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Cryptogenic fibrosing alveolitis: lack of association with Epstein-Barr virus infection

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Abstract

***Background* – Cryptogenic fibrosing alveolitis (CFA) is a well defined clinical entity of unknown aetiology. An association between CFA and the presence of protein indicating Epstein-Barr virus (EBV) replication within epithelial cells of the respiratory tract has recently been suggested, leading to speculation for a role for EBV in the pathogenesis of CFA.**

***Methods* – Lung tissue was obtained from patients in three groups: those with cryptogenic fibrosing alveolitis, either lone or associated with systemic sclerosis; patients with other pulmonary disorders; and patients with normal lung. Paraffin blocks were stained using three antibodies raised against well defined EBV antigens. In addition, EBER-1 and EBER-2 antisense nucleotide probes were used in an attempt to identify EBV RNA. DNA was also extracted from the tissue sections and evaluated for evidence of EBV DNA using the polymerase chain reaction.**

***Results* – Immunohistochemistry showed inconsistent focal positive staining with anti-EBV antibodies in all three groups, but there was no evidence of EBV RNA using in situ hybridisation. None of the samples from patients with pulmonary fibrotic disorders was found to contain EBV DNA following gene amplification.**

***Conclusion* – Contrary to an earlier report, these results do not support the hypothesis that EBV has a role in the pathogenesis of CFA.**

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Keywords: cryptogenic fibrosing alveolitis, Epstein-Barr virus.

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Cryptogenic fibrosing alveolitis (CFA), also known as idiopathic pulmonary fibrosis, is a condition of unknown aetiology characterised by inflammation and fibrosis of the pulmonary interstitium and alveolar tissues. According to one theory it occurs in susceptible individuals following some unknown stimulus which damages the epithelium and evokes a cascade of events involving factors controlling inflammatory, immune, and fibrotic processes in the lung. However, ultrastructural studies have shown both epithelial and endothelial injury in the early stages of CFA.[1] A role for viruses, especially Epstein-Barr virus (EBV), in the pathogenesis of CFA has been suggested.[2] Increased serum antibody to EBV detectable IgA against viral capsid antigen has been observed in samples from patients with CFA,[3] with the respiratory tract proposed as a possible reservoir for EBV by others.[4] However, there have been few attempts to localise the EBV genome in cells and there was a poor correlation between the titres of serum EBV antibodies and whether cells from patients were positive or negative for EBV DNA. Recently, evidence of EBV replication within pulmonary epithelial cells in patients with CFA was suggested using immunochemistry,[5] although the study did not confirm the presence of EBV by examining samples for the presence of DNA or RNA specific for EBV. In this study we sought to confirm previous observations which have implicated EBV in the pathogenesis of CFA by examining lung tissues from patients with CFA and comparing these with tissue from patients with other pulmonary disorders and tissue from normal lung. Three well characterised antibodies raised against EBV were used to identify protein expression and polymerase chain reaction (PCR) and in situ hybridisation were used to identify EBV DNA and RNA, respectively.

Methods

SUBJECTS

All patients in the study had been evaluated clinically and investigated at the Royal Brompton Hospital by one clinician (RdB). Paraffin blocks of lung tissue were obtained from the pathology department. Slides were reviewed and cases were selected as follows: lone CFA (n = 12), CFA associated with systemic sclerosis (n = 2), diffuse alveolar damage (n = 3), sarcoidosis (n = 3), idiopathic haemosiderosis (n = 1), extrinsic allergic alveolitis (n = 1), desquamative interstitial pneumonitis (n = 1), foregut cyst (n = 1), and normal lung (n = 3). Apart from the foregut cyst which was removed incidentally at the time of lobectomy for a carcinoma, tissue was taken either from open lung biopsy specimens or from uninvolved lung in resection specimens.

IMMUNOHISTOCHEMISTRY

Serial sections of 3–4 μm thickness were cut from each block, dewaxed, and treated with 3% hydrogen peroxide in methanol to quench the activity of endogenous peroxidase. The sections were washed in phosphate buffered saline (PBS, pH 7.2) and were microwaved in citrate buffer for 30 minutes. Non-specific protein binding was blocked with 1:5 normal rabbit serum (Dako Ltd, High Wycombe, Bucks, UK) in PBS. Primary antibodies used

Table 1 Immunohistochemistry, in situ hybridisation, and PCR analysis on samples from patients with cryptogenic fibrosing alveolitis, other interstitial lung diseases and normal lung

Case no.	*Diagnosis*	*CS1–4*	*BZ-1*	*VCA*	*EBER*	*EBV DNA*
1	CFA	++	+	++	−	−
2	CFA	++	−	+	−	−
3	CFA	−	−	+	−	−
4	CFA	+	+/−	+	−	−
5	CFA	−	−	−	−	−
6	CFA	++	−	−	−	−
7	CFA	++	+	+	−	+/−/−
8	CFA	++	−	+	−	−
9	CFA	−	−	−	−	−
10	CFA	++	+	−	−	−
11	CFA	−	−	−	−	+/−/−
12	CFA	−	−	−	−	−
13	SSCFA	++	−	+	−	−
14	SSCFA	+	+	+	−	−
15	EAA	+	−	−	−	−
16	DIP	+	−	−	−	−
17	Sarcoidosis	+	+	++	−	−
18	Sarcoidosis	+	−	−	−	−
19	Sarcoidosis	++	++	++	−	−
20	DAD	−	−	−	−	−
21	DAD	+	±	++	−	−
22	DAD	−	−	−	−	−
23	Haemosiderosis	−	−	−	−	−
24	Foregut cyst	+++	+++	+++	−	+/−
25	Normal	−	−	+/−	−	−
26	Normal	−	−	+	−	−
27	Normal	+	+	++	−	−

CFA = cryptogenic fibrosing alveolitis; SSCFA = CFA associated with systemic sclerosis; EAA = extrinsic allergic alveolitis; DIP = desquamative interstitial pneumonitis; DAD = diffuse alveolar damage.

in this study were a mixture of monoclonal anti-EBV LMP-1 antibodies (CS1–4),[6] monoclonal anti-EBV BZLF-1 protein,[7] and monoclonal anti-EBV viral capsid antigen (VCA) (Chemicon International Inc, Temecula, California, USA). The antibodies were diluted in 1:20 rabbit serum in PBS. The sections were incubated with the optimal dilution of the primary antibodies (1:100 for CS1–4, 1:2000 for BZ-1, and 1:1600 for VCA antibody) overnight in a humidifying chamber at 4°C. The sections were washed with PBS and incubated with biotinylated rabbit anti-mouse (Dako Ltd) for 30 minutes, washed, and then incubated with avidin biotin peroxidase complex (Dako Ltd), washed for 15 minutes, and visualised with 3, 3′-diamino benzidine (DAB) substrate (Dako Ltd). The sections were counterstained with Mayer's haematoxylin solution (Sigma Diagnostics, St Louis, USA) for one minute. After colour development, slides were examined for positive staining and scored for intensity of staining in coded fashion by one observer (AGN) on a semiquantitative scale of −, +/−, +, ++, +++, with − = no staining, + = definitive staining, ++ = prominent staining, and +++ = strong staining. In control sections (EBV positive lymphoma and EBV positive cultured cells) specificity was demonstrated by either omission of the primary antibody step or treatment of sections with purified normal rabbit serum (Dako Ltd).

IN SITU HYBRIDISATION

The procedure for in situ hybridisation was modified from previously described methods.[8] Briefly, 3–4 μm sections were cut from lung biopsy specimens embedded in paraffin and were mounted on poly-L-lysine coated slides. Tissue sections were dewaxed and rehydrated through an alcohol series to PBS, treated with proteinase K followed by acetylation with 0.1% acetic anhydride in 0.1 M triethanolamine to reduce non-specific interaction between DNA probes and tissue. Slides were washed in PBS, dehydrated, air dried, and used for hybridisation on the same day or stored at −20°C.

For EBV RNA the following three EBER antisense oligonucleotide sequences were used as a probe: (1) 5′-AAAACATGCGGACC-ACCAGC-3′ (6795-6776, EBER-1), (2) 5′-TCCTGACTTGCAAATGCTCT-3′ (7080-7061, EBER-2), and (3) 5′-CGGACAAG-CCGAATACCCTT-3′ (7120-7101, EBER-2) and were deduced from the published EBER-1 and EBER-2 sequences.[9] These probes have previously been used successfully for the determination of EBER by in situ hybridisation or PCR.[10 11] The three oligonucleotides were 3′-end labelled with deoxyadenosine 5′-thiotriphosphate (α-^{35}S). Hybridisation was performed overnight at room temperature. After incubation the sections were washed, air dried, and dipped in Ilford K5 nuclear track emulsion. After exposure periods of about two weeks the sections were developed and counterstained in Mayer's haematoxylin and mounted.

EBV positive lymphoma and EBV positive cultured cells were used as positive controls and one section from both the control and each specimen were treated before hybridisation with 20 μg/ml of ribonuclease A (Sigma, Poole, UK) and 80 units/ml of ribonuclease T1 in PBS at 37°C for 30 minutes in order to confirm specificity.

EBV DNA PCR

DNA was extracted from paraffin-embedded samples as previously described.[12] PCR amplification of the BamHIW repeat of EBV was carried out using the primers described by Brocksmith *et al* and the products were analysed on 3% agarose gels.[13] All samples were amplified in duplicate. DNA extracted from a paraffin section of a B cell lymphoma with Southern blot proven EBV was used as a posi-

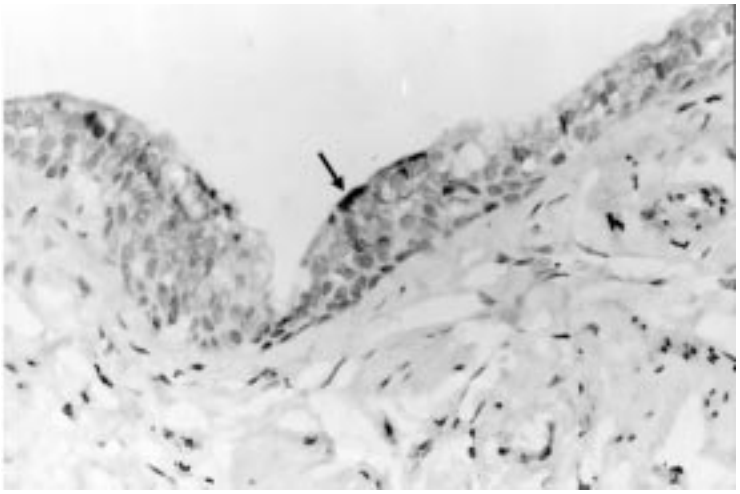

Figure 1 Bronchiolar epithelium from a patient with cryptogenic fibrosing alveolitis showing focal positive staining (arrow) towards the luminal aspect of the cells using antibody to CS1–4. Haematoxylin was used as a counterstain (original magnification × 400).

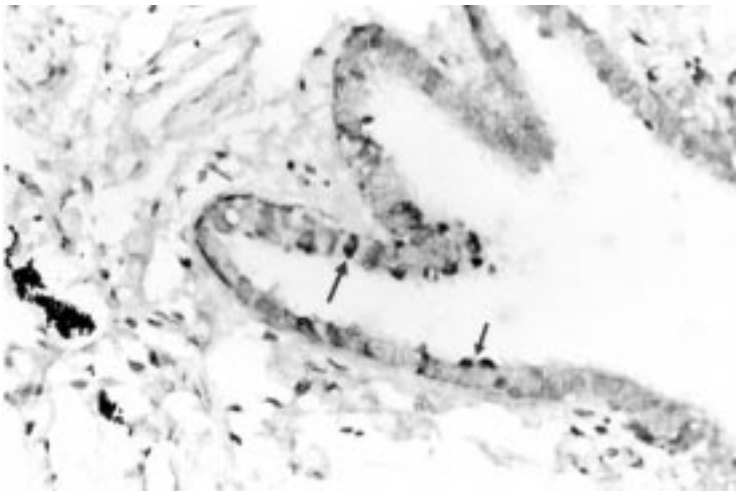

Figure 2 Bronchiolar epithelium from normal lung showing focal positive staining of several epithelial cells using antibody to VCA. Some cells stain only towards their luminal aspect (smaller arrow) whilst others show diffuse cytoplasmic staining (larger arrow). Haematoxylin was used as a counterstain (original magnification × 400). In this case staining was not present with the other two antibodies at this site.

tive control with each experiment. We used approximately 25 ng of this DNA and 50–100 ng of test sample DNA, and 40 cycles of PCR were used.

Results

The results are summarised in table 1. Immunohistochemical analysis yielded localised positive staining, not only in cases of CFA (fig 1) but also in normal lung (fig 2) and lung affected by other disease processes. Staining was present in respiratory type epithelium, alveolar epithelium and, in two cases (nos 19 and 24), seromucinous glands where the staining was at its most intense (fig 3). Serial sections were used to assess the distribution of the staining with all three antibodies within the tissue. A few cases showed positive staining at the same foci on adjacent sections, but most showed wide variability of staining with the different antibodies, not only in distribution but also in intensity. Also, when in situ hybridisation for EBER RNA was used, none of the samples from any of the patients was positive in any area showing positivity by immunohistochemistry. However, EBV positive lymphoma and EBV positive cultured cells showed presence of EBER RNA. Three cases, two of CFA and one of a foregut cyst removed from the mediastinum of a patient with a squamous cell carcinoma, gave a positive result for EBV DNA using PCR amplification on a single occasion. Duplicate specimens in these three cases and a further amplification in two were negative.

Discussion

The present study was conducted to evaluate a possible role for EBV in the pathogenesis of CFA. The hypothesis that EBV infection was associated with any form of interstitial lung disease was first suggested by Liebow and Carrington in relation to lymphocytic interstitial pneumonia.[14] Their hypothesis was supported by work using in situ hybridisation which showed the presence of EBV in type 2 epithelial cells in these patients. Interestingly, this work by Barbera *et al* failed to show evidence of EBV DNA in patients with fibrosing alveolitis whom they used as controls.[15]

Vergnon *et al*[3] have shown a high prevalence of serum antibodies to EBV and IgA against the viral capsid antigen (VCA), suggesting that EBV may play a part in the aetiology of CFA, but citing the caveat that raised levels of IgG and IgA may be related to non-specific depression of cell mediated immunity.[3] In another study of 61 necropsy cases with secondary diffuse interstitial pneumonia, EBV was detected in seven patients using immunohistochemistry. However, no viral inclusions were observed.[2] In another study tuboreticular structures were observed in interstitial lung disease associated with collagen vascular disease and viral pneumonia, although this study failed to identify such structures in 30 patients with CFA.[16] Most recently, Egan *et al* have suggested that EBV replicates in epithelial cells of patients with CFA after positive findings in 14 out of 20 patients.[5] Keeping in view the discrepancy between the studies of Barbera *et al* and Egan *et al*, it was emphasised by Hogg in 1995 that experiments that examine lung tissue for viral DNA, RNA and proteins will be needed to clarify why the EBV replicative proteins might be present when the internal repeat DNA is not.[17]

In the present study we set out to confirm these findings by immunohistochemistry, and also to look for EBV RNA and EBV DNA using in situ hybridisation and gene amplification, respectively, as support for the concept that EBV is present at disease sites. We investigated samples from 27 patients and controls, including 12 cases of CFA. Some staining was found in cases of CFA using an antibody raised against VCA which was used in the study by Egan *et al*,[5] and also with antibodies BZLF-1 and CS1–4. However, staining was also present

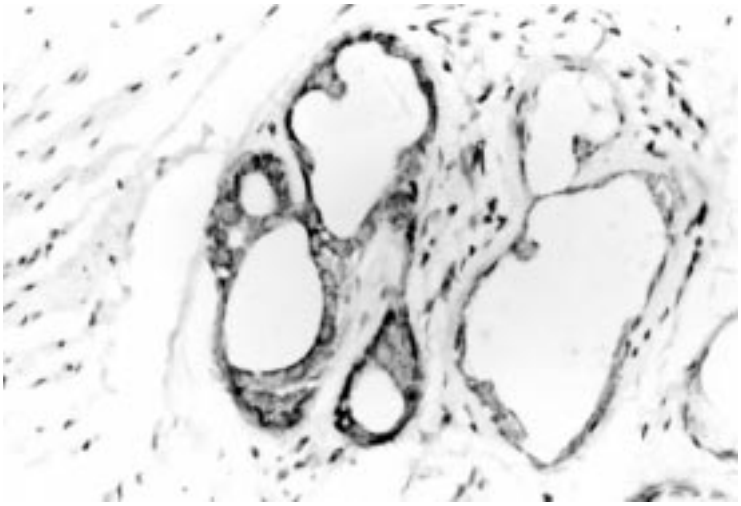

Figure 3 Seromucinous gland within a foregut cyst showing strong positive cytoplasmic staining using the antibody to BZ-1. Haematoxylin was used as a counterstain (original magnification × 400). In this case similar staining was seen with both the other antibodies in serial sections at the same site.

in most of the cases with other interstitial lung disease, a foregut cyst, and also in one of three cases of normal lung, and frequently the cellular appearance of this staining was different from that observed in EBV infected tissue culture cells. Serial sectioning occasionally showed some staining at similar locations with all three antibodies, but there was more variability than homogeneity. In particular, CS1–4 staining tended to be predominantly in the respiratory epithelium whilst BZLF-1 staining was more focal and less intense, apart from those cases where seromucinous glands showed positivity. Antibody against VCA stained both respiratory type epithelium and a higher proportion of alveolar epithelium. However, the absence of any evidence of EBV DNA in all samples except in one of three pairs of duplicates makes it likely that these antibodies may be cross reacting with native epitopes of cell proteins. The method used for EBV DNA is vastly oversensitive for detection of moderate numbers of EBV infected cells, as each copy of EBV contains 12–15 repeats of BamW fragment and infected cells often contain multiple copies of the virus. Forty cycles of PCR were used, which usually allows detection of 5–10 copies of target sequences. The method has permitted us to detect the virus in 100 pg of DNA extracted from paraffin sections of B cell lymphomas with EBV involvement. As we have used 500–1000 times more DNA in these experiments, sensitivity should not be a problem. We were more worried about false positive signals, which is why multiple amplifications were performed on each sample in order to eliminate both sporadic false positives and samples with few target sequences such as occasional EBV infected B cells unrelated to the disease under investigation. Indeed, amplification in a single specimen with failure to duplicate the result implies that EBV is probably present at a very low copy number, such as within an occasional cell, possibly a lymphocyte. Unfortunately, because the tissues are archival, there was no access to blood to look for serological evidence of EBV infection but the normal incidence of infection in the population would lead us to expect that at least 90% of the cases would be seropositive for EBV. Furthermore, the absence of positivity in these lung samples using in situ hybridisation demonstrates the difficulties associated with relying on a single reagent to test for the presence of EBV in tissue samples of this type. If EBV was replicating in its lytic cycle, coincident expression of BZLF-1 and VCA would be expected, and if it was latent the EBER and LMP1 (CS1–4) probes should have detected this type of infection. Whatever the type of EBV infection, the viral DNA should have been detectable. In conclusion, the lack of consistent viral antigen expression and general failure to detect EBV DNA with this series of well defined CFA patients indicate that cryptogenic fibrosing alveolitis does not appear to be associated with EBV infection.

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