# The Detection of Epstein-Barr Virus DNA in Lung Tissue from Patients with Idiopathic Pulmonary Fibrosis

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**Idiopathic pulmonary fibrosis (IPF) is a clinical syndrome in which the precipitating factors are unclear. An association between Epstein-Barr Virus (EBV) and IPF had previously been suggested using serology and immunohistochemistry. This study sought confirmation of the presence of EBV DNA in the lung tissue of patients with IPF. Lung tissue obtained surgically from 27 patients with IPF and 28 control subjects was investigated for the presence of EBV by immunohistochemistry and polymerase chain reaction (PCR) analysis. Immunohistochemistry used antibodies specific for EBV lytic cycle antigens (gp340/220 and VCA). Nested PCR analysis used oligonucleotide primers specific for EBV and was sensitive to one copy of EBV DNA. Twelve of the 27 patients with IPF (44%) and three of the 28 control subjects (10%) were EBV positive by immunohistochemistry ($p = 0.005$). Thirteen of the patients with IPF (48%) and four of the control subjects (14%) were EBV positive by PCR ($p = 0.007$). Eleven of the patients with IPF (41%) and none of the control subjects were EBV positive by both immunohistochemistry and PCR ($p = < 0.001$). These data further suggest an association between EBV and IPF. In addition it defines a novel method for detecting EBV in lung tissue. EBV may be involved in the pathogenesis of the disease; however, further studies are required to establish a causal relationship.** Stewart JP, Egan JJ, Ross AJ, Kelly BG, Lok SS, Hasleton PS, Woodcock AA. The detection of Epstein-Barr virus DNA in lung tissue from patients with idiopathic pulmonary fibrosis.

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Idiopathic pulmonary fibrosis (IPF) is a clinical syndrome characterized by clubbing, bibasal late inspiratory crackles, and reticular shadowing on chest radiographs. The causes of IPF are unclear. Environmental and infective factors have been implicated in the disease process; however, there is no clear causal association (1–3). The Epstein-Barr virus (EBV) is a ubiquitous Herpesvirus that has been associated with a number of diseases. These include B-cell lymphoproliferative disease, Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma. Although normally associated with infection of the upper respiratory tract and B-lymphocytes, EBV both infects and replicates in the lower respiratory tract (4, 5). An association, based on serology between EBV infection and IPF, was made some years ago (6). Vergnon and colleagues (6) identified elevated immunoglobulin A (IgA) levels against viral capsid antigen (VCA) in a study of 12 patients with IPF. In a recent report (7), we demonstrated productive EBV replication in type II alveolar epithelial cells in IPF by performing immunohistochemistry (IHC). Fourteen of 20 patients with biopsy-proven IPF had evidence of EBV lytic cycle antigen expression (VCA and gp340/220) in type II alveolar cells in comparison with two of 21 control subjects. However, it was felt that confirmatory evidence of the existence of EBV DNA within lung tissue was required. To test this hypothesis we have developed a sensitive EBV-specific nested PCR assay. This study examined lung tissue from patients with IPF and a group of control subjects by IHC and PCR analysis to determine the prevalence of EBV DNA and antigen expression in both groups.

## METHODS

### Patient Selection

Twenty-seven archived, surgical lung biopsies were randomly selected for study. Lung tissue was fixed in buffered formalin. In each case, 5-μM sections stained with hematoxylin-eosin were reviewed blind by the pulmonary histopathologist (PSH) in order to confirm a variegate pattern of fibrosis consistent with usual interstitial pneumonia (UIP) and to exclude nonspecific interstitial pneumonia (NSIP) and desquamative interstitial pneumonia. Corresponding clinical data were collected and assessed blind (SSL/JJE). Patients were designated as having IPF based on lung histology and a typical radiologic pattern on high resolution computer tomography (HRCT). On histology, a diagnosis of IPF was made if the biopsy met the following criteria. (*1*) A variegate

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picture of interstitial fibrosis, inflammation, and normal tissue. (*2*) Tendency for fibrosis to subpleural and peripheral distribution. (*3*) Exclusion of features indicating other pathology, e.g., asbestos bodies and granulomas. HCRT was performed using a picker PQ scanner at 10-mm intervals with 1.5-mm sections using the long bone algorithm. Criteria for diagnosing IPF were as follows. (*1*) Symmetrical bilateral interstitial infiltrate. (*2*) Pulmonary infiltrate affecting the bases of the lung and distributed subpleurally. (*3*) Presence of honeycombing of the lung with a subpleural distribution. (*4*) A predominant picture of fibrosis. Patients who had undergone pneumonectomies or lung biopsies for other reasons were randomly selected as control subjects (n = 28). All the tissue blocks were coded and sent to a separate laboratory (J.P.S., A.R., B.K.) for EBV studies.

Eleven of the 27 patients with IPF and 16 of the 28 control subjects had been used in our previous study (7). These were as follows. Patients with IPF: Nos. 1–4, 6, 7, 11–13, 17, 18, and 20 (*see* Table 2). Control patients: Nos. 1–5, 7–12, and 14–18. All patients irrespective of previous use were independently reassessed and analyzed blind.

### Patient Characteristics

The IPF group consisted of 27 patients (19 male) with a mean age of 57 yr (range, 26 to 75) (Table 1). The control group consisted of 28 patients (18 male) with a mean age of 54 (range, 21 to 74) (Table 1). The final diagnosis in the control group included: non-small-cell carcinoma (n = 9), sarcoidosis (n = 9), normal (n = 5), chemotherapy-induced fibrosis (n = 2), bronchiectasis after tuberculosis (n = 1), cystic fibrosis (n = 1), extrinsic allergic alveolitis (n = 1). Twenty-one of 27 (77%) patients with IPF and 17 of 28 (61%) control subjects were ex-smokers. Sixteen patients with IPF had received immunosuppressive therapy prior to biopsy (Table 2).

### Immunohistochemistry

Sections 5 μm thick were cut from formalin-fixed, paraffin-embedded blocks prepared from lung tissue. Sections were first incubated overnight at 4° C in a solution containing 20% (wt/vol) bovine serum albumin to block nonspecific binding of antibodies. Staining for EBV lytic cycle antigens and cellular antigens was then performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and using diaminobenzidine (Sigma Fast; Sigma, St Louis, MO) as substrate. Primary antibodies used were all mouse monoclonal antibodies and included: anti-EBV gp340/220 (clone 72A1), anti-EBV VCA (Mab 817; Chemicon International, Temecula, CA), antihuman cytokeratin (MNF116; Dakopatts, Copenhagen, Denmark) and antisheep MHC Class II antigen (clone VPM36). After immunostaining, sections were counterstained with hematoxylin. The specificities of both of the anti-EBV antibodies were ascertained by reaction against the EBV-positive B-cell line B95-8 and the EBV-negative B-cell line BL41. Both reagents were found to be entirely specific in that they reacted positively with B95-8 and failed to react with BL41. Antisheep MHC Class II is known not to bind to human antigens and was included as a negative control. Samples were randomized and analyzed blind, and the results decoded later.

### PCR Analysis

DNA was extracted from sections 5-μm thick that were serially adjacent to those analyzed by IHC using Qiamp tissue kits (Qiagen, Hilden, Germany). Control DNA was extracted from the B-lymphocyte cell lines AM (8) (EBV-positive) and BL41 (9) (EBV-negative).

**TABLE 1**

**PATIENT DEMOGRAPHICS**

| | Patients with IPF | Control Subjects | p Value |
|---|---|---|---|
| Patients, n | 27 | 28 | |
| Age, yr | 7 | 54 | 0.3 |
| Male, n/% | 19/70 | 18/64 | 0.4 |
| Smokers, n/% | 27/77 | 17/61 | 0.2 |

*Definition of abbreviations*: IPF = idiopathic pulmonary fibrosis.

Pilot studies were performed to analyze the ability of extracted DNA to be amplified by PCR using primers specific for the human p53 gene (10). This control PCR yielded a product of 180bp. Amplifiable DNA was best obtained from formalin-fixed lung tissue if proteinase K digestion of the sample was extended from 12 h to 5 d. Amplifiable DNA (100 ng) was subjected to nested PCR analysis using oligonucleotide primers specific for EBV and recombinant *Taq* DNA polymerase (GIBCO/BRL, Paisley, Scotland, U.K.). The first round of PCR used the primers 5′-GGTCCCGTAGTGACAACTATGCTG and 5′-GAGTGCACCACAGCCAACTCCATG at an annealing temperature of 60° C for 40 cycles as described by Lees and colleagues (11). The second (nested) round of PCR used the primers 5′-GGCTTTGGGTTCCATTGTGTGC and 5′-TGTACAGAACCAAAGAGGTGGC at an annealing temperature of 60° C for 25 cycles. PCR products were electrophoresed through 2% agarose gels and DNA visualized by ethidium bromide staining. Molecular weight determinations were made using the 1Kb ladder (GIBCO/BRL). Positive signal for EBV yielded a 284bp product. As for the PCR assay for the P53 gene (above), specificity of the EBV PCR analysis was determined using DNA extracted from EBV-positive (AM) and negative cell lines (BL41). It can be seen in Figure 2 that a positive PCR signal was obtained from EBV-positive cells and no PCR signal was obtained from EBV-negative cells. Thus, the PCR assay was entirely specific for EBV DNA. Limiting dilution of a known amount of cloned target DNA showed that the PCR was sensitive to one copy of EBV DNA. Sections were randomized and analyzed by PCR blind, and the results were decoded afterwards.

### Statistical Analysis

The proportions of patients in each group with positive results were compared using the chi-square test. The probability (p) value is quoted. Significance was set at the 5% level.

## RESULTS

### IHC Analysis

Lung tissue sections that were positive for EBV by IHC showed positive staining for both gp340/220 and VCA. Conversely, samples negative by IHC showed no staining for either antigen. IHC staining appeared in small, random patches throughout the sections and was present in bronchiolar and alveolar epithelial cells. A representative example of IHC staining is shown in Figure 1. EBV gp340/220 antigen (*panel A*) was present in cells bordering airways. *Panel B* is an adjacent serial section and shows that the cells expressing EBV gp340/220 were cytokeratin-positive and were therefore epithelial cells. No staining was seen with antisheep MHC class II (*panel C*) demonstrating the specificity of the positive antibody reactions. Of the patients with IPF, 12 of 27 had EBV-specific staining by IHC compared with three of 28 in the control group (p = 0.005).

### PCR Analysis

A representative gel with the products of PCR amplifications from both IPF and control groups are shown in Figure 2. To check the quality of extracted DNA, an aliquot was first amplified by PCR for a control human gene (P53). P53 sequences were amplified from all samples, indicating that the extracted DNA was of sufficient quality for analysis by PCR. A second aliquot of DNA was then analyzed by PCR for the presence of EBV DNA. This showed that 14 of 27 patients with IPF were positive compared with four of 28 control subjects (p = 0.007).

### Comparison of IHC and PCR Assays

The results of both analyses are collated and compared in Tables 2, 3, and 4. In the majority, 46 of 55 cases (81%) the PCR assays confirmed the result of the IHC analysis. In a small percentage of cases the IHC and PCR results were not concordant. Samples that were positive by both PCR and IHC were

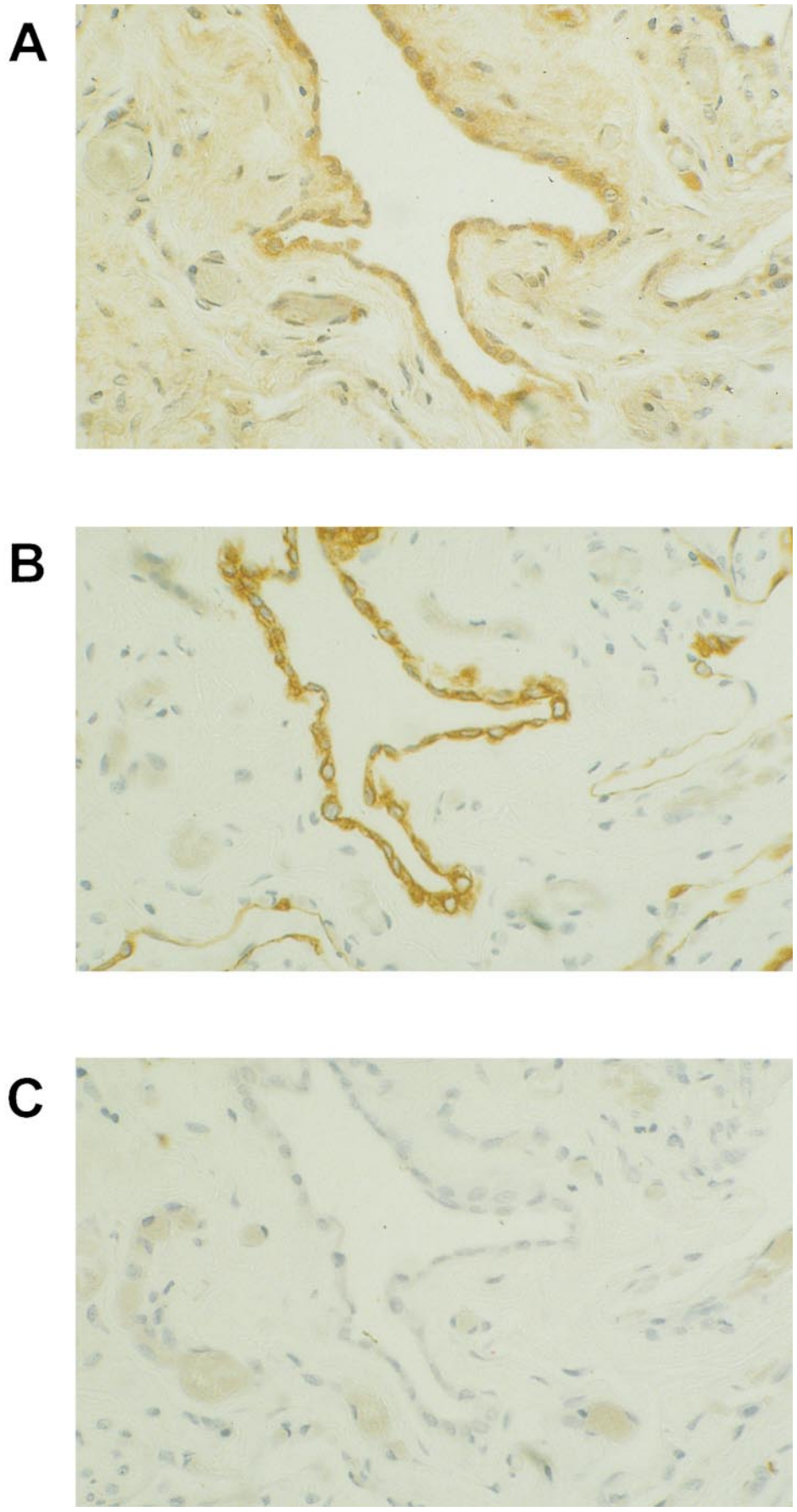


***Figure 1.*** IHC analysis of lung section. Adjacent serial sections were stained individually for (*A*) EBV gp340/220, (*B*) cytokeratin, and (*C*) sheep MHC Class II.

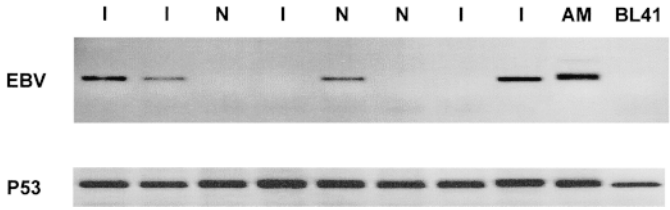


***Figure 2.*** PCR analysis of lung sections. DNA was amplified by PCR using primers specific for either the human P53 gene (as a positive control) or EBV. Products were electrophoresed through 2% agarose gels and visualized using a UV transilluminator. Images are shown with the colors reversed for clarity. DNA was amplified from biopsies from patients with IPF (labeled I), control subjects (labeled N), or DNA derived from the B-lymphocyte lines AM (EBV-positive) and BL41 (EBV-negative).

identified as "confirmed positives." Eleven of the 27 patients with IPF were identified as EBV "confirmed positive" as compared with none of the 28 control subjects ($p < 0.001$). Three patients with IPF were EBV PCR positive in isolation (IHC negative) and four of the 28 control subjects.

### Impact of Immunosuppression

Analysis of the results in relation to treatment received by patients is shown in Table 5. 57% of patients received immunosuppression. Eight of 14 (57%) patients who were EBV positive had received immunosuppression, whereas eight of 13 (61%) patients who were EBV negative had received immunosuppression ($p = 0.81$). Of 15 patients with IPF receiving immunosuppression, six were EBV "confirmed positive" and nine were EBV negative.

## DISCUSSION

The presence of IgA antibodies to EBV VCA has been correlated with a diagnosis of IPF (6). This marker is an indicator of active EBV replication at an epithelial surface. Our previous (7) and current finding of active EBV replication in the lung therefore corroborates the IgA serology, and this study confirms previous immunohistochemical observations (7). EBV antigens and DNA in the lower respiratory tract were significantly associated with a diagnosis of IPF.

The PCR assay confirmed IHC staining positive in 45 of 55 cases (81%). With any assay there is a finite number of false positive and negative results. False negative results could have stemmed from extended fixation times or prolonged storage. Fixation of tissue in formalin is detrimental to the quality of both antigen and DNA. An increased fixation time causes excessive crosslinking of protein, a consequent loss of antigenicity, and hence loss of detection by IHC staining. Increased fixation of tissues and storage of blocks also causes greater degradation of DNA, causing a decrease in the ability to detect target sequences by PCR amplification (12). All blocks studied were archived and were of variable (up to 8 yr) age. Variable fixation time and fixatives between tissue blocks may therefore have been factors underlying the discrepancies between samples.

Adjacent serial sections of tissue were used for the IHC and PCR assays. The pattern of EBV-specific IHC staining within the lungs suggests a localization of EBV into small, randomly distributed foci (5, 7). Discrepancies between IHC and

**TABLE 2**

**PROFILES OF PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS**

| Patient No. | Age (*yr*) | Smoking Status | Drug Treatment | IHC for EBV gp340/220 | IHC for EBV VCA | PCR for EBV |
|---|---|---|---|---|---|---|
| 1 | 62 | NS | – | + | + | + |
| 2 | 62 | ES | – | + | + | + |
| 3 | 59 | ES | CycloP, Pred | + | + | + |
| 4 | 60 | ES | Az, Pred | + | + | + |
| 5 | 68 | S | – | + | + | + |
| 6 | 69 | ES | – | + | + | + |
| 7 | 61 | NS | Pred | + | + | + |
| 8 | 48 | ES | CyA, Pred | + | + | + |
| 9 | 60 | S | CycloP, Pred | + | + | + |
| 10 | 56 | ES | CyA, Pred | + | + | + |
| 11 | 61 | S | – | + | + | + |
| 12 | 47 | ES | – | – | – | + |
| 13 | 38 | NS | – | – | – | + |
| 14 | 56 | ES | CyA | + | + | – |
| 15 | 50 | S | Pred | – | – | – |
| 16 | 67 | ES | Az, Pred | – | – | – |
| 17 | 53 | NS | – | – | – | – |
| 18 | 61 | ES | Pred | – | – | – |
| 19 | 59 | ES | – | – | – | – |
| 20 | 64 | ES | CycloP, Pred | – | – | – |
| 21 | 57 | S | CycloP, Pred | – | – | – |
| 22 | 41 | NS | CyA, Pred | – | – | – |
| 23 | 53 | S | CycloP | – | – | – |
| 24 | 53 | NS | – | – | – | – |
| 25 | 75 | NS | – | – | – | – |
| 26 | 65 | S | CycloP, Pred | – | – | – |
| 27 | 26 | NS | – | – | – | – |

*Definition of abbreviations*: Az = azathioprine; CyA = cyclosporin A; CycloP = cyclophosphamide; EBV = Epstein-Barr virus; ES= ex-smoker; IHC = immunohistochemistry; NS = nonsmoker; PCR = polymerase chain reaction; Pred = prednisolone; S = smoker; VCA = viral capsid antigen.

**TABLE 3**

**PROFILES OF CONTROL PATIENTS**

| Patient No. | Age (*yr*) | Diagnosis | Smoking Status | IHC for EBV gp340/220 | IHC for EBV VCA | PCR for EBV |
|---|---|---|---|---|---|---|
| 1 | 47 | Normal | NS | + | + | – |
| 2 | 55 | Normal | ES | + | + | – |
| 3 | 71 | NSCLC | S | + | + | – |
| 4 | 74 | NSCLC | ES | – | – | + |
| 5 | 70 | Fibrosis | ES | – | – | – |
| 6 | 66 | EEA | NS | – | – | – |
| 7 | 71 | NSCLC | ES | – | – | – |
| 8 | 56 | NSCLC | S | – | – | – |
| 9 | 31 | CF | NS | – | – | – |
| 10 | 61 | NSCLC | ES | – | – | – |
| 11 | 69 | NSCLC | S | – | – | – |
| 12 | 49 | NSCLC | S | – | – | – |
| 13 | 57 | NSCLC | S | – | – | – |
| 14 | 61 | NSCLC | ES | – | – | – |
| 15 | 48 | Bronchiectasis | ES | – | – | – |
| 16 | 70 | Normal | S | – | – | – |
| 17 | 59 | Normal | ES | – | – | – |
| 18 | 49 | Normal | NS | – | – | – |
| 19 | 63 | Sarcoidosis | NS | – | – | + |
| 20 | 55 | Sarcoidosis | NS | – | – | + |
| 21 | 39 | Sarcoidosis | S | – | – | – |
| 22 | 43 | Sarcoidosis | NS | – | – | – |
| 23 | 30 | Sarcoidosis | NS | – | – | – |
| 24 | 64 | Sarcoidosis | ES | – | – | – |
| 25 | 35 | Sarcoidosis | S | – | – | – |
| 26 | 42 | Sarcoidosis | NS | – | – | – |
| 27 | 21 | Sarcoidosis | S | – | – | – |
| 28 | 64 | Fibrosis | NS | – | – | – |

*Definition of abbreviations*: CF = cystic fibrosis; ES = ex-smoker; Fibrosis = radiation induced fibrosis; Normal = no abnormal diagnosis; NS = nonsmoker; NSCLC = non-small-cell lung cancer; S = Smoker. For other definitions, *see* Table 2.

**TABLE 4**

**EPSTEIN-BARR VIRUS IMMUNOHISTOCHEMICAL AND PCR RESULTS**

| Group | Patients (*n*) | IHC +ve (*n*) | IHC +ve (%) | PCR +ve (*n*) | PCR +ve (%) | IHC and PCR +ve (*n*) | IHC and PCR +ve (%) | IHC +ve PCR −ve (*n*) | IHC +ve PCR −ve (%) | IHC −ve PCR +ve (*n*) | IHC −ve PCR +ve (%) | IHC −ve PCR −ve (*n*) | IHC −ve PCR −ve (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IPF | 27 | 12 | 44 | 13 | 48 | 11 | 41 | 1 | 3 | 2 | 7 | 14 | 51 |
| Control | 28 | 3 | 10 | 4 | 14 | 0 | 0 | 3 | 10 | 4 | 14 | 21 | 75 |

**TABLE 5**

**TREATMENT RECEIVED BY IPF PATIENTS AT THE TIME OF THE SURGICAL PROCEDURE**

| Drugs | Immunohistochemistry | PCR |
|---|---|---|
| Pred | + | + |
| Pred | + | + |
| CyA, Pred | + | + |
| CyA, Pred | + | + |
| Cycloph, Pred | + | + |
| Cycloph, Pred | + | + |
| Az, Pred | + | + |
| CyA | + | − |
| Pred | − | − |
| Pred | − | − |
| CyA, Pred | − | − |
| Cycloph | − | − |
| Cycloph, Pred | − | − |
| Cycloph, Pred | − | − |
| Cycloph, Pred | − | − |
| Az, Pred | − | − |

For definition of abbreviations, *see* Table 2.

PCR assays could therefore also be due to cells containing EBV in one section being absent in the adjacent serial section or vice versa.

The IHC staining and PCR assays used are both EBV-specific. However, each assay measures a distinct biologic facet of EBV infection. EBV exists in either a latent or productively replicating form. In latency there is a restricted pattern of virus gene expression. During productive replication some 60 proteins are produced. The monoclonal antibodies used here were specific for productive cycle proteins and would therefore not detect cells latently infected by EBV. In contrast, the PCR analysis is able to detect latent and productive EBV. Biopsies that were EBV positive by PCR analysis but negative by IHC could therefore have contained latent EBV.

B-lymphocytes that contain latent EBV are known to traffic in the blood of all infected persons at a frequency of 1 in $2 \times 10^5$ to 1 in $10^7$ mononuclear cells (13). Because EBV has a seroprevalence of 95% in the age range studied, each group studied might be expected to contain a low number of samples that are PCR positive (14). The precise serologic status of the patients was not available. Such latently infected B-lymphocytes should be negative by IHC. This pattern was identified in six biopsies studied that were positive by PCR but negative by IHC (Table 4). These samples may therefore represent the background of latent EBV normally present in the lung tissue of infected persons.

Two recent reports have studied EBV in IPF, one confirming and one conflicting with our findings (15, 16). Wangoo and colleagues (16) were unable to demonstrate specificity of monoclonal antibodies for EBV in lung biopsies. This lack of specificity was not observed either by ourselves in our present or former study or by others (7, 15). The reasons underlying the differing results obtained by Wangoo and colleagues are therefore likely to be technical and related to the reagents used in their study.

The inability of Wangoo and colleagues to identify any EBV DNA in lung tissue is perhaps more surprising. Even if EBV were not associated with IPF one would expect to find a low-level background of EBV DNA in lung tissue simply because of the blood volume in this organ. Controls from a study of Human Herpesvirus 8 (HHV-8) in sarcoidosis had a high prevalence of EBV DNA (17). Using PCR described by Wangoo and colleagues (16) one would have expected to identify EBV DNA in some cases. This difference may be rationalized on the basis of difficulty in extraction of DNA from paraffin blocks. In our work, we optimized the DNA extraction procedure from lung tissue and included a positive control PCR for a single-copy human gene (P53). This ensured that each extracted DNA sample was capable of being amplified. Both this study and that of Quddus and colleagues (15), who included an internal amplification control, were able to detect EBV sequences in DNA extracted from lung biopsies obtained from patients with IPF. Wangoo and colleagues did not include this essential control. It is therefore difficult to assess whether the inability of Wangoo and colleagues to detect EBV DNA was due to genuine absence of EBV sequences or to the lack of amplifiable DNA.

Appraisal of patient selection is important in evaluating our results. In the IPF group, no cases of DIP or NSIP were identified by histologic review. This may be explained by the fact that cases of steroid responsive disease (DIP and NSIP) are unlikely to be referred to a tertiary center in the United Kingdom. The control groups predominantly constituted pneumonectomy lung tissue remote from a tumor and sarcoidosis. Our data confirm that of Di Alberti and colleagues (17) who demonstrated that EBV DNA is not detectable in patients with sarcoidosis. However, the rate of EBV PCR positivity was lower in the remainder of our control subjects than in this study. An underestimation of the EBV identified in the control group is unlikely to have occurred as P53 positive controls for DNA extraction was complete for each subject.

Recurrent EBV disease may follow immunosuppressive therapy (14). Some patients in this study had received immunosuppression that might have influenced the frequency of EBV detection. Analysis of the data showed that there was no difference in the frequency of EBV detection between patients who received and those who did not receive treatment. However this does not preclude the importance of immunosuppression as a factor in determining the detection of EBV.

Samples positive by only one assay may be the result of false positive or false negative reactions. Therefore, absolute positives were considered to be both PCR and IHC positive. The dual assay detection of EBV gave an absolute and statistically significant discrimination between patients with IPF and control subjects. Eleven of 27 (41%) patients with IPF were EBV positive in lung tissue by both assays. It seems likely that this is a true representation of the rate of "IPF associated EBV." These data suggest that there may be at least two distinct groups of patients with IPF, those EBV-associated and with the other showing no association with EBV. EBV as cofactor in disease progression may explain why immunosuppression therapy alone has uniformly poor results in patients with IPF. Recent preliminary data suggest that EBV is associated with disease progression (18). Further study is required to determine whether EBV is an important cofactor in disease progression.

This study describes an improved method for identifying EBV DNA in lung tissue and confirms an association between EBV and IPF. However, this does not prove a causal relationship between IPF and EBV. To establish whether Herpesviruses are cofactors in patients with IPF, many of whom receive immunosuppression, demands the demonstration of a clinical response to an antiviral agent. The presence of potentially pathogenic viruses in the lung tissue of patients with IPF may explain why immunosuppressive therapy has uniformly poor results in IPF.

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