Localisation of a pulmonary autoantigen in cryptogenic fibrosing alveolitis

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Abstract

Background – Cryptogenic fibrosing alveolitis (CFA) is believed to have an immunological pathogenesis with a persisting inflammatory reaction to an as yet unidentified pulmonary antigen(s). A high frequency of IgG autoantibodies has previously been found in the plasma of patients with CFA to an extractable 70–90 kDa lung antigen by Western blotting. Preliminary immunohistochemical studies with patient IgG had indicated that the target protein(s) might be associated with alveolar epithelial lining cells which have previously been suggested as the site of immunological attack in CFA.

Methods – In order to confirm this finding immunohistochemical analysis and Western blotting were performed on a human type II alveolar cell line (A549) using CFA patient plasma. In order to study further the distribution of the antigen, antibodies were raised in a rabbit to the partially purified 70–90 kDa CFA lung protein.

Results – The results showed that the human CFA autoantibody recognised a 70–90 kDa protein with a cytoplasmic distribution present in the A549 cells, confirming previous observations. The immune rabbit IgG recognised a protein of similar molecular weight by Western blotting of protein derived from lung biopsy samples of patients with CFA and A549 cells. In addition it immunoprecipitated protein(s) of this molecular weight from lung biopsy protein extracts from patients with CFA. The precipitated protein(s) were found to cross-react with the autoantibody found in the plasma of patients with CFA. Immunohistochemical analysis with the immunised rabbit antibody revealed positive staining of type I and II alveolar epithelial lining cells in CFA. A similar pattern of epithelial staining was also observed with the rabbit IgG on biopsy specimens of lung from patients with sarcoidosis and control lung tissue, although this was more focal and less intense. No positive staining was seen on sections from a number of non-pulmonary tissues (colon, liver, kidney, tonsil, lymph node, skin, cervix). Cytoplasmic staining of the A549 cell line was also detected.

Conclusions – The 70–90 kDa protein recognised by autoantibodies in patients with CFA is associated with pulmonary epithelial lining cells. The immune rabbit IgG produced appears to recognise antigen by Western blotting and immunohistochemical staining of lung tissue in a similar pattern to the patient autoantibodies. Immunohistochemical data obtained with this antibody suggest that the putative autoantigen against which patients with CFA mount a humoral immune response may be endogenous and specific to the lung.

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Cryptogenic fibrosing alveolitis (CFA) is characterised by progessive pulmonary fibrosis[1] often leading to death within five years from the time of diagnosis.[2] Although the aetiology remains uncertain, CFA is believed to have an immunologically mediated pathogenesis with a persisting inflammatory reaction to as yet unidentified antigen(s) within the lung.[3] Research interest has focused principally on the role of the cellular arm of the immune system and the interaction of T lymphocytes, monocytes, and lung fibroblasts in this disease process.[4]

We have recently described the presence of circulating IgG autoantibodies in patients with CFA which recognise a 70–90 kDa lung antigen on Western blot analysis.[5] These studies indicated that the antigen was likely to be endogenous as it was detectable in lung tissue from patients with sarcoidosis and in those with no evidence of interstitial lung disease. Preliminary immunohistochemical studies using IgG purified from patient and control plasma samples suggested that the antigen was associated with the alveolar lining cells of the lung. Whilst the role of these antibodies in the pathogenesis of the disease is unclear, morphological studies have suggested that alveolar lining cells may be the principal target of immunological attack in CFA[6] and previous reports have detected immune complexes at this site.[7]

In this study we report the results of experiments performed using an immunised rabbit IgG raised against the partially purified human 70–90 kDa lung protein. These experiments were designed to confirm our initial observations that the antigenic protein(s) detected by patient autoantibodies are associated with alveolar epithelial lining cells by studying tissue sections and using a human alveolar epithelial cell line (A549).[8]

Methods

CFA PATIENT AND CONTROL PLASMA SAMPLES
Twenty ml heparinised venous blood was obtained from 17 patients with histologically con-

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firmed CFA (11M:6F, median age (range) 68 (49–80) years) following approval by the local medical ethics committee. All the samples were obtained at the time of biopsy and none of the patients were on medication specific for their disease. The plasma fraction from each sample was recovered, aliquoted, and stored at −70°C.

Plasma samples were obtained in an identical fashion from 17 healthy volunteers within the Department of Pathology who had no evidence of respiratory symptoms and were on no medication (10M:7F, median age (range) 42 (28–58) years).

PREPARATION OF CFA LUNG BIOPSY PROTEIN EXTRACTS

Protein extracts were produced from tissue obtained at open lung biopsy from patients with histologically confirmed CFA by a method we have previously described.[5] The tissue was obtained fresh and snap frozen in lysis buffer containing 1% Nonidet P-40. It was then mechanically disrupted using a mortar and pestle and transferred to a universal container. A further 1 ml aliquot of lysis buffer was added and the container placed in an ultrasonic water bath for three minutes. The sample was then ultracentrifuged at 32 000 rpm for 10 minutes and the protein-containing supernatant removed and kept on ice. The protein content was then estimated using a commercially available colorimetric assay (BioRad UK).

A549 CELL LINE: PREPARATION OF PROTEIN EXTRACTS AND CYTOSPINS

The human A549 type II alveolar cell line[8] was grown in RPMI medium (Gibco BRL Ltd, UK) supplemented with 10% heat inactivated fetal calf serum (Gibco BRL Ltd, UK) in 5% CO_2 at 37°C. When confluent the monolayers were washed with phosphate buffered saline and harvested using a non-enzymatic cell dissociation buffer (Gibco BRL Ltd, UK). The harvested cells were then pooled and protein extracts produced for immunohistochemical analysis by making cytospin preparations at a density of 5×10^4 per slide followed by acetone fixation and storage at −20°C.

POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

The methods used in these experiments have been described in detail elsewhere.[5] Briefly, protein extracts produced from CFA open lung biopsy specimens and A549 cell pellets were run on 7·5% acrylamide gels under reducing conditions according to the manufacturer's protocol (BioRad Ltd, UK). The separated proteins were transferred to nitrocellulose sheets (Amersham, UK) and blotted with either human plasma or rabbit IgG.

Interaction between antibodies in human plasma and protein on the nitrocellulose was detected by an avidin–biotin alkaline phosphatase detection system which we have described elsewhere.[5] Interaction between rabbit IgG and the protein was carried

out by a similar method using a biotin conjugated swine anti-rabbit antibody (1:500; Dako UK Ltd) followed by the avidin–biotin-alkaline phosphatase complex (Dako UK Ltd). Visualisation in both cases was performed using the NBT/BCIP substrate (Sigma UK).

PRODUCTION OF ANTI-HUMAN SERUM

Partially purified antigen was obtained from lung biopsy material from patients with CFA for immunisation in two separate ways as we were unsure how antigenic the material produced would be.

(a) Protein extracts pooled from open lung biopsies from patients with CFA were separated by molecular weight on 7·5% polyacrylamide gels and transferred to nitrocellulose sheets as described above. The position of the 70–90 kDa protein(s) membrane on the membrane was identified by blotting one edge. The strip containing the protein(s) was then excised, solubilised in dimethylsulphoxide at 37°C, and reprecipitated with phosphate buffered saline (PBS) to give a fine particulate suspension of protein(s) bound to nitrocellulose.[9]

(b) IgG was purified from patient plasma using protein G (Pharmacia) in a manner we have previously described.[5] This was then used to construct an affinity purification column by linking the IgG to cyanogen bromide activated Sepharose beads (Pharmacia Ltd, UK) according to the manufacturer's protocol. The beads were then incubated at 4°C with protein extracts derived from CFA open lung biopsy samples overnight on a roller mixer. This technique resulted in the production of particulate Sepharose-IgG-protein(s) complexes which were recovered by centrifugation and washed with PBS.

The protein-nitrocellulose and Sepharose-IgG-protein complexes were combined and mixed with an equal volume of alum adjuvant (Pierce and Warriner) for immunisation. One female Dutch White rabbit (obtained from Hyline Commercial Rabbits Ltd, UK) was immunised by administering 1·5 ml of the mixture subcutaneously at two sites following the collection of baseline serum samples to act as non-immune controls. A total of three such injections were given at six weekly intervals following which test serum samples were obtained.

The control (baseline) and immunised serum samples were absorbed against human IgG-coated agarose beads to remove any anti-human IgG reactivity. IgG was then purified from both samples using a Sepharose-protein A column (Pharmacia Ltd, UK) and adjusted to give a final total IgG concentration of 1 mg/ml.

IMMUNOPRECIPITATION

Immunoprecipitation of the antigenic protein from lung tissue obtained from patients with CFA was performed using the immunised rabbit serum according to an established protocol.[10] One hundred and fifty μl (2·25 mg) of
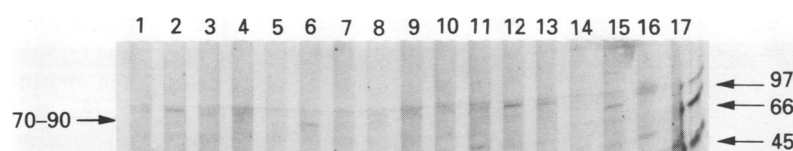
Figure 1 Western blot showing the results obtained by blotting 17 plasma samples obtained from patients with CFA on a protein extract produced from the A549 human type II alveolar epithelial cell line. The molecular weight markers are indicated in kDa on the right. Positive bands are identified in the 70–90 kDa range in 12 of the 17 plasma samples (lanes 1, 2, 3, 4, 6, 8, 11, 12, 13, 15, 16, and 17).

pooled protein extract, derived from four CFA open lung biopsy samples as described above, was incubated on ice with 7·5 µl control rabbit serum to preclear the extract. The rabbit immunoglobulin and any complexes that had formed were removed by adding 100 µl of a 10% w/v suspension of protein A-conjugated Sepharose beads and incubating on a rocker at room temperature for 30 minutes. The mixture was then ultracentrifuged at 13 000 rpm for 10 minutes, the supernatant retained, and the beads discarded. Immunised rabbit serum was added to give a 1:10 dilution of serum in the protein extract solution. This was incubated for two hours on ice. A further 100 µl of the 10% protein A-Sepharose suspension was added to recover the immunised rabbit immunoglobulin antigen complexes. The mixture was incubated for two hours at room temperature on a rocker and the beads with attached immunoglobulin and complexes were recovered by ultracentrifugation at 13 000 rpm for 10 minutes. The beads were then washed twice in high salt buffer, twice in low salt buffer, and once in a no salt buffer prior to boiling for five minutes in a polyacrylamide gel electrophoresis sample buffer (3·6 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) to release the immunoglobulin from the beads and break the complexes with the precipitated cellular proteins. The sample was centrifuged at 13 000 rpm for 10 minutes, the supernatant removed and run on 7·5% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose membranes as described above. The polyacrylamide gels were then stained with Coomassie Blue (Sigma UK) to determine whether any protein had been precipitated.

To determine if antigen precipitated from the lung biopsy extracts with the rabbit serum samples was recognised by the human autoantibody the membrane was blotted with patient plasma. Since the detection system employed utilised a monoclonal anti-human IgGγ which we knew to crossreact with rabbit IgG (WAH Wallace, personal observation), a second parallel blot was performed omitting the patient plasma. This step was required to identify the position of the rabbit IgG on the blot and to differentiate precipitated human pulmonary proteins from rabbit immunoglobulin.

IMMUNOHISTOCHEMISTRY WITH IgG FROM PATIENTS WITH CFA AND CONTROLS
Acetone-fixed cytospin preparations of A549 cells were stained with purified IgG from serum

samples from patients with CFA and healthy controls using a technique we have previously described.[5] Briefly, the specimens were incubated with the IgG at a standard concentration (0·02 µg/100 µl) and adherent antibody was detected using a protein A-alkaline phosphatase conjugate (Sigma UK) followed by visualisation with Vector Red (Vector Laboratories UK). Sections were then viewed on a confocal laser microscope (Zeiss).

IMMUNOHISTOCHEMISTRY WITH RABBIT IgG
Acteone-fixed cryostat sections were obtained from eight cases of CFA, two cases of sarcoidosis, and from two specimens from pneumonectomy procedures performed for malignant disease and showing no histological evidence of interstitial lung disease. Sections were also obtained and processed in an identical manner from histologically normal skin, tonsil, lymph node, colonic mucosa, cervix, liver, and kidney. Cytospin preparations of A549 cells, peripheral blood mononuclear cells, human T cell lines (H9 and Jurkat), and primary cultured chondrocytes were obtained in a similar fashion from other groups in the laboratory and prepared as described above.

All the cases were stained with the control and immunised rabbit IgG diluted 1:10 using a standard ABC-alkaline phosphatase method. Visualisation was performed using the Vector Red substrate with the inclusion of 1 mmol/l levamasole to block endogenous alkaline phosphatase activity. For each case a further section was included where the rabbit IgG was omitted to act as a negative control. The sections were counterstained, dehydrated, mounted, and viewed by light microscopy.

IMMUNOHISTOCHEMICAL DEMONSTRATION OF IMMUNE COMPLEXES IN LUNG TISSUE FROM PATIENTS WITH CFA
Identification of immune complexes in lung biopsy specimens obtained from patients with CFA was carried out using an antibody to C1q, an activated fragment of complement. Formalin-fixed paraffin embedded biopsy tissue was obtained from the departmental archives from 12 cases of CFA. Sections from these cases were stained using a standard indirect immunoperoxidase method with an antibody to C1q (1:500, Dako UK) and visualisation with diaminobenzidene (Sigma UK). Endogenous peroxidase activity was blocked with H_2O_2.

Results
DETECTION OF ANTIGEN IN A549 CELLS BY WESTERN BLOTTING AND IMMUNOHISTOCHEMISTRY WITH PLASMA OBTAINED FROM PATIENTS WITH CFA
Figure 1 shows the results obtained from blotting plasma samples from 17 patients against protein derived from the A549 cell line. Of the 17 patients 12 (71%) had IgG which recognised a protein in the 70–90 kDa range on the blot compared with only three (18%) of the 17 healthy control plasma samples ($p<0·01$, χ^2
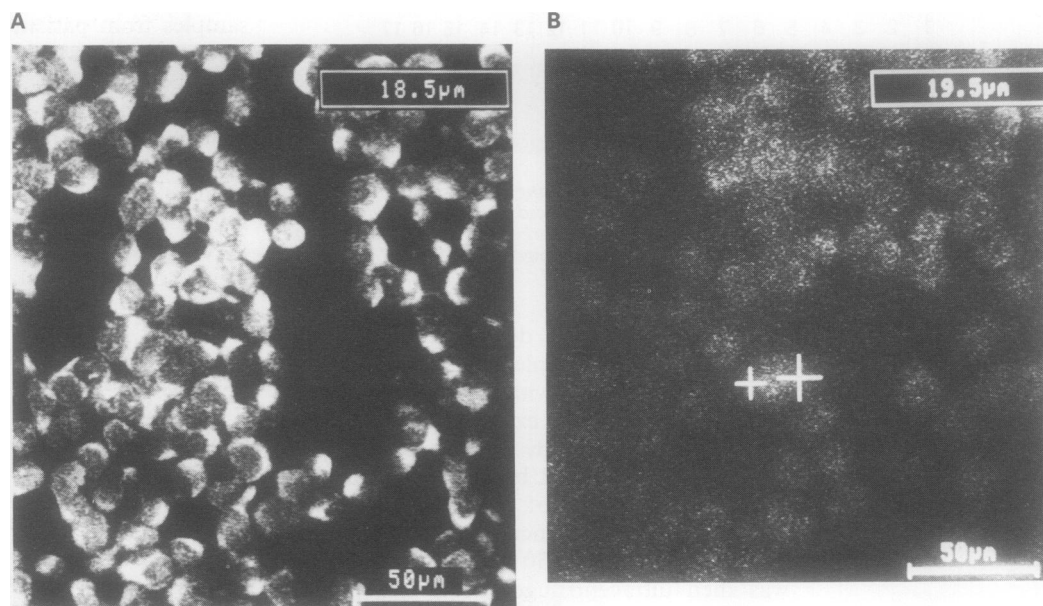
Figure 2 Photomicrograph obtained using the Confocal laser microscope in ultraviolet mode of A549 cells stained with purified IgG (20 µg) from (A) plasma obtained from patients with CFA and (B) control plasma. Strong cytoplasmic staining of the cells can be seen with the CFA IgG which is not present in the slide synchronously stained with the control IgG.
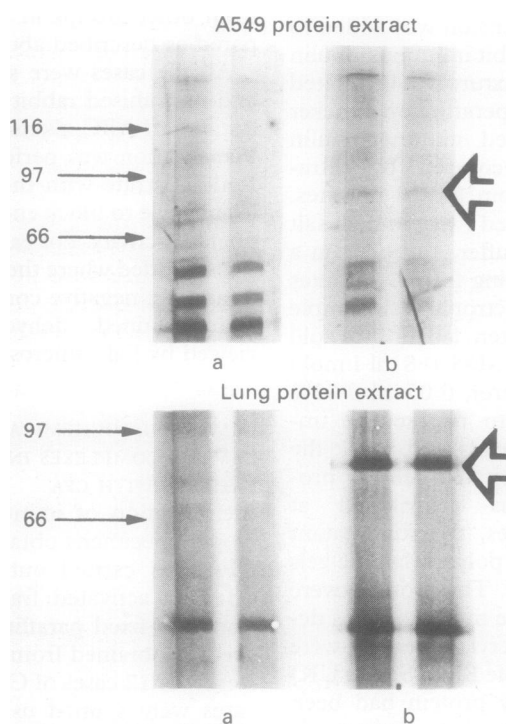

Figure 3 Western blots obtained with the non-immunised control rabbit IgG (a) and the immunised rabbit IgG (b) with protein extracts obtained from A549 cell pellets and lung biopsy tissue obtained from patients with CFA. The molecular weight markers are shown on the left. Positive bands can be seen on both blots with the immune IgG in the 70–90 kDa range (open arrows) that are absent with the control IgG.

Figure 4 Polyacrylamide gel stained with Coomassie Blue. The left lane shows the position of bands following disassociation of the precipitated human proteins from the immune rabbit IgG. The banding pattern obtained with the protein extract used in the experiment is shown alongside for comparison. The molecular weight markers are shown to the right. Bands of 55 and 110 kDa are observed which are believed to correspond to the rabbit IgGγ chains. In addition, however, fainter bands at around 80 and 160 kDa are present which may represent monomeric and dimeric forms of the pulmonary protein to which the rabbit antibody was raised.

test). Immunohistochemical staining of A549 cell line cytospin preparations are shown in fig 2. IgG derived from plasma samples from patients with CFA, but not controls, gave strong positive cytoplasmic staining of the cells.

WESTERN BLOTTING WITH RABBIT IgG

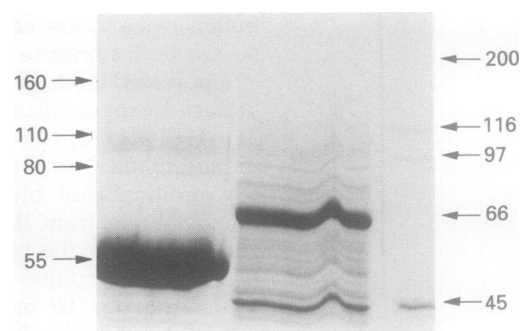Figure 3 illustrates the results obtained from blotting the control and immunised rabbit IgG on protein extracts from lung biopsy specimens obtained from patients with CFA and A549 cells. Immune IgG, but not control IgG, recognised a protein in the 70–90 kDa region of blots produced with protein from both sources.

IMMUNOPRECIPITATION

Staining of polyacrylamide gels containing the precipitated human lung protein(s) and disassociated rabbit IgG revealed the presence of several distinct bands (fig 4). The most prominent band was identified at around 55 kDa with another at around 110 kDa. These appear to represent monomeric and dimeric
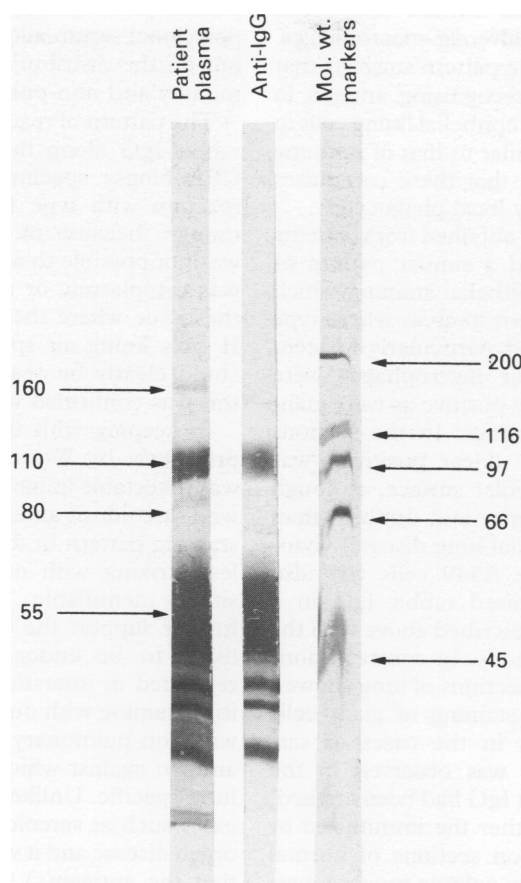
Figure 5   Western blotting of protein immunoprecipitated from lung biopsy material obtained from patients with CFA using immune rabbit IgG with and without patient plasma. The molecular weight markers are indicated on the right. The bands in the 80 and 160 kDa range are only detected in the presence of patient plasma indicating that they are not fragments of the rabbit IgG used in the experiment. The bands in the 50 and 110 kDa range, however, are positive in both blots confirming their nature as immunoglobulin.

IgG heavy chain (55 kDa) of the rabbit immunoglobulin used to precipitate the antigen. Further smaller bands were also noted at lower molecular weights and may represent either light chains or digestion fragments of the larger proteins.

In addition to bands attributable to the rabbit IgG, a band was identified in the 70–90 kDa region with a second around 150–160 kDa. The molecular weights of these bands correspond to the predicted position of monomeric and dimeric forms of the protein(s) which the rabbit and human autoantibodies recognise on Western blots performed with the lung protein extracts.

In order to confirm the nature of the various bands, the precipitated protein(s) were blotted in parallel, with and without patient plasma (fig 5). This was necessary as we were aware that the anti-human IgG antibody used in our detection system crossreacted with rabbit IgG. Using this strategy we were able to exclude the possibility that all the bands were due to rabbit immunoglobulin as the 70–90 and 150–160 kDa bands were only detected when the patient plasma was present. In addition, this confirmed that the 55 and 110 kDa bands were due to the rabbit immunoglobulin.

This result confirmed our results obtained by Western blotting which showed that the immunised rabbit IgG recognised a 70–90 kDa lung protein and that the protein to which the animal had raised antibodies was also recognised by the human CFA autoantibody.

IMMUNOHISTOCHEMISTRY

Sections from biopsy specimens obtained from patients with CFA stained with the immunised rabbit IgG (fig 6) showed strong positive staining along the edge of the alveolar surface in a manner suggestive of reaction with type I alveolar lining cells. In addition, strong cytoplasmic staining of hyperplastic type II cells was apparent and focal positivity was noted along the surface of ciliated bronchial mucosal cells. Alveolar macrophages were also noted to show cytoplasmic staining. This pattern of staining was very similar to that seen with the antibody to C1q (fig 7) which showed patchy
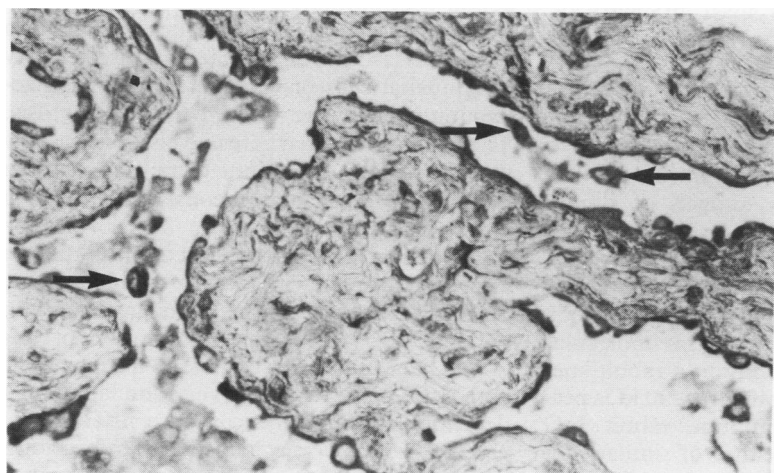

Figure 6   Cryostat section of an open lung biopsy specimen from a patient with CFA stained using immunised rabbit IgG and an immuno-alkaline phosphatase method with visualisation using the Vector Red substrate (original magnification × 250). Positive staining of alveolar epithelial lining cells and some alveolar macrophages (arrows) can be identified.
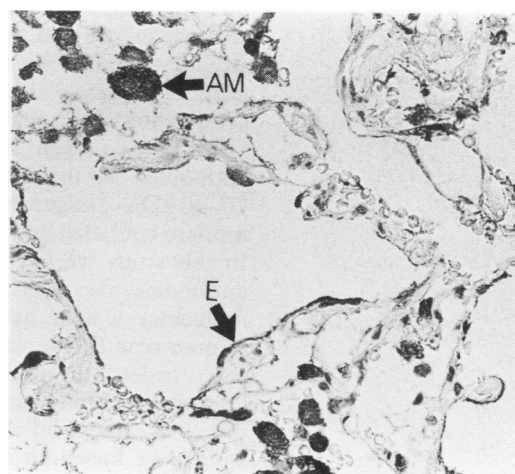

Figure 7   Formalin-fixed paraffin-embedded tissue from an open lung biopsy specimen obtained from a patient with CFA stained using an antibody to C1q in order to demonstrate the distribution of immune complexes in the lung (original magnification × 200). Positive staining can be identified along the surface of the air space associated with the alveolar epithelial lining cells (E). In addition there is prominent cytoplasmic staining of alveolar macrophages present in the air spaces (AM).

positivity on the alveolar surface and dense cytoplasmic staining of alveolar macrophages. This similarity of staining pattern suggests that the rabbit antibody is recognising antigen in association with alveolar epithelial lining cells in a distribution that is similar to that of immune complex formation and that these complexes may be being cleared by local phagocytes.

Sections of lung tissue obtained from patients with sarcoidosis showed a similar pattern of reactivity with linear epithelial staining which appeared more prominent in areas where type II cells could be identified, particularly adjacent to granulomas. Alveolar macrophages were again noted to be focally positive as were giant cells within the granulomas. In the sections of control lung patchy linear positivity was observed along the alveolar surface, although this was much less intense and uniform than in patients with interstitial lung disease. Cytoplasmic staining of the A549 cells was also noted with the immunised rabbit IgG in a similar pattern to that described above with the native patient autoantibody. In contrast, non-immunised IgG on the sections of lung showed only faint cytoplasmic staining of giant cells within the granulomas in the cases of sarcoidosis. No positivity was observed in the sections where no rabbit IgG had been applied.

No positivity with either the immunised or control IgG was seen on sections of normal skin, tonsil, lymph node, colonic mucosa, cervix, liver, and kidney or cytospins of peripheral blood mononuclear cells, human T cell lines (H9 and Jurkat), and primary cultured chondrocytes. All were stained under identical conditions with the inclusion of A549 cell cytospins as a positive control in each experiment.

Discussion

CFA is believed to have an immunologically mediated pathogenesis. The nature and location of the antigen(s) are unknown but are believed to be associated with pulmonary epithelial lining cells and, perhaps, with capillary endothelial cells on the basis of ultrastructural studies[6] and the previous demonstration of immune complexes at these sites.[7] We have recently shown that patients with biopsy proven CFA have a high frequency of IgG autoantibodies in their plasma which recognise 70–90 kDa antigen(s) associated with pulmonary epithelial lining cells in tissue sections.[5] In this study we have shown that these autoantibodies also recognise antigen of similar molecular weight associated with the A549 human type II alveolar cell line.

In order to confirm that the immunochemical staining was due to an antibody directed against an antigen of this molecular weight we raised an antibody in a rabbit specifically to the partially purified 70–90 kDa pulmonary antigen. The immune rabbit IgG obtained recognised protein(s) of similar molecular weight to the patient autoantibody in protein extracts produced from lung tissue from patients with CFA and A549 cells. In addition, the ~80 kDa protein precipitated by the rabbit IgG showed crossreactivity with the patient

plasma. The development of this anti-human polyclonal serum allowed studies to be carried out on the distribution of the antigen in pulmonary and non-pulmonary sites.

The pattern of reactivity with the immunised rabbit IgG along the alveolar surface of the CFA biopsy specimens was suggestive of a reaction with type I alveolar lining cells although, because of their flat morphology, it was not possible to assess whether the staining was cytoplasmic or membranous. In areas of the tissue where there were hyperplastic type II cells lining air spaces the staining pattern could clearly be seen to be cytoplasmic and this was confirmed with the A549 cells.

In keeping with the data we had obtained previously by Western blotting,[5] the antigen was detectable in biopsy material from patients with sarcoidosis and in control lung tissue. The staining pattern in the control lung tissue was less striking with only patchy epithelial positivity identifiable. This result did, however, further support the view that the antigen was likely to be endogenous and may be upregulated in interstitial lung disease. No positive staining with our rabbit IgG was obtained with non-pulmonary tissues suggesting that the antigen against which it was directed may be lung-specific. Unlike other interstitial lung diseases such as sarcoidosis, CFA is not a multi-organ disease and it would therefore seem likely that the antigen(s) which are responsible for the maintenance of the inflammatory process are restricted to the lung. Although the nature of the antigen is not yet known, pulmonary epithelial cells are undoubtedly highly specialised and it is therefore not surprising that they express unique antigens not present at other sites.

The significance of the autoantibody response in the pathogenesis of CFA is unclear. The antigen we have shown appears to have a cytoplasmic distribution, although we can detect it in bronchoalveolar lavage samples from some patients with CFA (unpublished data). This suggests that the antigen may be released from epithelial cells, possibly following cell injury. The resulting antibody–antigen interaction with immune complex formation could have a significant role in the perpetuation of the disease process, either by direct injury of epithelial cells or via local macrophage activation as they are cleared by phagocytosis. The results presented here and elsewhere[7] show that the pattern of immune complex formation in CFA has a distribution consistent with such a hypothesis.

We believe that this study confirms that an antigen associated with alveolar epithelial lining cells is the target for IgG autoantibodies in patients with CFA. The distribution of this 70–90 kDa antigen in lung tissue from patients with CFA appears to be very similar to that described for immune complexes. This suggests a possible mechanism whereby autoantibodies combining with this antigen may, at least in part, account for some of the persisting immunological reaction observed in these patients, perhaps via complement activation and epithelial cell injury.

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