliferation [39]. Indeed, PBMC of patients with PTLD or with a high EBVL more often express EBNA2 or EBNA3C-specific transcripts, and this correlates with a high number of Ki67-positive proliferating B cells [15]. The observation that liver and kidney transplant recipients very frequently display signs of increased EBNA1 and EBNA2 transcription shortly before and after the development of PTLD also supports this concept [40]. In contrast, infected B cells of transplant recipients with no PTLD tend not to express EBNA2 and only 1% of the EBV-infected cells in the blood of transplant recipients were engaged in the cell cycle [41]. We also found many patients with absent or very low EBNA2 expression. Our observation that many patients had a considerably lower burden of EBV-positive B cells in the blood than suggested by the EBVL and that these cells were EBNA2-negative could provide an explanation for the previous description of patients who displayed a constantly increased EBVL for several months without developing signs of PTLD [42,43]. These observations could also explain why an increased EBVL is not predictive of PTLD [28,44]. This situation draws a parallel with CMV infection where the DNA load was found to be less informative than a staining for pp65 [45].

Interestingly, our data suggest that even the presence of EBNA2-positive cells in the peripheral blood does not predict the development of PTLD, probably because the total number of infected B cells remains low. Therefore, we hypothesize that the most important parameter is the number of infected B cells per ml of blood. This can be determined by an EBER staining that is routinely performed in histopathology departments and can be also analyzed by FACS [46]. We suggest considering the predictive value of the number of EBER-positive cells for the development of PTLD and its usefulness to guide preemptive rituximab therapy. Our study shows the feasibility of such an approach, even with relatively low blood volumes. Other assays could be performed to complement the information conveyed by the EBVL. Immunostains for BZLF1 and gp350 on PBMC or purified B cells would also identify patients undergoing lytic replication that might benefit from treatment with virostatics such as valganciclovir, although the efficacy of these drugs remains controversially debated [47,48]. The percentage of EBNA2-positive cells among the infected cell population is also likely to be an important parameter. Future prospective studies will determine the value of these tests to identify patients with subclinical PTLD.

Authorship

SF and HJD designed the study wrote the paper. All other co-authors provided clinical data and samples.

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Conflicts of interest

PW declares honoraria and membership on advisory boards of Sanofi-Aventis and Hexal AG. The authors declare no other conflict of interest.

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