Balance between herpes viruses and immunosuppression after lung transplantation

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Chapter 10

Epstein-Barr virus reactivation and lung transplant dysfunction

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Abstract

Since 1997 we have studied the relation between Epstein-Barr virus (EBV) infection and post transplant lymphoproliferative disease (PTLD) after lung transplantation. During these studies we noticed a relation between EBV DNA load and transplant dysfunction. This study reports the analysis of this relation in a cohort of lung transplant recipients before EBV monitoring was introduced as a diagnostic routine in our program. Whole blood samples, prospectively collected, were retrospectively tested for EBV DNA in 29 patients.

Changes in pulmonary function were related to the onset of detectable EBV DNA, and showed in the majority of cases a decrease in FEV1, when EBV DNA became detectable.

Episodes of rejection when EBV DNA was detectable and episodes when EBV-DNA was undetectable were compared with regard to the response to rejection therapy. Rejection treatment given during EBV reactivation was not effective in contrast with rejection treatment given in the absence of EBV DNA.

Rejection treatment given in presence of EBV DNA was always given on the basis of clinical suspicion of rejection and biopsies taken when EBV DNA was detectable never confirmed acute rejection. EBER positive cells could be in a minority of the biopsy samples, only when EBV-DNA load was very high.

We conclude from this that retrospective analysis that EBV reactivation is frequently associated with transplant dysfunction in our lung transplant patients. This EBV associated transplant dysfunction was in most of the cases mistaken for rejection and treated accordingly without success.

Introduction

The high incidence of Epstein-Barr virus associated Post Transplant Lymphoproliferative Disease (PTLD) after lung transplantation (LTx), with frequencies up to 10%, underlines the importance of Epstein-Barr virus (EBV) infection after lung transplantation (1); (2-4). Due to iatrogenic suppression of EBV-specific T-cell function immune surveillance of EBV is impaired, leading to EBV induced B-cell proliferation and a subsequent emergence or increase of circulating EBV positive B-cells (5).

Sensitive techniques for the detection of EBV-DNA in peripheral blood have shown their value for early diagnosis of and possible intervention in PTLD (6,7). The high seroprevalence (>90%) of EBV in the general population and the presence of detectable EBV-DNA in blood up to months before the clinical manifestations of PTLD (6,8) suggest that PTLD is the tip of the iceberg of EBV reactivation and that EBV reactivation is more common than previously recognized.

A striking feature of PTLD is, on the one hand, its correlation with increase in immunosuppression and, on the other, its treatment that consists primarily of reduction of immunosuppression. The usual explanation is that the increase in immunosuppression, given because of rejection, results in EBV reactivation and PTLD (9).

In our previous observations, however, we frequently observed that EBV infection was already present before rejection therapy was started, and suggestions of a relation between transplant dysfunction and Epstein Barr virus have been made (10-12). Therefore, we suggest an alternative explanation, namely that EBV reactivation, if unrecognized, might mimic rejection, and thus, leads to rejection treatment, over-immunosuppression and, ultimately, PTLD.

The implementation of routine EBV-DNA detection in blood samples has allowed us to explore this hypothesis. The present report describes the retrospective analysis of blood levels of EBV-DNA in relation with transplant function and histolopathological findings in 29 lung transplant recipients.

Materials and Methods

Patients

The initial study population consisted of 34 consecutive recipients of a primary bilateral or unilateral lung transplant. Five patients were excluded

because of early death (primary graft failure in 3 patients, lethal hemorrhage and complicated relapse of tuberculosis in one patient each). The remaining 29 patients all survived for more than one year and a complete follow up was available from all of them.

Induction treatment included 3 gifts of rabbit-antithymocyte-globulin (Merieux, France), 3 mg/kg, during the first 10 days after transplantation. Maintenance immunosuppressive regimen consisted of cyclosporine-A (Neoral, Novartis, Switzerland), azathioprine (2 mg/kg/d), and prednisolone (0.2 mg/kg/d with subsequent tapering to 0.1 mg/kg/d). Cyclosporine-A treatment aimed at a trough level of 400 ng/ml directly after transplantation, as determined by high-performance liquid chromatography, which was tapered in 3 weeks to reach a trough level of 150 ng/ml. Acute rejection was treated with a 3 days course of 500 to 1000 mg methylprednisolone intravenously daily. All patients received acyclovir, 4 daily doses of 200 mg orally, for herpes prophylaxis, and co-trimoxazole, 960 mg orally, on alternate days for Pneumocystis Jerovici (formerly Carinii) prophylaxis. No Cytomegalovirus (CMV) prophylaxis was given at that time.

Diagnostic protocol and routine follow-up

Graft function was determined by formal spirometry according to ATS/ERS guidelines, with emphasis on forced-expiratory-volume-in-one-second (FEV1). Measurements were done at least twice weekly during hospitalization and at every outpatient visit, which were initially weekly and gradually reduced to once every three months one year after lung transplantation (Ltx). Acute allograft rejection was diagnosed clinically in case of deteriorating pulmonary function after exclusion of infection. Histologic diagnosis of rejection was defined according to ISHLT criteria (13). EBV-DNA load was measured retrospectively in, for that purpose, stored blood samples. Results were not available at the time of treatment, and, as a consequence, did not influence clinical management. CMV infection or reactivation was monitored by testing for CMV antigenemia (14) and CMV serology (15). Active CMV infection was defined as the presence of pp65-positive cells in the CMV antigenemia test.

Bronchoscopies were routinely performed within 6 weeks after lung transplantation, at 6 months intervals, and when clinically indicated. Bronchoscopy included bronchoalveolar lavage (BAL) and transbronchial biopsies, as described previously(16). All BAL samples were cultured for (myco)bacteria, fungi and yeast, and subjected to auramine and silver staining for detection of Mycobacterium Tuberculosis and Pneumocystis Jerovici, respectively.

Blood sampling for EBV-DNA

From 1997 until 2001, whole blood samples of all lung transplant recipients were prospectively collected by venapuncture during the first postoperative year, weekly during admission and at every outpatient visit. For EBV PCR analysis 1 ml of EDTA-treated whole blood was mixed thoroughly with 9 ml of NASBA-lysisbuffer (Organon Technika,Oss, The Netherlands) and stored at -80°C until use (11). Serially obtained samples of our study population were retrospectively tested for EBV DNA.

Quantitative EBV-DNA PCR

The quantitative EBV-DNA PCR was carried out as described previously (6). In short, one ml of freshly obtained unfractionated whole blood was lysed in 9 ml of lysisbuffer and stored at -80°C until use. DNA was isolated from 1 ml of lysate by silica-based extraction. The DNA equivalent of 5 μl whole blood or serum was amplified in a qualitative EBNA-1 PCR. When the qualitative EBNA-1 PCR tested positive EBV-DNA load was subsequently determined by quantitative competitive EBNA-1 PCR (Q-PCR). The cut-off value used in Q-PCR was 2000 copies/ml blood of EBV-DNA load, which is the detection limit of this assay. DNA quality of whole blood samples was checked by ¤-globin PCR(6).

EBV-DNA load in relation to transplant (dys)function

EBV reactivation was considered present when the number of EBV-DNA copies exceeded the detection limit. As a marker of the severity of EBV reactivation the peak DNA load of an EBV reactivation episode was used. Peak values of EBV reactivation were related to changes in FEV1 (defined as the difference between the average of the last two FEV1 values measured within 3 months before EBV-DNA tested positive and the FEV1 value measured at the time EBV-DNA became positive). Care was taken to exclusively include FEV1 values taken prior to, i.e. not influenced by, a diagnostic or therapeutic intervention (e.g. bronchoscopy or rejection treatment). For this part of the analysis blood samples and routine lung function data available after LTx were used.

To further substantiate the relation between EBV and transplant (dys-)function we evaluated the response to rejection treatment, that is pulse methylprednisolone, in the presence or absence of positive testing for EBV DNA.

EBV-DNA load and histopathology

All routine biopsies were re-evaluated, especially for EBER positive cells in biopsies taken during the presence of positive blood tests for EBV-DNA. EBV in

biopsies was analyzed by EBER1/2 in situ hybridization (8). When necessary, additional EBER-ISH was performed. Biopsy specimens of lesions suspected of PTLD were stained by immunohistochemistry as described before (8).

Statistical analysis

SPSS software (release 12.0.2) was used (Spearmans rho test) to determine the correlation between EBV-DNAemia and changes in FEV1.

Results

EBV-DNA load

Twenty-nine out of the 34 consecutive LTx recipients survived the initial phase after transplantation and were included in the study. Patient data are given in table 1. Of these 29 patients, 18 patients remained EBV-DNA negative throughout the observation period. The median number of samples tested per patient in this group was 9 (range 8-17). Eleven patients became EBV-DNA positive. These patients were divided in a group with 'low' EBV DNA load and a group with 'high' EBV DNA load. This was arbitrarily based on the peak level of EBV DNA load (Table 1). The EBV DNA 'low' group consisted of 6 patients with 13 EBV DNA positive samples out of 96 samples tested, and an EBV-DNA load up to 14,600 genomes/ml whole blood. The EBV 'high' group consisted of 5 patients with 67 EBV DNA positive samples out of 100 samples tested, and peak levels of EBV DNA ranging from 22,900 to 137,600 EBV-DNA genomes/ml (Table 1).

EBV-DNA and transplant function

The 11 patients who became EBV DNA positive presented 20 episodes of EBV reactivation. Five episodes started shortly after transplantation before routine lung function testing had started. For one episode the first EBV DNA positive sample had been taken elsewhere and lung function testing was not available from the start of the EBV DNA positive episode. From the remaining 14 episodes, lung function data were available from before the start of the episode, and, thus, the relation between EBV reactivation and changes in pulmonary function could be evaluated.

Appearance of EBV DNA coincided in time with decrease in FEV1 in 10 out of the 14 episodes (in 8 patients) (median 19%, range 2-35%) (representative examples are given in Fig. 1a-c).

***Table 1**: Patient data and results of EBV DNA testing. (AT-def= Alpha-1-antitrypsin deficiency). Other indications were Bronchiectasies (3), Pulmonary fibrosis (1), Primary pulmonary hypertension (1) and Lymfangioleiomyomatosis (1) No=number, pt=patient.*

	EBV neg	EBV pos	
		Low	high
No of patients	18	6	5
Indications for lung transplantation			
Emphysema (AT-def)	13 (5)	3 (2)	1
Cystic Fibrosis	4		2
Other	1	3	2
Median age (range, yrs)	39 (13-55)	47 (34-55)	48 (21-61)
No of patients with EBV mismatch	0	0	1
No of EBV DNA pos. samples (samples tested)	0(187)	13(96)	67(100)
Median no of EBV DNA pos samples /pt (range) Median no of samples tested per patient (range)	0 9 (8-17)	1 (1-6) 14(10-29)	13 (4-23) 20(13-29)
peak EBV DNA load (genomes/ml whole blood)(range)	NA	2,400-14,600	22,900-137,600
Median EBV DNA load	NA	6,500	98,400
No of PTLD	1	0	3
No of patients with CMV mismatch	1	1	3
No of episodes with rejection treatments (mean/pt)	31(1.7)	14(2.3)	15(3)

Response to Rejection treatment and EBV DNA load

Overall, from one month after Ltx on, 60 courses of rejection treatments were given to the 29 patients.

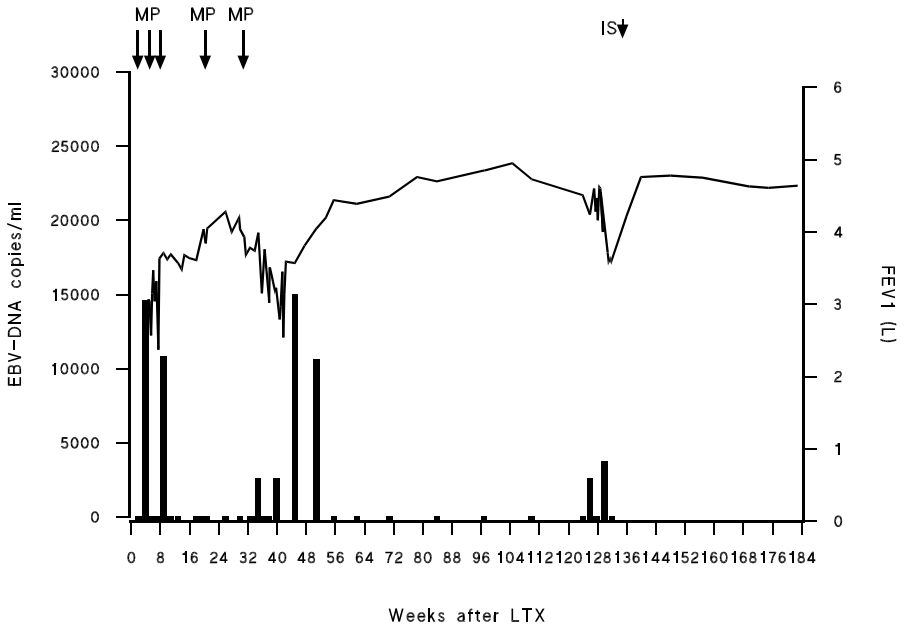
There was a gradual increase in the number of rejection treatment courses given in the "high" EBV DNA group (1.7, 2.2 and 3.0 rejection treatment episodes per patient in the EBV DNA negative, the EBV DNA "low" and the EBV DNA "high" group, respectively, although this was not statistically significant).

Twenty-nine episodes of rejection treatment were recorded in the EBV DNA positive groups.

Response to rejection treatment was defined as increase of FEV1 within a period of one month after starting treatment. Failure was defined as a decrease in FEV1 below the level of the FEV1 value at the time rejection therapy was started or as ongoing rejection based on the clinically judged necessity to give another rejection treatment within one month.

Results are depicted in figure 2a. The patients with EBV reactivation received 29 rejection treatment couses. Twelve were given at a time that EBV DNA was present and resulted in only 3 responses. In contrast, 17 courses of rejection treatment were given to 7 of the former 12 patients at a time that EBV DNA was absent. A response to treatment was noted after 12 out of these 17 courses ($p<0.05$, Chi-square). 31 rejection treatment courses were given to patients who did not have an EBV reactivation during the observation period. These resulted in 24 responses ($P<0.01$, Chi-square, as compared to the number of responses to rejection treatment given when EBV DNA was present).

***Figure 1A**: Shows the relation in time between FEV1 (line) and EBV DNA load (bars). MP,rejection treatment with methylprednisolone; IS arrow, reduction of immunosuppression based on elevated EBV DNA load.*



The impact of EBV reactivation on the functional outcome after rejection treatment is shown in figure 2b. Here, the first episode of EBV reactivation in a patient is depicted in time in relation to the delivery of rejection treatment. The influence of rejection treatment on FEV1 is given for the group of patients positive for EBV DNA and the group negative for EBV DNA at the time that rejection treatment was started.

***Figure 1B**: Shows the relation in time between FEV1 (line) and EBV DNA load (bars). MP,rejection treatment with methylprednisolone.*

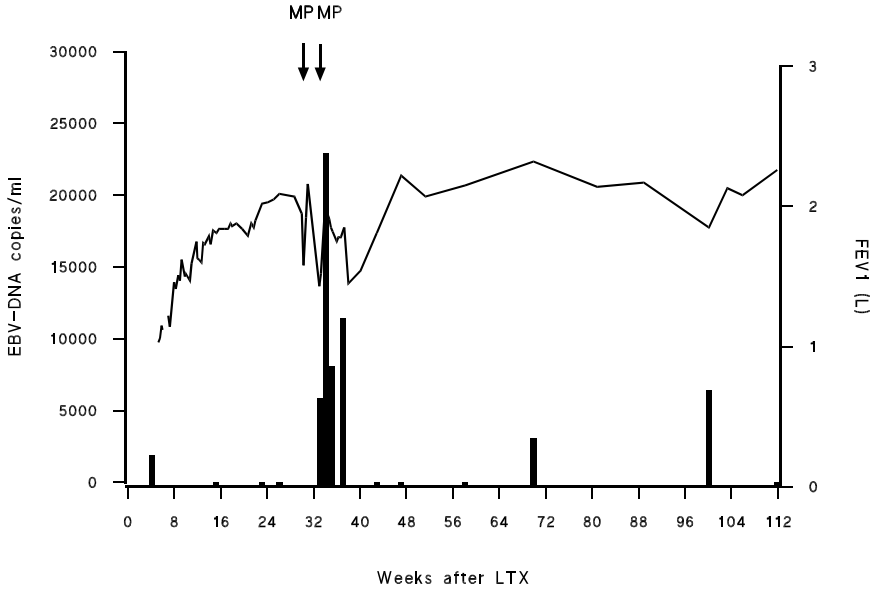
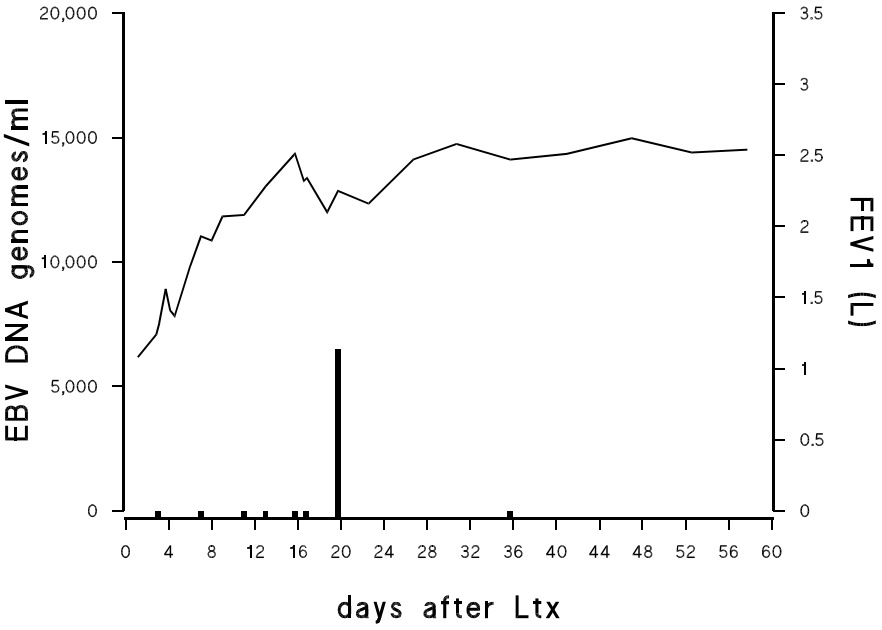


***Figure 1C**: Shows the relation in time between FEV1 (line) and EBV DNA load (bars). No rejection treatment was given.*



Of the 11 patients who had an EBV reactivation, 7 received rejection therapy before they became EBV DNA positive. Five of these patients received another rejection therapy thereafter and lung function did not improve in these patients as depicted in figure 2b (P=0,41 NS, T-Test). Four patients became EBV DNA positive during stable immunosuppression and 3 of these 4 patients received a course of rejection therapy shortly thereafter. Pulmonary function decreased significantly in these 3 patients after the course of rejection therapy (P<0.05, T-Test). Of the 18 patients who remained EBV DNA negative, 16 received rejection therapy, resulting in an improvement of pulmonary function (P<0.0001, T-Test). From these data we conclude that decrease in pulmonary function when EBV DNA is detectable should not be treated as rejection but should lead to further diagnostic tests to prove whether or not rejection is present.

***Figure 2A**: Total number of rejection treatment courses given and the response in FEV1. Rej Tr = Rejection Treatment. During EBV= rejection treatment given when EBV DNA was detectable, Absence of EBV= rejection treatment given to patients who developed an EBV reactivation during follow-up but received rejection treatment at a time EBV DNA was not detectable. Response to rejection treatment was defined as increase of FEV1 within a period of one month after starting treatment. Failure was defined as a decrease in FEV1 below the level of the FEV1 value at the time rejection therapy was started or as ongoing rejection based on the clinical necessity to give another rejection treatment within one month.*

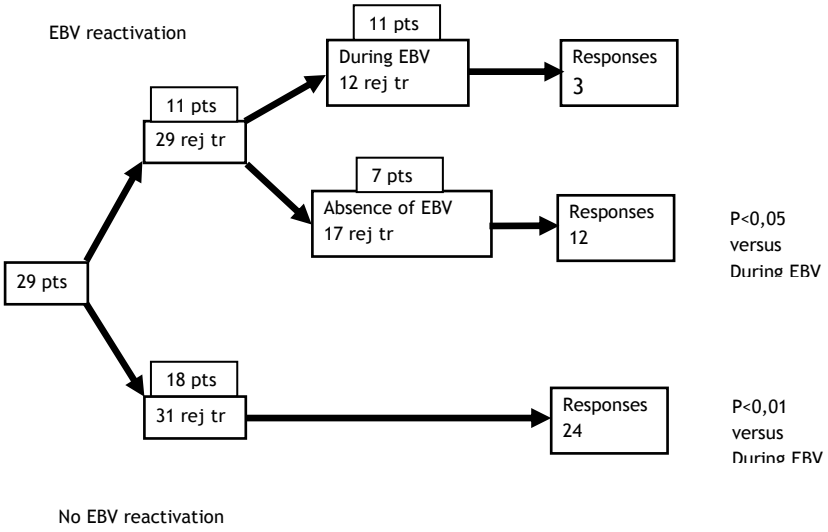
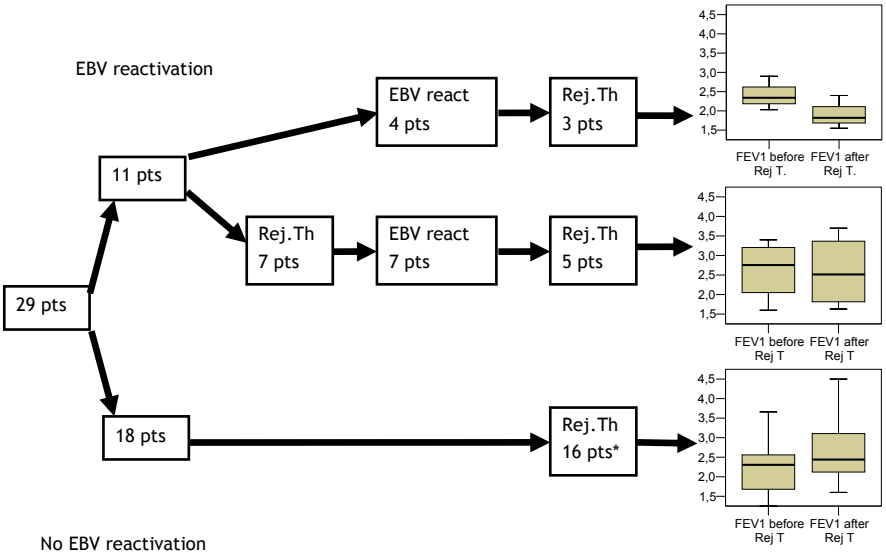


***Figure 2B**: Relation in time between the first EBV DNA positive episode and rejection therapy given within one month after start of this EBV DNA positive episode. Furthermore, the influence of rejection therapy given after EBV DNA became detectable on FEV1. Values are depicted. *Two patients who remained EBV DNA negative never received rejection therapy.*



EBV-DNA load and histopathology

In total, 110 bronchoscopies were performed in the 29 patients (Table 2). Histologically confirmed acute rejection was found in biopsies taken from 9 of the 68 bronchoscopies that were performed in patients without EBV reactivation. Fourty-two bronchoscopies were performed in the 11 patients with EBV reactivation. In these patients with EBV reactivation 4 biopsies showed acute rejection but this was never found when EBV DNA was detectable in these patients at the time of the biopsy. EBER positive cells, in the absence of PTLD, were only seen in 3 biopsy specimens obtained at peak levels of EBV DNA (24.800, 95.400 and 137.588 EBV DNA copies/ml respectively) (Fig. 3). Additionally, all biopsies obtained during detectable EBV DNA load were re-evaluated. No additional EBER positive cells were found in these biopsies.

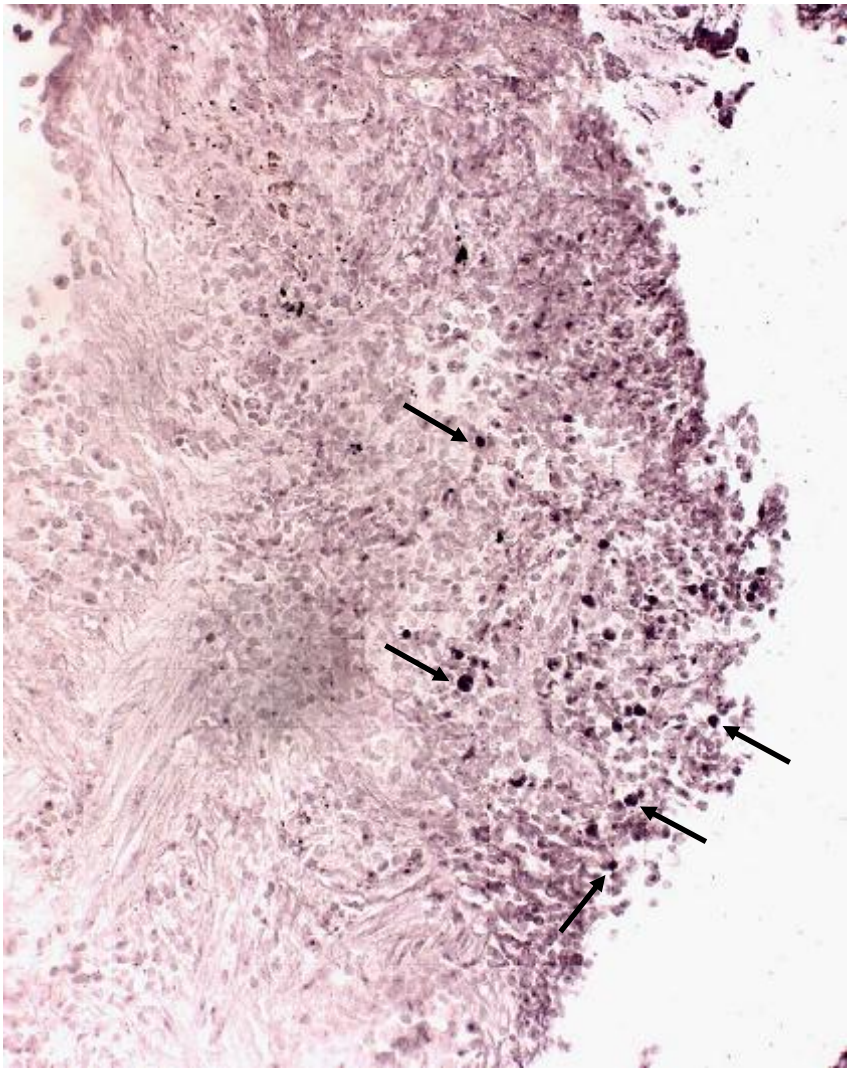
In spite of the fact that EBV-DNA remained undetectable in 18 patients, one of these patients developed a PTLD 6 months after Ltx. HLA-typing of PTLD cells demonstrated that the PTLD was of donor B-cell origin as described earlier (8).

***Table 2**: Results of the 110 bronchoscopies take during the first year after lung transplantation. Acute rejection was never seen in the group with EBV reactivation at the time EBV DNA was present in the peripheral blood. There was a tendency to a lower rate of acute rejection and a higher rate of suspected infection in the biopsies taken from patients with EBV reactivation.*

	Patients		
histology	without EBV reactivation (n=18)	with EBV reactivation (n-11)	
		EBV DNA negative at the time of biopsy	EBV DNA detectable at the time of biopsy
No. of bronchoscopies	68	28	14
Acute rejection	9	4	0
OB/vasculopathy	4	5	2
PTLD/ EBER-pos. cells	1	0	4
Infection	7	4	4
No clear diagnosis	44	15	4

OB= obliterative bronchiolitis, EBER= Epstein Barr encoded RNA,

***Figure 3**: sporadic EBER positive cells in a lung biopsy from a patient obtained by flexible bronchoscopy taken because of transplant dysfunction during peak levels of EBV DNA load (95.400 copies/ml). Arrows indicate EBER positive cells.*



Discussion

In previous studies (6,8,17), we have noticed that rejection treatment was given on several occasions when, in retrospect, peak levels of EBV DNA were reached. Also, EBV DNA was, retrospectively, often detectable before rejection therapy was given, especially in patients who developed PTLD. It led to the

suspicion that in these patients, we mistakenly were treating EBV reactivation as rejection. To further explore this possibility we retrospectively analyzed EBV DNA load in relation to rejection in a cohort of lung transplant patients.

High levels of immunosuppression and frequent courses of rejection therapy, especially with anti-T-cell antibodies, form a major risk factor for the development of PTLD (18). Indeed, in this study we observed that patients with EBV reactivation received more courses of rejection treatment and over half of the episodes of EBV reactivation were preceded by a course of rejection treatment. So, these EBV reactivations seem to be induced by increased immunosuppression.

We, however, also noticed the opposite situation, i.e. an EBV reactivation preceding a rejection treatment, strongly suggesting that EBV reactivation is associated with transplant dysfunction. The subsequent rejection treatment may have led to the overimmunosuppression associated with PTLD, in three of our patients.

EBV infection, both in the normal and the immuno-compromised host, are usually mildly symptomatic. So, it comes as no surprise that EBV reactivation after lung transplantation showed little symptoms. The symptoms we could identify retrospectively were limited and consisted mostly of a low grade fever, fatigue, a slight decrease in transplant function and eosinophilia. All these symptoms are non-specific and can easily be mistaken for rejection.

There are several arguments that made us conclude that EBV reactivation was mistaken for rejection. The first relates to, the time EBV DNA became detectable in relation to the occurrence of rejection. EBV DNA was detectable in all 11 patients before rejection therapy was given. EBV reactivation was preceded by rejection therapy in 7 patients, and again followed by a second course of rejection in 5 of these patients, suggestive of mimicking steroid resistant rejection. Secondly, we found a relation between peak levels of EBV DNA load and transplant dysfunction, which suggests a causal relationship. In contrats, we seldom (only at 3 episodes of peak levels of EBV DNA load) found EBER positive cells in lung biopsies. For this reason a relationship between presence of EBV in tissue and transplant dysfunction should be further examined. Thirdly, when rejection therapy was given on clinical ground at the time EBV DNA appeared to be detectable, the histology of the transbronchial biopsies never confirmed the rejection. Moreover, rejection therapy never had a prolonged beneficial effect on transplant function.

Summarizing, rejection therapy was started in a considerable number of lung transplant recipients during EBV reactivation. As we did not measure EBV DNA load at that time, only in retrospect we could recognize a presumable cause

of these episodes of transplant dysfunction. This is supported by the lack of beneficial outcome, even worsening of lung function by these rejection treatments given at times EBV DNA was detectable. In contrast, patients without EBV reactivation overall responded well to rejection therapy.

A clinical example of the hypothesis that transplant dysfunction might be the result of over-immunosuppression driven EBV reactivation is the observation in one of our patients that reduction of immunosuppression led to complete recovery of lung function and disappearance of EBV DNA from the blood (Fig. 1a). This outcome would not have been expected when the transplant dysfunction was caused by rejection. Reduction of immunosuppression as described here, would be the logical consequence of EBV DNA positivity and is currently under investigation.

In conclusion, we here describe a group of 29 sequential lung transplant patients who were retrospectively evaluated for EBV DNA load and transplant (dys-)function. We suggest that EBV reactivation after lung transplantation is associated with transplant dysfunction. This EBV associated transplant dysfunction was several times mistaken for rejection and treated as such.

This could be one of the reasons for the high number of PTLD reported after lung transplantation. EBV associated transplant dysfunction can be recognized by routinely measuring EBV DNA load, which in our view should be a routine diagnostic after lung transplantation. Further controlled prospective studies are needed to evaluate the role of EBV and its role as a biomarker of over-immunosuppression in the clinical approach to patients with a lung transplant.

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