Circulating antibodies to lung protein(s) in patients with cryptogenic fibrosing alveolitis

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Abstract

Background – It has been hypothesised that cryptogenic fibrosing alveolitis has an immunological pathogenesis mediated by T lymphocytes. It is, however, recognised that patients may show dysregulation of the humoral immune system and that the presence of large numbers of B lymphocytes in open lung biopsies may be associated with a poor prognosis. Evidence of a role for the humoral immune system in the pathogenesis of cryptogenic fibrosing alveolitis has been suggested, but attempts to demonstrate circulating immunoglobulin to antigen within the lung have been inconclusive.

Methods – Plasma samples from 22 patients with cryptogenic fibrosing alveolitis, 22 patients with sarcoidosis, and 17 healthy controls were screened by SDS-PAGE and Western blotting for the presence of autoantibodies to lung proteins derived from cryptogenic fibrosing alveolitis, sarcoid and control lung tissue, as well as four normal non-pulmonary tissues. Possible site(s) of target protein(s) within the lung tissue were identified by immunohistochemical examination using IgG purified from the plasma of six patients and two controls.

Results – Eighteen of the plasma samples from patients with cryptogenic fibrosing alveolitis had reactive IgG to lung protein(s) in the 70–90 kDa molecular weight range compared with five of 18 plasma samples from patients with sarcoidosis and one of 17 controls. Plasma from patients with cryptogenic fibrosing alveolitis recognised antigen(s) of the same molecular weight in control and sarcoid lung tissue, but not non-pulmonary tissues, with a similar frequency. Immunohistochemical staining of cryptogenic fibrosing alveolitis biopsy material using IgG purified from plasma samples from patients with cryptogenic fibrosing alveolitis, but not control samples, revealed fine linear positivity in the lung parenchyma in a pattern suggestive of reaction with alveolar lining cells. The pattern was cytoplasmic/membranous and not nuclear.

Conclusions – Patients with cryptogenic fibrosing alveolitis have a high frequency of plasma IgG autoantibodies to protein(s) within lung tissue associated with alveolar lining cells. This is believed to be the site where immunological injury occurs in cryptogenic fibrosing alveolitis, but the significance of these antibodies to the aetiology and pathogenesis is as yet unclear.
and immunoglobulin in bronchoalveolar lavage fluid from patients with cryptogenic fibrosing alveolitis compared with normal subjects. This indicates possible functional significance for the humoral immune system.

Other better characterised inflammatory disorders of the pulmonary interstitium which may result in scarring, such as sarcoidosis, are also believed to have a T cell mediated pathogenesis. In sarcoidosis there is similar evidence of systemic immune dysregulation, hypergammaglobulinaemia, and accumulation of B lymphocytes and plasma cells within the lung, and it has been shown that such patients have circulating autoantibodies against T lymphocyte epitopes although the significance is unknown.

The presence of autoantibodies in patients with cryptogenic fibrosing alveolitis has been predicted and previously sought but the results have been inconclusive. The most recent study found that 40% of controls and patients with cryptogenic fibrosing alveolitis were positive by an indirect immunofluorescence technique with whole serum on cryostat sections of lung tissue. Other workers using ELISA techniques have found antibodies to the nuclear antigen topoisomerase II in 38% of patients and hepatitis C virus in 28% of patients.

We have readdressed the question of whether cryptogenic fibrosing alveolitis is associated with circulating antibodies to lung proteins by screening the plasma of patients with cryptogenic fibrosing alveolitis, sarcoidosis, and healthy controls using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. In addition we attempted to find the tissue distribution of protein(s) to which patients with cryptogenic fibrosing alveolitis may have autoantibodies.

**Methods**

**Patients**

Twenty two newly diagnosed patients with cryptogenic fibrosing alveolitis (14 men, eight women of mean (SE) age 66-2 (1-5) years) were studied. Nineteen of the individuals had undergone open lung biopsy and the remaining three were diagnosed by clinical, radiological, and physiological features. Twenty ml samples of heparinised venous blood were obtained from each of the patients following approval by the local ethics committee. None were on any specific treatment for their disease. The blood sample was centrifuged at 1800 rpm on a Ficol-Hypaque gradient and the plasma was removed and stored at −70°C.

**Controls**

Plasma samples were obtained in a similar manner from 17 healthy subjects in the laboratory who had no evidence of respiratory symptoms and who were on no medication (10 men and seven women of mean (SE) age 41-5 (2-5) years), and 22 patients with histologically confirmed sarcoidosis (13 men and nine women of mean (SE) age 43-4 (2-2) years).

**Preparation of Protein Extracts**

Protein extracts were prepared from open lung biopsy material obtained from eight patients with cryptogenic fibrosing alveolitis and three with sarcoidosis. Control tissues were obtained from histologically normal lung resection tissue, colonic mucosa, skin, liver, and tonsil.

The tissue was collected fresh in theatre, snap frozen in 1 ml 1% Nonidet P-40 lysis buffer (a non-ionic detergent), and stored at −70°C. The specimen, still in lysis buffer, was then mechanically disrupted while still frozen with a mortar and pestle, and transferred to a universal container. A further 1 ml aliquot of lysis buffer was then added and the container placed in an ultrasonic water bath for 3 minutes. The sample was then placed in an ultracentrifuge, spun at 32 000 rpm for 10 minutes, and the protein containing supernatant removed and kept on ice.

The protein content of the supernatant was assayed using a colorimetric technique (BioRad Labs Ltd, UK) and diluted 1:2 with sample buffer (3 ml distilled water, 1.0 ml 0.5 mol/l Tris-HCl, pH 6.8, 10% w/v SDS, 0.8 ml 1 mol/l dithiothreitol, 0.8 ml glycerol, 0.05 ml 0.05% w/v bromophenol blue) and stored at −70°C.

**SDS-PAGE Electrophoresis**

The Mini-Protein II polyacrylamide gel electrophoresis system (BioRad Labs Ltd, UK) was used throughout and the manufacturer’s protocol followed. 7.5%-acrylamide running gels with 4% acrylamide preparative (with one reference well) stacking gels were produced according to the manual. The gels were loaded with 2 mg of a single protein extract alongside markers of known molecular weight in the reference well. The sample was run under reducing conditions using a constant 200 volt supply until the bromophenol blue band was seen to reach the bottom of the well. The proteins were then electrophoretically transferred to nitrocellulose paper (Hybond C, Amersham, UK) and stored at 4°C. The gels were stained with 0.2% w/v Coomassie blue to ensure that protein separation had occurred.

**Western Blotting**

Before blotting the nitrocellulose sheets were blocked for one hour at room temperature (5% w/v dried milk powder in Tris buffered saline (TBS)) followed by three five minute washes in Tris buffered saline with 0.1% Tween 20 (TTBS). The test sera were diluted 1:50 with antibody buffer (1% w/v dried milk powder in TTBS). The nitrocellulose sheets were then incubated for 30–60 minutes with the patient and control plasma at room temperature in a multichannel developing clamp (BioRad Labs Ltd, UK) which divided the membrane into distinct channels allowing up to 20 different sera to be tested simultaneously against one protein extract. The monoclonal antibodies against the leucocyte common antigen, CD45, or vimentin (both Dako UK Ltd), at 1:500
Summary of Western blotting data for the experiments presented indicating the frequency of plasma samples from each test group which contain autoantibodies recognizing a 70-90 kDa antigenic protein derived from the different tissue extracts

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Frequency of plasma samples recognising a 70-90 kDa protein in tissue extracts</th>
<th>CFA plasma</th>
<th>Sarcoïd plasma</th>
<th>Control plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA lung biopsy</td>
<td>18/22 (82%)*</td>
<td>5/18 (28%)</td>
<td>1/17 (6%)</td>
<td></td>
</tr>
<tr>
<td>Sarcoïd lung biopsy</td>
<td>18/19 (95%)*</td>
<td>6/22 (27%)</td>
<td>5/17 (23%)</td>
<td></td>
</tr>
<tr>
<td>Control lung</td>
<td>12/15 (80%)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colonic mucosa</td>
<td>3/13 (23%)†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
<td>3/14 (21%)†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tonsil</td>
<td>3/14 (21%)†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skin</td>
<td>4/14 (29%)†</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

CFA = cryptogenic fibrosing alveolitis; ND = not done.
* Frequency of positive bands in the 70-90 kDa range is significantly more frequent (p<0.001) with the plasma samples from patients with cryptogenic fibrosing alveolitis than the sarcoïd or control plasma samples.
† Frequency of positive bands in the 70-90 kDa range is significantly less frequent than on the control lung extract (p<0.05) or the interstitial lung disease extracts (p<0.001).

dilution were used as positive controls and antibody buffer alone as a negative.
The membranes were removed from the clamp, given three five minute washes in TTBS, and then developed for adherent antibodies by incubation with murine antihuman IgG₆ (Sigma Chemical Co, UK, clone no. GG5 at 1:1000), antihuman IgM₆ (clone no. DA16127, kindly donated by Dr K G Guy), or antihuman IgA₆ (Sigma Chemical Co, UK, clone no. GA-112). The membranes were washed as above, incubated with biotinylated rabbit anti-mouse antibody (Dako UK Ltd), diluted 1:1000 for 30 minutes, and washed again. This was followed by a further 30 minute incubation with an alkaline phosphatase conjugated avidin–biotin complex (Dako UK Ltd) diluted 1:30 and further washing. Visualisation was carried out using the NBT/BCIP substrate (Sigma Chemical Co, UK) in an alkaline phosphatase buffer. The membranes were then blotted dry with tissue paper and stored.

The molecular weights for the positive bands in each sample were calculated from a standard curve produced from the molecular weight markers.

PURIFICATION OF IgG FROM PLASMA
The IgG fraction in plasma samples from six patients and two controls was purified from the whole sample using protein G (Pharmacia) following the manufacturer’s protocol. The concentration of the purified IgG was measured as described above, aliquoted, and stored at -70°C.

STAINING OF TISSUE SECTIONS WITH PURIFIED IgG
Cryptostat sections of lung from patients with cryptogenic fibrosing alveolitis obtained at open lung biopsy were cut, fixed with acetone, and washed with TBS. The sections were blocked with protein A (Sigma Chemical Co, UK) for 30 minutes, washed with TBS, and then further blocked with fresh normal human serum for one hour. They were then incubated with 20 μg of the purified IgG from either a patient with cryptogenic fibrosing alveolitis or healthy control for one hour at room temperature, followed by further washing with TBS. A final incubation was then performed with a protein A-alkaline phosphatase conjugate (Sigma Chemical Co, UK). Visualisation was performed using Vector Red (Vector Lab, UK) with levamisole to block endogenous alkaline phosphatase activity. This substrate is visible by both light and ultraviolet microscopy. The sections were counterstained, mounted, and viewed with a confocal laser microscope (Zeiss) in ultraviolet mode with filters to reduce autofluorescence from collagen and elastin.

STATISTICS
All statistical analyses were performed with the χ² test.

Results
The results obtained for IgG autoantibodies are summarised in the table. No evidence of reactive IgM or IgA autoantibodies was found.

CRYPTOGENIC FIBROSING ALVEOLITIS PROTEIN EXTRACT
Cryptogenic fibrosing alveolitis (fig 1) and control (fig 2) plasma samples were blotted against protein derived from cryptogenic fibrosing alveolitis biopsy material to identify any evidence of autoantibodies that were specific for cryptogenic fibrosing alveolitis. The results showed bands extending across the blot as the result of non-specific reactivity of the detection system with immunoglobulin and biotin present in the original lung tissue. In addition many individuals showed antibodies reacting with several proteins of differing molecular weights. These represent naturally occurring autoantibodies which are also found in the controls and as such have no pathological significance.

Figure 1  Illustration of Western blot results obtained by blotting the 22 plasma samples from patients with cryptogenic fibrosing alveolitis on a protein extract produced from cryptogenic fibrosing alveolitis lung biopsy material. The molecular weight markers are indicated in kDa on the right. Positive bands of varying intensity are identified in the 70-90 kDa range for 18 of the 22 plasma samples (lanes 2, 4, 5, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, and 22). The biopsy material in the blot shown corresponds to the plasma shown in lane 22. Other bands visible on the blot are the result of non-specific reactions between the detection system and immunoglobulin or biotin in the tissue extracts, or the presence of naturally occurring autoantibodies which are also found in the controls and as such have no pathological significance.
occurring autoantibodies with no pathological significance as they are seen in both patient and control groups. Only bands in the 70-90 kDa range were found to have a significant correlation with the cryptogenic fibrosing alveolitis samples (cryptogenic fibrosing alveolitis plasma samples 18 of 22; control plasma samples one of 17, p < 0.001). Similar results were obtained using eight different cryptogenic fibrosing alveolitis biopsy samples as a source of protein extract.

To determine whether autoantibodies to these protein(s) were specific to the plasma samples from patients with cryptogenic fibrosing alveolitis or represented a non-specific response to inflammation in the pulmonary interstitium, 18 plasma samples from patients with sarcoidosis were blotted against the same extract. Only five of these 18 samples contained autoantibodies which recognised protein(s) in the 70-90 kDa range. This was significantly fewer than for the cryptogenic fibrosing alveolitis plasma samples (p < 0.001), but not significantly different from the controls.

**SARCOID PROTEIN EXTRACT**

As we had found evidence that cryptogenic fibrosing alveolitis plasma samples contained a high frequency of autoantibodies to 70-90 kDa lung protein(s) present in lung tissue from patients with cryptogenic fibrosing alveolitis, it was of interest to determine whether the same protein(s) were present in lung tissue from other sources. We chose to use extracts from lung biopsies taken from patients with sarcoidosis as this represented another inflammatory condition affecting the pulmonary interstitium with a different pattern of disease and prognosis.

The same plasma samples were blotted onto protein extracts derived from biopsy material from patients with sarcoidosis (fig 3). Eighteen of 19 cryptogenic fibrosing alveolitis plasma samples were shown to react with 70-90 kDa protein(s) compared with only six of 22 sarcoidosis plasma samples (p < 0.001). The frequency of positivity to 70–90 kDa protein(s) was not significantly different from that obtained using the cryptogenic fibrosing alveolitis extract for either group. Five of 17 control plasma samples were positive on the 70–90 kDa region on the sarcoid extract, and this was found to be significantly more frequent than on the cryptogenic fibrosing alveolitis extract (p < 0.05).

**CONTROL LUNG EXTRACT**

To determine whether the protein(s) detected by the autoantibodies in the plasma from patients with cryptogenic fibrosing alveolitis were normal lung constituents or only present in patients with inflammatory lung disease, cryptogenic fibrosing alveolitis plasma samples were blotted against protein extracts from control lung tissue. Twelve of the 15 cryptogenic fibrosing alveolitis plasma samples were positive, recognising 70–90 kDa protein(s) in the extract (fig 4). The bands obtained were very
much fainter than with the diseased lung extracts but were still present at a frequency which was not significantly different from that obtained on the cryptogenic fibrosing alveolitis or sarcoid protein extracts.

NON-PULMONARY TISSUE EXTRACTS
Plasma samples from patients with cryptogenic fibrosing alveolitis were blotted against extracts of non-pulmonary tissues (fig 5) to ascertain if the protein(s) detected were lung specific. The frequency of positivity obtained (normal colonic mucosa three of 13; liver three of 14; skin four of 14) was significantly lower than on the control lung extract (p < 0.05) and lung extracts from patients with interstitial lung disease (p < 0.001).

IMMUNOLOCALISATION OF THE TARGET PROTEIN(S)
Examination by light microscopy of the cryostat sections stained with purified IgG from patients with cryptogenic fibrosing alveolitis and controls was difficult to interpret because of the fine linear staining pattern obtained. When the sections were viewed with ultraviolet light using a confocal laser microscope the IgG purified from the plasma of patients with cryptogenic fibrosing alveolitis was observed to show a fine linear staining pattern along the surface of alveolar septa (fig 6A). The distribution suggested that the target protein(s) detected may be associated with alveolar lining cells. The staining pattern was not nuclear but cytoplasmic or membranous, or both, in its distribution. Parallel sections of the same biopsy material stained with IgG purified from the controls did not show this pattern of positivity (fig 6B).

Discussion
In patients with cryptogenic fibrosing alveolitis there is evidence of a persistent immune reaction in the pulmonary interstitium with resulting fibrosis. The mechanisms which drive these chronic responses are unknown but theoretically could result from either persistence of an extrinsic triggering (possibly viral) antigen as is seen in viral myocarditis, or from the development of an autoimmune reaction either to altered or previously sequestered self-antigen which is exposed to the immune system as a consequence of cell damage.

Possible immunoglobulin production locally within the lung has been previously suggested in patients with cryptogenic fibrosing alveolitis but attempts to identify circulating autoantibodies have failed to provide conclusive results, principally because the lung contains large amounts of cell bound immunoglobulin to which labelled antihuman immunoglobulin sticks when applied to cryostat sections. The technique of Western blotting circumvents this problem as the proteins present in the lung, including immunoglobulins, are separated according to molecular weight. This allows identification of specific reactions between antibodies in plasma with non-immunoglobulin antigenic proteins.

In our study we have shown that plasma samples from patients with cryptogenic fibrosing alveolitis have a high frequency (82%) of circulating IgG autoantibodies to 70-90 kDa protein(s) present within cryptogenic fibrosing alveolitis biopsy lung tissue. The presence of this autoantibody appears to be a feature of the plasma samples from patients with cryptogenic fibrosing alveolitis, in comparison with the sarcoid and control plasma samples. The 70-90 kDa protein(s) within the tissue extracts, however, were not specific to the cryptogenic fibrosing alveolitis extract and could be seen also in extracts of sarcoid and control lung tissue.

As might be expected, the positive bands in the 70-90 kDa range obtained with the cryptogenic fibrosing alveolitis plasma samples showed a wide variation in intensity between individuals even on the same blot (fig 1). This would seem to indicate differing autoantibody titres in different individuals. The significance of this in terms of disease severity, stage, prognosis, and possible response to treatment is at this stage unclear and must await the development of a quantitative ELISA technique.

The control plasma samples recognised 70-90 kDa protein(s) significantly more frequently in the sarcoid protein extract than the
Circulating antibodies to lung protein(s) in patients with cryptogenic fibrosing alveolitis.

The explanation for this is unclear, but may relate to the relative amounts of lung epithelial tissue present in the two biopsy types. In biopsy material from patients with cryptogenic fibrosing alveolitis a larger proportion of the tissue volume is likely to be occupied by scar tissue than in biopsy material from patients with sarcoidosis; thus the relative contribution of lung epithelial protein may be greater in the sarcoid extract than the cryptogenic fibrosing alveolitis extract. Since most autoantibodies occur at low titre in normal individuals, this possible relative increase in the amount of epithelial cell derived protein in the extract may explain the small rise in background frequency observed against sarcoïd extracts. Interestingly, the sarcoïd plasma samples did not show such an increase in frequency of positivity against the sarcoïd extract compared with the cryptogenic fibrosing alveolitis extract. Whilst cryptogenic fibrosing alveolitis is recognised as having associations with other diseases, in most cases the lung is the sole organ affected by the inflammatory process, unlike sarcoïdosis which may present as a multiorgan disease. We hypothesised that if the autoantibodies we have detected in cryptogenic fibrosing alveolitis have a pathogenic role, then the target protein(s) are likely to be lung specific. We have shown that most patients with cryptogenic fibrosing alveolitis who have autoantibodies recognising 70–90 kDa protein(s) in lung tissue fail to recognise protein(s) of similar weight in four non-pulmonary tissues.

In order to identify possible site(s) of the antigenic protein(s) in biopsy material from patients with cryptogenic fibrosing alveolitis we used IgG purified from the plasma of patients with cryptogenic fibrosing alveolitis and controls. By doing this we were able to apply a standard amount of IgG (20 µg) to each section, thus standardising the immunohistochemical technique and circumventing any non-specific effect of hypergammaglobulinemia on the results in the cryptogenic fibrosing alveolitis patients. The tissue staining with purified IgG was obtained by using a protein A-alkaline phosphatase conjugate which binds IgG specifically by the Fc portion of the molecule as a second layer. This technique allowed the omission of an antihuman immunoglobulin and thus removed one of the technical problems that has prevented such studies being successful in the past.

Even when the technical problems of non-specific background had been overcome, assessment by light microscopy was difficult because of problems of image definition when examining stained alveolar lining cells of only 2–3 µm in diameter on a section which is 6–8 µm in thickness. The confocal laser microscope is a high definition instrument which views an optical section within the tissue being examined. This resulted in removal of many of the optical limitations presented by the sections and optimised the image obtained. The fine, linear pattern of staining observed with the cryptogenic fibrosing alveolitis derived IgG (but not the control IgG) strongly suggested that the protein(s) recognised by the autoantibodies were in association with alveolar lining cells. Previous studies have suggested that this is the site of immunological damage in cryptogenic fibrosing alveolitis and immune complexes have been reported on the alveolar surface.

We hypothesised that the autoantibodies we had detected in patients with cryptogenic fibrosing alveolitis could be directed against either self-antigen or exogenous antigen expressed in the lungs of patients with cryptogenic fibrosing alveolitis. The data we have presented here show that the protein(s) detected by the cryptogenic fibrosing alveolitis autoantibody are present in both patients with sarcoïdosis and control non-interstitial lung disease tissue, suggesting that they are endogenous rather than exogenous. The staining pattern obtained on the control lung tissue extracts was weaker than on the interstitial lung disease lung extracts. This could be due to an increase in the result of the disease process, or an increase in the number of cells in the tissue expressing it. Recent studies of rat type I and II lung epithelial cells in culture have shown the presence of a variably glycosylated cell surface protein of 74–88 kDa which is present on the surface of both cell types. Inflammatory conditions of the lung, particularly cryptogenic fibrosing alveolitis, are known to be associated with proliferation and increased numbers of type II pneumocytes and this may be relevant to our findings.

We have also considered the possibility that the autoantibodies are detecting heat shock proteins of which there are many in this weight range. Patients with sarcoïdosis are, however, reported as having a high frequency of detectable antibodies to heat shock proteins, but in our study plasma samples from patients with sarcoïdosis did not show significant reactivity to the 70–90 kDa protein(s). Nor do we believe that the antigen is directed against immunologically active molecules such as MHC class II, which may be inducible on epithelial cells at sites of inflammation or immunoglobulin, as the frequency of positivity of plasma from patients with cryptogenic fibrosing alveolitis on tonsillar extracts, which contains an abundance of such molecules, was significantly lower than on the control lung tissue.

Our results have shown that patients with cryptogenic fibrosing alveolitis have a high incidence of circulating IgG autoantibodies to endogenous lung protein(s) which appear to be associated with alveolar lining cells. Most of these cases fail to recognise protein(s) of similar weight in non-pulmonary tissues, suggesting that it may be relatively lung-specific in its distribution. The antigenic protein(s) would appear to be upregulated in both lung tissue from patients with cryptogenic fibrosing alveolitis and sarcoïdosis, although the latter group do not seem to mount a humoral immune response. This suggests that the development of these autoantibodies is not simply the result of upregulation or release of...
sequestered protein(s) due to inflammation in the lung, but that other factors – possibly reflecting differences in the immunological pathogenesis of the two diseases – are involved.

We believe that this study represents the first conclusive evidence of circulating antibodies to protein(s) present in the lung in patients with cryptogenic fibrosing alveolitis.

The significance of this finding with regard to our understanding of the aetiology and pathogenesis of cryptogenic fibrosing alveolitis is, at this stage, unclear, but it reinforces the view that the role of the humoral immune system in the pathogenesis of cryptogenic fibrosing alveolitis deserves further consideration.

We would like to thank Mr E W Cameron and Mr W Walker of the Regional Thoracic Unit, City Hospital, Edinburgh for their cooperation in allowing the collection of blood samples and biopsy specimens from patients with cryptogenic fibrosing alveolitis suggesting local expression of immune hypersensitivity. Thorax 1985;40:405–11.


