Original articles

Epstein-Barr virus replication within pulmonary epithelial cells in cryptogenic fibrosing alveolitis

Jim J Egan, James P Stewart, Philip S Hasleton, John R Arrand, Kevin B Carroll, Ashley A Woodcock

Abstract

***Background* – Cryptogenic fibrosing alveolitis (synonymous with idiopathic pulmonary fibrosis) is a clinically heterogeneous condition in which the precipitating factor is unclear. Both environmental and infective factors have been implicated. An association between Epstein-Barr virus (EBV) and cryptogenic fibrosing alveolitis was suggested over a decade ago by a study based on EBV serology, but the significance of this has been unclear.**

***Methods* – Lung tissue obtained surgically from patients (n = 20) with cryptogenic fibrosing alveolitis was investigated for evidence of EBV replication and compared with lung tissue from 21 control patients. Fourteen of the 20 patients had received no specific therapy for cryptogenic fibrosing alveolitis at the time of biopsy. Monoclonal antibodies directed against the EBV viral antigens, EBV viral capsid antigen (VCA) and gp 340/220 antigen, which are expressed during the lytic phase of the EBV life cycle, were studied.**

***Results* – Fourteen (70%) of the 20 patients with cryptogenic fibrosing alveolitis were positive for both EBV VCA and gp 340/220 compared with two (9%) of the 21 controls. In the patients with cryptogenic fibrosing alveolitis viral replication was localised to pulmonary epithelial cells using epithelial cell markers, and immunohistochemical analysis confirmed the staining to be within type II alveolar cells.**

***Conclusions* – This is the first report of in vivo EBV replication within epithelial cells of the lower respiratory tract in an immunocompetent human host. Furthermore, this suggests that EBV may be an immune trigger or contribute to lung injury in cryptogenic fibrosing alveolitis, thus offering a potential new avenue of treatment.**

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Keywords: Epstein-Barr virus, idiopathic pulmonary fibrosis, cryptogenic fibrosing alveolitis, pulmonary epithelial cells.

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Cryptogenic fibrosing alveolitis is a restrictive lung disease characterised by clubbing, basal late inspiratory crackles, and reticular shadowing on the chest radiograph. The disease is usually progressive, but the rate of progression and the response to treatment with steroids and immunosuppressive drugs are both variable with a mean life expectancy from diagnosis of 4–5 years.[1] The aetiology is unclear, with both environmental and infective factors being implicated, but causal relationships have not been established.[2 3] Little is known about the behaviour of the Epstein-Barr virus (EBV) in the lower respiratory tract, and controversy exists as to whether EBV persists within epithelial cells or B lymphocytes.[4] A previous study has suggested that the lower respiratory tract provides a reservoir for EBV.[5] Furthermore, an association between EBV and cryptogenic fibrosing alveolitis was suggested over a decade ago by a study based on serological tests on EBV,[3] but the significance of this has been unclear because of the high frequency of seropositivity in an ageing population. To investigate further a possible relationship between EBV and cryptogenic fibrosing alveolitis we have studied the lung tissue of patients with cryptogenic fibrosing alveolitis using monoclonal antibodies directed against lytic phase (replicating) EBV antigens, viral capsid antigen (VCA) and the membrane antigen gp 340/220.

Methods

PATIENT SELECTION

Surgically obtained lung tissue, fixed in buffered formalin, from cases labelled as "pulmonary fibrosis" (n = 22) and "pneumonectomy" or "open lung biopsy specimens" (n = 22) were obtained for study. In each case 5 μm haemotoxylin and eosin sections were reviewed blind by the pulmonary histopathologist (PSH) without knowledge of the clinical or radiological findings. The corresponding clinical data were collected and assessed blind (KBC). Occupational histories were independently assessed (Professor C A C Pickering). Only patients with a typical histological and clinical picture were designated cryptogenic fibrosing alveolitis (n = 20). Patients who had undergone pneumonectomies were used as controls (n = 21). Two patients from the cryptogenic fibrosing alveolitis group (one with histological extrinsic allergic alveolitis and one with histological pneumonoconiosis) and one patient from the control group (pul-

Table 1 Epstein-Barr virus (EBV) and histological profiles of patients with cryptogenic fibrosing alveolitis

Patient no.	*Fibrosis (0–8)*	*Inflammation (0–3)*	*Macrophage score (1–2)*	*Cuboidal epithelium*	*EBV gp 340/220*	*EBV VCA*
1	7	2	2	Diffuse	+	+
2	7	2	1	Focal	+	+
3	8	1	1	Diffuse	–	–
4	7	1	1	Diffuse	–	–
5	5	1	1	Focal	+	+
6	7	3	1	Focal	++	++
7	5	1	1	Focal	–	–
8	7	3	1	Diffuse	+	++
9	7	2	1	Focal	+	+
10	5	1	2	Focal	+	+
11	7	1	2	Focal	++	++
12	7	1	2	Diffuse	++	++
13	4	3	1	Focal	–	–
14	7	2	1	Diffuse	–	–
15*	8	2	1	Diffuse	++	++
16*	7	1	1	Focal	+	+
17*	7	1	2	Focal	+	+
18*	7	1	1	Diffuse	–	–
19*	7	2	1	Diffuse	++	++
20*	7	1	1	Focal	+	+

* Patients who received immunosuppression (see table 2).
VCA = viral capsid antigen; + = staining restricted to 1–2 patches within the section; ++ = multiple patches of intense staining throughout the section.

monary fibrosis considered to be related to asbestos) were excluded. The tissue blocks were coded and sent to a separate laboratory (JPS, JRA) for EBV studies.

PATIENTS

The cryptogenic fibrosing alveolitis group consisted of 20 patients (17 men), mean age 58 years (range 39–69). The control group contained 21 patients (14 men), mean age 61 years (range 32–75). The final diagnosis in the control patients included: non-small cell carcinoma (n = 11), adenocarcinoma (n = 3), cystic fibrosis (n = 1), testicular carcinoma (n = 1), non-Hodgkin's lymphoma chemotherapy-induced pulmonary fibrosis (n = 1), bronchiectasis following tuberculosis (n = 1), cystic bronchiectasis (n = 1), emphysema (n = 1), and sarcoidosis (n = 1). No patient was diagnosed as having extrinsic allergic alveolitis, lymphangioleiomatosis, systemic sclerosis, or a collagen disorder. Occupational histories and details of current and previous immunosuppressive therapy were obtained from the case notes and by telephone. Fifteen of the 20 patients with cryptogenic fibrosing alveolitis and 16 of the 21 control patients were ex-smokers.

HISTOPATHOLOGY

In control subjects where resections had been carried out for malignancy, lung blocks some distance from the tumour were examined. For each patient in both groups the numbers of slides examined were recorded. The degree of fibrosis was scored histologically on a scale of 0–8; inflammation and the extent of macrophage proliferation were scored on a scale of 0–3.[6] The presence of cuboidalised epithelium was identified and noted as diffuse or focal. Asbestos bodies were sought by examination of the histological sections using a ×10 objective and ×10 eyepiece. Only typical asbestos bodies were accepted and their presence as either single asbestos bodies or aggregates was noted. The presence or absence of a vasculitis, with or without neutrophil infiltration of the vascular wall or fibrinoid necrosis, was also noted as was smooth muscle proliferation and hyaline membranes in the alveoli.

EBV IMMUNOHISTOCHEMISTRY

Expression of viral and cellular markers within the tissue blocks was analysed by immunofluorescence using a streptavidin-biotin-FITC amplification step as previously described.[7] In order to identify which epithelial cell type was expressing EBV antigens, cases positive for EBV immunofluorescent staining were further examined by (a) an alkaline phosphatase monoclonal anti-alkaline phosphatase (APAAP) technique,[8] or (b) an immunoperoxidase method using Vectorstain Elite ABC kit (Vector Laboratories) using diaminobenzadine as a substrate. These sections were counterstained with haematoxylin. Primary antisera were all mouse monoclonal reagents and included: anti-EBV membrane antigen gp 340/220[9]; anti-EBV VCA (MAB 817; Chemicon International Temecula, California, USA); anti-cytokeratin (MNF116; Dakopatts, Copenhagen, Denmark), anti-epithelial membrane antigen EMA (M613; Dakopatts); anti-CD20, pan B cell (M755; Dakopatts). Purified anti-gp 340/220 was used at a concentration of 10 μg/ml and anti-VCA was used at a 1:400 dilution. All EBV specific reagents were tested against EBV positive and EBV negative B cell lines and were found to be entirely specific. In addition, isotype-matched control monoclonal antisera did not react with tissue sections. Sections

Table 2 Treatment received by patients with cryptogenic fibrosing alveolitis at the time of the surgical procedure

Patient no.	*Drugs*	*Duration (months)*	*EBV gp 340/220*	*EBV VCA*
15	Az, Pred	7	++	++
16	Csp, Pred	10	+	+
17	Cycloph, Pred	13	+	+
18	Csp, Pred	19	–	–
19	Az, Csp, Pred	28	++	++
20	Pred	Unknown	+	+

Az = azathioprine; Csp = cyclosporin; Pred = prednisolone; Cycloph = cyclophosphamide.

Table 3 Epstein-Barr virus (EBV) and histological profiles of control patients

Patient no.	*Fibrosis (0–8)*	*Inflammation (0–3)*	*Macrophage score (0–3)*	*Histology*	*EBV gp 340/220*	*EBV VCA*	*Primary disease*
21	2	0	1	Emphysema	+	+	Adenocarcinoma
22	1	0	1	Bronchiectasis	–	–	Bronchiectasis
23	1	0	1	Normal	–	–	NSC
24	1	1	2	Normal	–	–	NSC
25	1	0	1	Normal	–	–	Secondary testicular carcinoma
26	2	0	1	Emphysema	–	–	NSC
27	3	3	1	Bronchiectasis	–	–	Cystic fibrosis
28	2	1	1	Emphysema	–	–	NSC
29	0	0	1	Normal	–	–	NSC
30	1	0	1	Normal	+	+	NSC
31	1	0	1	Normal	–	–	NSC
32	3	1	1	Normal	–	–	NSC
33	3	0	1	Emphysema	–	–	NSC
34	1	1	1	Emphysema	–	–	NSC
35	1	0	1	Normal	–	–	Adenocarcinoma
36	2	0	1	Emphysema	–	–	NSC
37	2	0	1	Normal	–	–	Adenocarcinoma
38	2	1	1	Emphysema	–	–	Emphysema
39	7	1	1	Fibrosis	–	–	Chemotherapy-induced fibrosis
40	3	0	1	Fibrosis	–	–	Sarcoidosis
41	7	2	2	Aspiration pneumonitis	–	–	Previous tuberculosis

VCA = viral capsid antigen; NSC = non-small cell carcinoma.

positive for gp 340/200 and VCA were scored as follows: "+" = staining restricted to one or two patches within the section or "++" = multiple patches of intense staining throughout the biopsy specimen.

DATA ANALYSIS

The proportion of patients in each group with positive staining for EBV was compared using Fisher's exact test. The two tailed probability (by summation) p value is quoted. Significance was set at the 5% level.

Results

All the patients in the cryptogenic fibrosing alveolitis group had typical histological features of cryptogenic fibrosing alveolitis (table 1). Two patients (nos 10 and 11) were ex-coal miners but neither had histological evidence of pneumoconiosis; two (nos 12 and 13) gave an occupational history of brief asbestos exposure which was not deemed to be clinically important and in neither patient was there concomitant radiological (plaques) or histological (asbestos bodies, pleural fibrosis) evidence of asbestos-related disease; one other patient (no. 14) had significant occupational asbestos exposure but as no asbestos bodies were identified histologically he was characterised as having cryptogenic fibrosing alveolitis. Fourteen of the 20 patients with cryptogenic fibrosing alveolitis had never received immunosuppression before the acquisition of lung tissue. The remaining six patients had received a combination of either prednisolone, azathioprine, cyclophosphamide, and cyclosporin (table 2). Asbestos bodies were not identified in any biopsy specimen.

In the control group, of the 14 cases whose tissue blocks were taken from a malignancy, the histological examination was completely normal in eight patients and showed a minor degree of emphysema in six (table 3). Two control patients had pulmonary fibrosis, attributable to chemotherapy (no. 39) and previous tuberculosis (no. 41).

In all subjects whose lung tissue was positive for EBV staining both antigens (gp 340/220 and VCA) were identified, and in all negative patients neither antigen was detected. In the cryptogenic fibrosing alveolitis group 14 of 20 patients had positive staining for EBV lytic phase antigens (table 1, fig 1) compared with two of 21 patients in the control group (table 2; $p = 0{\cdot}0001$). Nine of the 14 cryptogenic fibrosing alveolitis patients who had received no immunosuppressive treatment were positive for EBV ($p < 0{\cdot}005$ compared with controls). Of the six previously treated cryptogenic fibrosing alveolitis patients five were positive for EBV ($p = 0{\cdot}001$ compared with controls). There was no significant difference in the proportion of EBV positive cryptogenic fibrosing alveolitis patients who had (five of six) or had not (nine of 14) received immunosuppressive treatment.

The EBV specific staining was localised within epithelial tissue and not in B lymphocytes as evidenced by the use of virus and tissue-specific markers on serial sections. Figure 1 illustrates EBV VCA positive staining at $\times 100$ magnification.

Figure 1 EBV VCA staining in alveolar epithelial cells. A 7 μm section cut from an open lung biopsy specimen taken from a patient with cryptogenic fibrosing alveolitis (patient 12) was stained with monoclonal anti-VCA using biotin-streptavidin-FITC. The fluorescent image shows a punctate, intracellular localisation characteristic of VCA-specific staining. Magnification ×100, reduced to 62% in origination.

Figure 2 EBV VCA origination

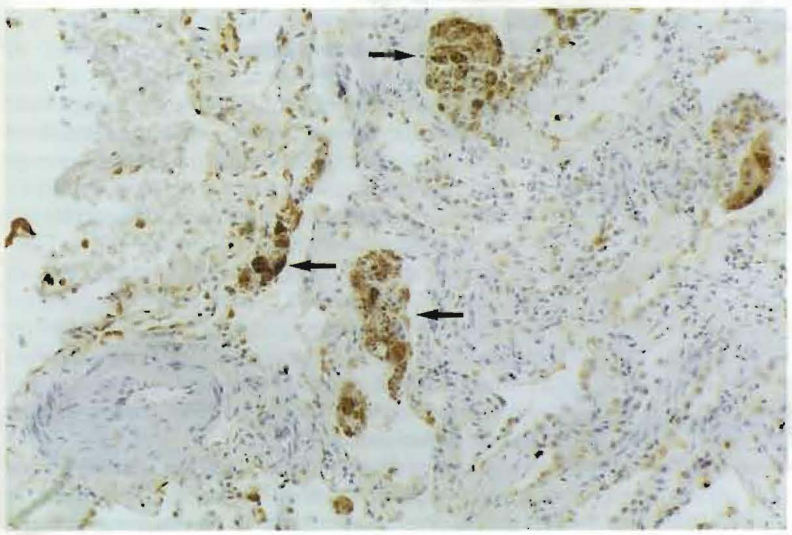
A

B

Figure 3 VCA using cytokeratin origination

Figure 2 Fibrosing alveolitis with hyperplastic type II cells some of which are positive for EBV VCA antigen (arrows) (APAAP, VCA). Magnification × 500 reduced to 72% in origination.

A



B

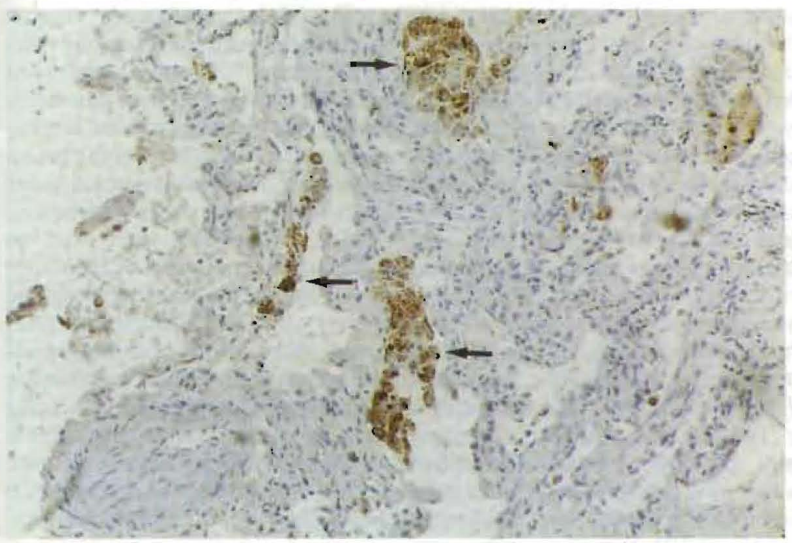


Figure 3 Adjacent serial sections stained individually for (A) cytokeratin and (B) EBV VCA using an immunoperoxidase technique. Three nests of cells expressing both cytokeratin and VCA can be seen (arrowed). Magnification × 160, reduced to 70% in origination.

Detailed histological analysis of sections stained by the APAAP technique revealed that the EBV antigens were present in type II alveolar cells, some lymphocytes and, additionally, in a proportion of macrophages (fig 2). Serial sections stained with the cytokeratin marker MNF116 and EBV VCA consecutively showed the EBV positive staining to be localised to epithelial cells (fig 3A and 3B). Immunoperoxidase staining showed EBV VCA cytoplasmic staining of type II alveolar cells (fig 4) and additional EBV VCA nuclear staining in bronchiolar epithelial cells (fig 5).

Discussion

We have described the presence of productively replicating EBV within the epithelial cells (type II alveolar cells) of the lower respiratory tract of patients with a clinical and histological diagnosis of cryptogenic fibrosing alveolitis. A previous serological study suggested that EBV is associated with cryptogenic fibrosing alveolitis but did not establish a causal relationship.[3] In the age range of patients studied here, up to 90% EBV seropositivity would be expected in both the cryptogenic fibrosing alveolitis group and the control group.[10] It is recognised that considerable variation between laboratories can occur when antibody titres of identical sera are tested.[11] Abnormally elevated serological titres to EBV specific antigens are often found in conditions of unknown aetiology including rheumatoid arthritis, chronic lymphatic leukaemia, and ataxia telangiectasia.[12] This has given rise to the suggestion that EBV plays a part in the aetiology of these immune related conditions, but no tissue based studies have confirmed this. In the current study replicating EBV has been identified within type II alveolar cells. As these cells may have a critical role in the deposition of type I collagen in the lung,[13] this study suggests a direct relationship between viral injury and cryptogenic fibrosing alveolitis and represents an advance on serological studies. The absence of historical EBV serological data does not detract from the immunohistochemical findings within the lung.

The immunohistochemistry techniques used in this study are sensitive and specific for the EBV antigens VCA and gp 340/220.[9] These antigens are expressed during the lytic or productive phase of the virus life cycle and not during viral latency.[9] Hence, this study is distinct from others in different clinical settings which have used EBER in situ probes (small RNA transcribed by the EBER-1 gene during latent EBV infection)[14] and polymerase chain reaction (PCR) for the identification of latent EBV within tissue. EBER expression occurs in all EBV positive cells whenever latently infected and in almost all supporting viral replication, with the exception of oral hairy leukoplakia (OHL) which supports extensive productive EBV replication in the absence of EBER.[15,16] EBER expression is therefore probably not a prerequisite for viral replication because recombinant EBV with the EBER genes deleted can replicate in lymphoblastoid cells in vitro.[16] Although PCR is considered to be sensitive,

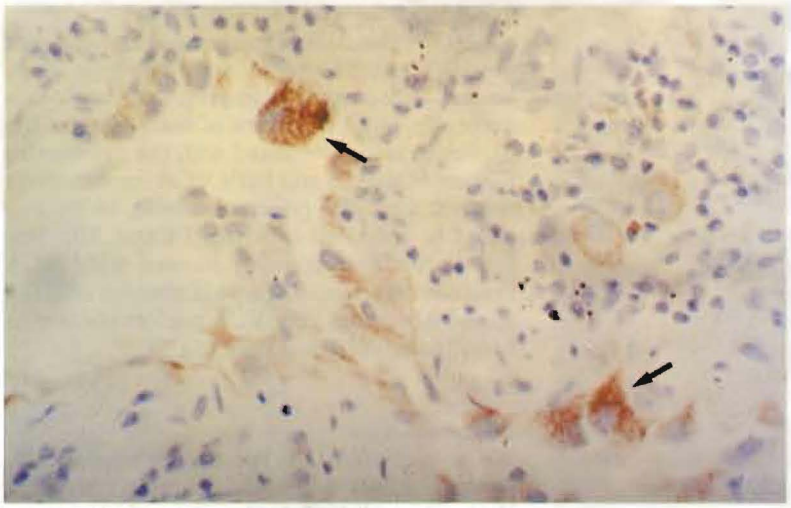


Figure 4 EBV VCA positive staining (brown, arrowed) within the cytoplasm of metaplastic type II alveolar cells from a patient with cryptogenic fibrosing alveolitis.

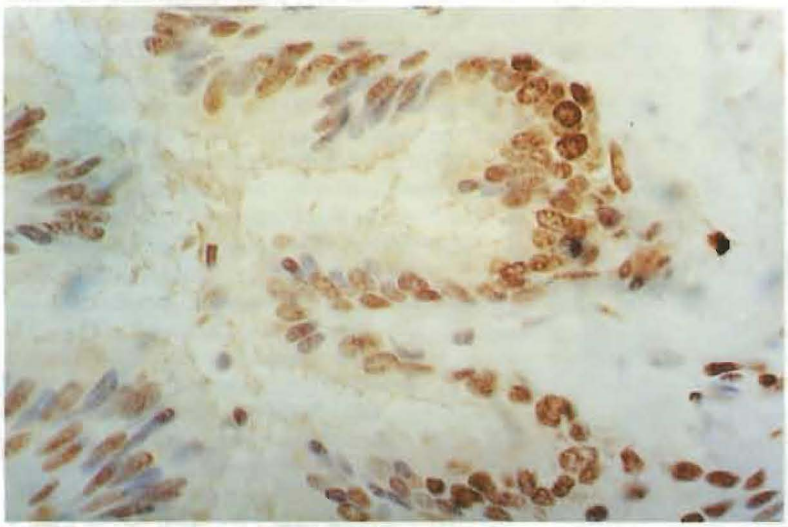


Figure 5 EBV VCA positive staining (brown) within the nuclei of bronchiolar epithelial cells.

the documentation of EBV DNA by PCR does not differentiate between latent or productively replicating virus and, furthermore, gives no information on the types of cells involved.[17] EBER in situ hybridisation and PCR therefore have certain limitations in evaluating actively replicating EBV compared with the immunohistochemistry techniques used in this study.

The application of immunohistochemistry has a particular advantage in allowing the localisation of EBV within the lung tissue. This is a critical issue to the findings of this study because positive EBV staining, for example, may merely reflect infiltration of the tissues by EBV positive lymphocytes or macrophages. We have demonstrated EBV VCA nuclear (fig 2) and cytoplasmic staining (fig 4) within type II alveolar cells. Consecutive staining of serial sections with cytokeratin and VCA antibodies, respectively, confirm the localisation to be within epithelial cells (fig 3A and 3B). It is also of interest that EBV VCA nuclear staining is documented within the epithelial cells of bronchiolar tissue (fig 5).

EBV has previously been identified within epithelial cells obtained by oropharyngeal washings, although the precise site of origin is unclear.[18] Whether EBV in oropharyngeal epithelial cells represents a pool of EBV persisting in epithelial tissue or periodic reinfection of epithelial cells from a B cell reservoir is controversial.[4] The only previously reported example of EBV productively replicating in epithelia in vivo has been in OHL, an AIDS associated condition in which human papillomavirus has also been identified.[19] This, we believe, is the first in vivo report of EBV replication within the epithelial cells of the lower respiratory tract in humans.

Histological analysis additionally identified EBV positive staining within alveolar macrophages. EBV has been shown to infect cells of monocytic lineage both in vivo[20] and in vitro.[21] However, the positive staining within macrophages in lung tissue may reflect phagocytosis of productively infected epithelial cells, as this finding was not evident in patients with cryptogenic fibrosing alveolitis who had no evidence of EBV staining in epithelial cells or in the control group.

A previous report suggesting that EBV was located within the lung was confounded by the fact that EBV was detected by in situ hybridisation from bronchoalveolar lavage fluid and this may have reflected contamination from the oropharynx.[5] Our data support the concept that the lower respiratory tract is a potential reservoir for EBV. Furthermore, our results are in concordance with the behaviour of the EBV related animal virus, murine gamma herpes virus 68, a pathogen localised to the epithelial tissue of the lower respiratory tract of the mouse model.[22] Thus, cryptogenic fibrosing alveolitis may provide a new biological model for the study of the interaction of EBV and epithelial cells in humans.

The pathogenicity of herpes viruses identified within diseased tissue is often difficult to resolve, particularly in immunosuppressed patients. Examples of this are seen with cytomegalovirus (CMV) in pulmonary tissue of AIDS patients[23] and human herpes virus 6 associated interstitial pneumonitis in bone marrow transplant patients.[24] It could be argued that in our patients EBV is merely a passenger within the lung tissue of patients with cryptogenic fibrosing alveolitis. However, in the group of patients studied most of those positive for EBV replication had never received immunosuppression (nine of 14) and there was no difference between the proportion of EBV replication positive patients with cryptogenic fibrosing alveolitis who had received immunosuppression and those who had not. Furthermore, the proportion of EBV replication positivity was significantly higher among immunocompetent patients with cryptogenic fibrosing alveolitis than in the control group. This suggests that the presence of productively replicating EBV may be of pathogenic significance.

It may be considered that the control tissue used in this study was not collected from normal subjects. However, the collection of

surgically obtained tissue from a normal population has obvious practical and ethical problems. The tissue blocks taken some distance from the tumours were analysed histologically and, in eight of the 14 cases, the tissue was histologically normal with the presence of a minor degree of emphysema in the remaining six patients (table 3). This is unlikely to be a confounding influence on the clear difference in tissue viral expression, particularly as the cryptogenic fibrosing alveolitis group and control group were well matched for age, sex, and smoking history. Indeed, both control patients who stained positive for EBV were smokers. We believe the histological features within the control group accurately represents "normal" control tissue from a population with a mean age of 61 years.

Cryptogenic fibrosing alveolitis is generally a condition found in older patients.[1] Age-related immune deregulation may be critical for a pathogenic immune response prompted by EBV replication in cryptogenic fibrosing alveolitis. Herpes viruses, particularly CMV, are recognised as having virus/host antigen homology and immune crossreactivity with the resulting potential to promote chronic inflammation and fibrosis.[25] Not only is it perceived that downregulation of the immune system occurs with ageing but, in addition, an increase in suppressor signals is recognised which may correlate with an increased inflammatory response to a normally quiescent antigen.[26]

Pulmonary fibrosis is an expression of lung injury recognised following many precipitating factors including infection, environmental agents, and drugs. Environmental factors have been implicated in the aetiology of cryptogenic fibrosing alveolitis.[2 27] However, a recent study has described a rat model in which retrovirus RNA was incorporated into the type II alveolar cell nucleus prompting type I collagen deposition.[28] The current study supports the concept of virus-induced injury, but this does not preclude the importance of environmental factors in some patients with cryptogenic fibrosing alveolitis. Within the heterogeneous group of patients labelled clinically as "cryptogenic fibrosing alveolitis" it is possible that subsets of patients exist, some with an environmental trigger and others with a viral trigger. In some patients a primary environmental injury may be potentiated at a later date by viral replication within the injured tissue. Ultimately each individual or combination of aetiological factors may result in a common clinical and histological response to injury.

This study shows that EBV replication is occurring within type II pulmonary epithelial cells of patients with cryptogenic fibrosing alveolitis and therefore may be an important aetiological agent in a significant number of patients. After further clarification of the role of EBV, specific antiviral therapy may become an important component of future treatment for cryptogenic fibrosing alveolitis, but we would caution that this should only occur within the context of controlled trials.

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