A Rearranged Form of Epstein–Barr Virus DNA Is Associated with Idiopathic Pulmonary Fibrosis

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An association between idiopathic pulmonary fibrosis (IPF) and productive Epstein–Barr virus (EBV) infection has been found previously. Productive EBV replication can be associated with a rearrangement in EBV genomes termed WZhet. We hypothesized that WZhet genomes might be present in patients with IPF. Thirty-nine patients with IPF, 26 lung transplant recipients, and 24 normal subjects were studied. When EBV DNA–positive lung tissue biopsies from IPF patients were analyzed, 11 of 18 (61%) were positive for WZhet. Buffy coat DNA analysis showed that 75–85% were EBV DNA–positive in both IPF and control groups. Buffy coat analysis for WZhet was positive in 16 of 27 (59%) IPF patients, compared with none of 32 lung transplant recipients and 1 of 24 (4%) normal blood donors ($p \leq 0.001$). There was thus a good correlation between the presence of WZhet in lung tissue and peripheral blood. However, there was no significant association between the presence of WZhet and immunosuppressive therapy. These data further confirm the association between active EBV infection and IPF and provide a potential marker in the peripheral blood for the tracking of EBV in this disease.

Keywords: Epstein–Barr virus infections; polymerase chain reaction; pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease characterized by thickened, fibrotic alveolar walls and focal cuboidalization of the alveolar epithelium. Clinically this results in shortness of breath and inspiratory crackles. Bilateral radiologic shadowing, mainly of the lower lung zone, impaired gas transfer, and a restricted ventilatory pattern are characteristic. Prognosis is poor, and lung transplantation in selected patients is the only current therapy with any degree of success. Both environmental exposure and viral infections have been associated with IPF, suggesting that different injuries may trigger the clinical disease in different individuals (1–3).

The Epstein–Barr virus (EBV) is a herpesvirus prevalent in all human populations and associated with a number of diseases. These include infectious mononucleosis, B cell lymphoproliferative disease, Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (4). Although normally associated with infection of the upper respiratory mucosa and B lymphocytes, EBV can infect and replicate in the lower respiratory tract (5, 6). Previous serologic analysis found an association between active EBV infection and IPF (7). Here, Vergnon and coworkers showed raised anti-EBV viral capsid antigen immunoglobulin A levels in a study of patients with IPF (7). We have reported immunohistochemical evidence of EBV-productive cycle antigens in Type II alveolar epithelial cells in IPF (8). This was confirmed by detecting EBV DNA by polymerase chain reaction (PCR) in the lung tissue of patients with IPF (9). More recent observations by Tsukamoto and colleagues (10) have confirmed and extended these observations showing that expression of the EBV latent membrane protein 1 in infected epithelial cells was associated with poor prognosis in patients with IPF.

Some conditions are associated with spontaneous, productive EBV replication. For instance, oral hairy leukoplakia, thymic carcinoma, and Hodgkin's disease have been shown to contain rearranged EBV genomes (11–14). These genomes were first identified *in vitro* in the Burkitt's lymphoma cell line P3J-HR-1 by the presence of aberrant DNA restriction fragments, termed heterogeneous (het) fragments (15–17). The deleted and rearranged genome (het DNA) in defective virus forms self-contained replicons that multiply independent of standard EBV and are capable of cell-to-cell spread (18). The most salient rearrangement in these het genomes is the juxtapositioning of sequences from the *Bam*HI W and Z restriction fragments that are separated in the standard genome by more than 55 kb (19) (*see* Figure 1). This so-called WZhet recombinant fragment contains the entire BZLF1 open reading frame encoding a product (ZEBRA) that *trans* activates not only the lytic (productive) origin of EBV DNA replication but also EBV lytic cycle promoter elements (20, 21). The ability of WZhet DNA to activate EBV lytic replication stems from dysregulation of expression of BZLF1 by the ectopic positioning during rearrangement of positive regulatory elements on either side of the gene (22). It has been postulated that this rearrangement may represent a novel mechanism by which a persistent virus can move, via recombinational events, from a latent to a productive phase of its life cycle (11).

As defective EBV is associated with productive EBV infection in other clinical lesions, we hypothesized that patients with IPF might also harbor defective EBV. Most human subjects are EBV-positive and contain EBV DNA in their peripheral blood, but almost none harbor EBV DNA in lung tissue (4, 9). The aim of this study was to assess whether the presence of rearranged EBV (WZhet) DNA in either the lung tissue or the peripheral blood was associated with IPF.

METHODS

Patient Selection

Two premises were used in patient selection. First, 90–95% of all human subjects are EBV-positive and harbor EBV DNA in their peripheral blood lymphocytes (4). Second, EBV DNA is almost never detected in the lung tissue of normal subjects (9).

In an initial pilot screen of lung tissue for WZhet-rearranged EBV DNA, 18 archived, surgical lung biopsies from patients with IPF (Patients 1 to 18; Table E1 in the online data supplement) were selected for study on the basis of lung tissue containing EBV DNA. This had been determined in a previous study (9). All patients were independently reassessed and analyzed blind.

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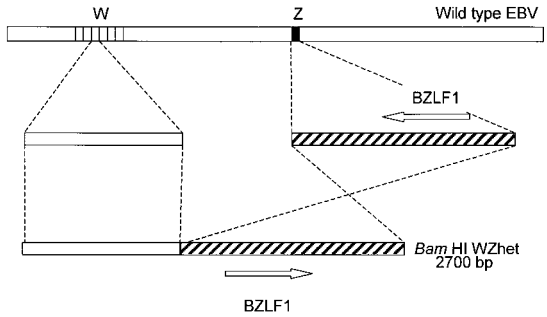

Figure 1. Map of the WZhet-rearranged DNA. The *top line* represents the intact wild type EBV DNA genome. The arrangement and position of the *Bam*HI W and Z restriction fragments within the genome are shown. The *Bam*HI Z fragment contains the BZLF1 open reading frame that encodes the ZEBRA immediate early transcription factor. In the WZhet-rearranged genomes, the orientation of the BamHI Z fragment is reversed with respect to the *Bam*HI W fragment, and the two fragments, normally 52 kbp apart, are juxtaposed (*bottom line*). This results in the abnormal positioning of regulatory elements that lead to the activation of ZEBRA expression and hence reactivation of EBV from latency.

In a subsequent analysis for the presence of WZhet-rearranged EBV DNA in peripheral lymphocytes, blood was taken from 21 patients in whom the diagnosis of IPF was made by high-resolution computer tomography (Patients 19 to 39). In addition, to assess the correlation between WZhet in the lung and peripheral blood, six patients from the aforementioned lung tissue analysis (Patients 13 to 18), who were still attending the North West Lung Centre, were recruited for analysis of peripheral blood. Two control groups were studied. First, randomly selected patients who had undergone lung transplants for various clinical conditions (n = 26). All transplant recipients were on an immunosuppressive regimen of cyclosporin A (5 mg/kg/day) and azathioprine (2 mg/kg/day), altered according to blood tests, and prednisolone (10 mg/kg/day). Second, normal control blood samples were obtained from the Scottish National Blood Transfusion Service (n = 24). These samples were from routine blood donors and were chosen randomly; therefore, no patient details were available. All the tissue blocks and blood samples were coded and sent to a separate laboratory (JPS, SSL, BK) for PCR analysis.

The patient demographics are as follows. Patients with IPF (n = 39) had a mean age of 56 years, and 20 (50%) were male. Lung transplant recipients (n = 26) had a mean age of 49, and 15 (57%) were male. There was no significant difference between these two groups with respect to these parameters (p = 0.03). The profiles of individual patients with respect to age, drug treatment, and EBV status in lung tissue and peripheral blood are shown in Tables E1 and E2 in the online data supplement for patients with IPF and lung transplant patients, respectively.

PCR Analysis

DNA was extracted using QIAmp tissue kits (Qiagen, Crawley, UK) according to the manufacturer's instructions. The extracted DNA (100 ng) was amplified either by nested PCR using oligonucleotide primers specific for all EBV DNA molecules as described by Stewart and colleagues (9) or by PCR–Southern analysis for rearranged WZhet EBV DNA as described by Patton and coworkers (11). In the latter case, PCR products were subjected to Southern blot analysis using oligonucleotide probes end-labeled with digoxigenin and visualized using a combination of alkaline phosphatase–conjugated anti-digoxigenin antibody (Roche) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The probes used were as described by Patton and coworkers (11). The sensitivity of each assay was assessed by the use of serially diluted, cloned template and found to be one copy. In addition, the quality of all DNA was assessed by PCR using primers specific for the P53 gene as described previously (9). All DNA samples in the study were positive in this assay confirming that they were of sufficient quality to be assessed for EBV DNA by PCR.

Statistical Analysis

The proportion of patients in each group with positive results was compared using the χ-square test with Yates' correction for continuity. Group comparisons were used in all cases. The p values are reported. Significance was set at the 5% level.

RESULTS

WZhet DNA Analysis of Lung Tissue from EBV-Positive Patients with IPF

Archival lung tissue sections from patients with IPF that had been used in our previous analysis (9) and that were EBV DNA–positive (n = 18; Patients 1–18, Table E1 in the online data supplement) were selected. They were reanalyzed for the presence of EBV DNA and WZhet DNA by PCR. As before, all were positive for EBV DNA, and 11 of 18 patients (61%) were positive for WZhet DNA.

Analysis of Buffy Coat

DNA was extracted from buffy coat cells from patients with IPF, lung transplant recipients, and normal blood donors and analyzed by PCR for both EBV DNA and WZhet DNA. The results are shown in Tables E1 and E2 in the online data supplement and summarized in Table 1. Twenty-three of 27 patients (85%) with IPF were positive for EBV compared with 21 of 26 (81%) transplant recipients (p = 0.94) and 20 of 24 (83%) normal blood donors (p = 0.83). In contrast, 16 of 27 patients (59%) with IPF were positive for WZhet DNA compared with none of 26 transplant recipients (p = 0.00001) and 1 of 24 (4%) blood donors (p = 0.000078). All of the patients with IPF who were WZhet-positive were also EBV-positive. Likewise, all of the patients who were EBV DNA–negative were also WZhet DNA–negative. A representative example of a PCR assay for WZhet DNA from patients with IPF is shown in Figure 2.

Subgroup Analysis of Data

In buffy coat samples, 16 of 23 (69%) patients with IPF who were positive for EBV DNA were positive for WZhet-rearranged DNA. This compares well with 11 of 18 (61%) patients in the case of lung tissue (p = 0.57). In six cases (Patients 13 to 18; Table E1 in the online data supplement), we were able to directly correlate the presence of EBV DNA with WZhet DNA in lung tissue and in the buffy coat. In these cases, there was a 100% correlation between the presence of EBV DNA and WZhet DNA in both the lungs and buffy coat.

Impact of Immunosuppression on the Presence of WZhet

Twenty-five (64%) patients with IPF had received immunosuppression. Sixteen of 21 (72%) patients with IPF who were WZhet-positive had received immunosuppression, compared

TABLE 1. DETECTION OF EPSTEIN–BARR VIRUS AND WZhet DNA BY POLYMERASE CHAIN REACTION IN BUFFY COAT FROM SUBJECTS WITH IDIOPATHIC PULMONARY FIBROSIS AND CONTROL SUBJECTS

Group	Patients (*n*)	EBV DNA+		WZhet DNA+		EBV+ WZhet+		EBV+ WZhet−		EBV− WZhet+		EBV− WZhet−	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
IPF	27	23	(85)	16	(59)	16	(59)	7	(26)	0	(0)	4	(15)
Transplant	26	21	(81)	0	(0)	0	(0)	21	(81)	0	(0)	5	(19)
BD	24	20	(83)	1	(4)	1	(4)	19	(79)	0	(0)	4	(17)

Definition of abbreviations: BD = blood donor; EBV = Epstein–Barr virus; het = WZhet; IPF = idiopathic pulmonary fibrosis; PCR = polymerase chain reaction.

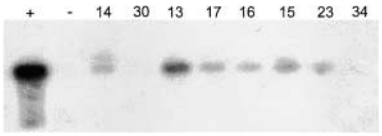

Figure 2. Detection of WZhet in buffy coat cells. DNA extracted from peripheral buffy coat cells was analyzed by PCR with primers specific for the WZhet rearrangement followed by Southern blotting using probes specific for *Bam*HI W and Z fragments. Representative results are shown for eight individual patients with IPF, the *numbers* above the tracks corresponding to the patient numbers described in Table E1 in the online data supplement. Control reactions were performed on DNA extracted from the P3-HR1 cell line that contains WZhet (+) and DNA extracted from the BL41 cell line, which is an EBV-negative Burkitt lymphoma line (−).

with 9 of 18 (50%) patients who were WZhet-negative and had received immunosuppression ($p = 0.08$). All the lung transplant recipients had received immunosuppression, and all were WZhet-negative. None of the blood donors had received immunosuppressive therapy, and 1 of 24 was WZhet-positive.

DISCUSSION

Our previous observations (9) confirmed a significant association between EBV DNA as well as markers of EBV-productive replication in lung tissue and IPF. Lung tissue from normal subjects did not contain EBV. Further study was limited by the absence of a peripheral blood marker of EBV-productive replication given that most people are EBV-positive and have EBV DNA in peripheral blood. WZhet-rearranged EBV is associated with EBV replication and virion production (15–17) and has been detected in other diseases where EBV-productive replication is found (11, 13). The aim of the present study was to ascertain whether the presence of WZhet-rearranged EBV DNA was associated with IPF. We found that the WZhet DNA rearrangement was common in IPF and rare in immunosuppressed lung transplant recipients and normal subjects. We also found that detection of WZhet in peripheral blood buffy coat correlated with tissue-based EBV replication.

In the present study we found that in blood-derived cells most (81–85%) of the IPF patients and control subjects tested were positive for EBV DNA and there was no significant difference in the frequency of detection of viral DNA between the groups. This is as expected because most subjects in the age ranges studied are EBV-positive and carry EBV in peripheral blood B cells (4). However, there was a highly significant association between the presence of WZhet-rearranged DNA in peripheral blood and IPF ($p = 0.00001$). In fact, only 1 of the 50 control samples, from a blood donor, was WZhet-positive. Blood donors by definition are a highly selected group of people with no obvious medical problems. However, we cannot exclude the possibility that in this case the donor had an underlying medical condition.

We analyzed both lung biopsy tissue and peripheral blood–derived buffy coat cells from patients with IPF for WZhet EBV DNA. The frequency of WZhet was not significantly different between the two sets of samples ($p = 0.57$). In addition, in six cases where both samples were available, there was an absolute correlation between the lung biopsy and buffy coat data. These results strongly suggest that the presence of WZhet-rearranged DNA in lung tissue is reflected by its presence in the peripheral blood of the same patients. The presence of WZhet DNA was also always associated with the presence of EBV DNA. Thus, the detection of WZhet DNA in the peripheral blood is a potential surrogate marker for the presence of WZhet DNA and EBV DNA in the lung tissue.

We were concerned about the impact of immunosuppression on our results because the clinical reactivation of other herpes viruses is associated with immunosuppressive therapy. Our previous data detected no significant association between the presence of EBV DNA or EBV antigens in lung tissue and immunosuppression in patients with IPF (9). Likewise, there was no significant association between immunosuppressive therapy and the presence of WZhet in either patients with IPF or lung transplant recipients. Thus, the detection of WZhet in IPF does not appear to be associated with the reactivation of EBV replication because of iatrogenic immunosuppression.

WZhet-rearranged DNA has been seen previously in tissue derived from diseases such as oral hairy leukoplakia (11), thymic carcinoma (13), and Hodgkin's disease (14). However, we also detect WZhet DNA in the peripheral blood. This suggests that WZhet-containing EBV may be generated in lung tissue and spread to the peripheral circulation. Conversely, it is also possible that WZhet-containing EBV is being produced or is multiplying in systemic lymphoid tissue and spreading to the lungs via the peripheral circulation. This is entirely feasible because it has recently been shown that distinct EBV strains can traffic in both directions between the epithelium and the peripheral blood in oral hariy leukoplakia (23). The resolution of this question in IPF would require a longitudinal study of WZhet in blood and pulmonary tissue.

WZhet-rearranged EBV is associated with productive EBV replication. Indeed, the introduction of DNA containing this rearrangement reactivates EBV from latency (19). Thus, WZhet EBV may contribute to the pathogenesis of disease where EBV production is seen. Infection with WZhet-containing EBV or random generation of the mutation within a patient may be responsible for triggering reactivation of EBV. However, the presence of WZhet may simply be a by-product of productive EBV replication.

This study confirms the association between EBV and IPF. More importantly, it describes a highly amenable method of detecting a form of EBV that is more than likely associated with productive viral replication in patients with IPF. The establishment of a causal association between EBV and IPF requires a clinical response to an antiviral agent combined with evidence of a reduction in viral replication. The assay described here provides the means with which to perform such a study.

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