Primary research

Private specificities can dominate the humoral response to self-antigens in patients with cryptogenic fibrosing alveolitis

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Abstract

Background: The pathogenetic mechanisms that underlie the interstitial lung disease cryptogenic fibrosing alveolitis (CFA) may involve an immunological reaction to unidentified antigens in the lung, resulting in tissue damage.

Method: In order to identify the range of target autoantigens, we used expression cloning, employing serum from an index patient as the probe against an expressed cDNA library that was derived from a tumour cell line. We screened over 5×10^5 recombinants and obtained sequence information on three antigens that had provoked strong responses with immunoglobulin heavy chain class switching, presumably as a consequence of T-cell recognition.

Results: All of the antigens were identifiable by comparison with sequence data from the US National Center for Biotechnology Information. Alanyl tRNA synthetase (ATS) was picked on six occasions; five of these incidences reflected independent recombination events, indicating that the library was not biased. Antibodies to ATS (anti-PL-12) represent the most common reactivity that defines the antisynthetase syndrome, which is typically expressed as polymyositis, dermatomyositis and interstitial lung disease (ILD). The index patient never showed symptoms other than those associated with alveolitis, even though sera obtained from him over a period of 2 years contained antibodies with the same specificity. Autoantibodies to ATS were never detected in serial bleeds from 11 other patients with CFA, and neither did we detect antibodies to the other two antigens identified from the serum of the index patient.

Conclusion: The humoral response in patients with CFA can be dominated by autoantibodies with private specificities. This suggests that the antibodies are epiphenomenal and are a secondary feature of tissue damage induced by some other mechanism.

Keywords: antisynthetase syndrome, cryptogenic fibrosing alveolitis, diagnostic autoantibodies

Introduction

The ILDs are a heterogeneous group of disorders, the most common of which is CFA. The latter is an inflammatory condition of the lungs that results in scarring, pulmonary failure and death. The aetiology of the disease is unknown, but the pathogenesis may involve an immunological reaction to unidentified antigens in the lung, resulting in tissue damage. Recent reports suggest a higher prevalence of CFA than was previously documented (13.2–20.2/100,000 population) and a rising mortality rate. The prognosis is universally

ATS = alanyl tRNA synthetase; CFA = cryptogenic fibrosing alveolitis; EST = expressed sequence tag; IGBP1 = α_4 immunoglobulin-binding protein; ILD = interstitial lung disease; SAGE = serial analysis of gene expression; SLE = systemic lupus erythematosus.

poor, with 50% of patients dying within 5 years. Although approximately 30% of patients may live for long periods (>10 years), morbidity is significant and quality of life in long-term survivors is poor [1].

Diagnosis is traditionally based on an open lung biopsy, but more recently high-resolution computed tomography has been used. It has been shown that CFA is associated with the production of circulating IgG autoantibodies to antigen(s) that are associated with alveolar lining cells, and there is evidence for a local humoral immune response associated with B-lymphocyte aggregates in the lungs [2–4]. The presence of large numbers of B lymphocytes in open lung biopsies may be associated with a poor prognosis, but it should be noted that B cells are not a prominent feature of this disease. Although serum levels of anticytokeratin 8 antibody are increased, it is not clear whether they are involved in the pathological process or are epiphenomenal [5]. Antinuclear antibodies have long been recognized as a feature of CFA [6]. Antitopoisomerase II antibodies have also been found in approximately 37% of sera from patients with CFA [7,8]. In addition, antibodies to poly (ADP-ribose) polymerase have been identified in up to 25% of patients [9].

Serological identification of antigens by recombinant expression cloning can readily be applied to define antigens at the molecular level. We applied the technique using serum from a patient with the canonical features of CFA.

Patients and method

Patients

Eleven patients with CFA (six male, five female), eight with sarcoidosis (five male, three female) and one with systemic lupus erythematosus (SLE; female) were recruited from the relevant clinics at the Sir Charles Gairdner Hospital, Perth, Western Australia. The diagnosis of CFA was based on the following: presence of diffuse mid-, late or pan-inspiratory crackles, with or without clubbing; the absence of clinical, radiological or laboratory evidence of any other ILD; no exposure to agents that are known to be able to induce ILD (ie dusts, such as asbestos; drugs, such as nitrofurantoin, amiodarone and bleomycin; and biological antigens, such as pigeon droppings and inhaled fungi); diffuse reticular shadowing on chest radiography without pleural or hilar disease; a high-resolution thoracic computed tomography scan pattern consistent with CFA; and lung function tests that revealed the presence of restriction with reduced gas transfer.

The index case had clubbing and crackles, the radiological pattern referred to above, negative antinuclear antibodies and negative rheumatoid factor, a transfer factor that was 56% of the predicted value and an arterial partial oxygen tension of 63 mmHg. All patients with sarcoidosis had a clinical pattern that was consistent with sarcoidosis plus histological evidence of noncaseating granulomata in the absence of any evidence for other granulomatous disease, such as tuberculosis or fungal disease. The patient with SLE had typical subacute cutaneous lupus with breathlessness and linear atelactases, but borderline interstitial disease. She exhibited high levels of circulating anti-SSA, but was negative for anti-DNA antibodies.

Sera

Blood was obtained by venipuncture from patients attending the respiratory clinic at the Queen Elizabeth II Medical Centre in Perth, Western Australia. Patients included 11 with CFA, eight with sarcoidosis, three with ILD and one with SLE. In addition, sera were obtained from four healthy laboratory volunteers aged between 29 and 58 years. Blood was allowed to clot at room temperature, and the clots were allowed to retract overnight at 4°C. Sera were clarified by centrifugation and stored in aliquots at –20°C. These procedures were all approved by the Human Rights Committee of the University of Western Australia, fulfilled National Health and Medical Research Council of Australia Guidelines on Human Experimentation, and informed consent was obtained from all patients before enrollment in the study.

Construction of λ expression library

cDNA was prepared from a tumour cell line, AB1, using *Xho* I tagged oligo dT as a primer. cDNA was blunted, capped with *Eco* RI adapters and ligated into the λzap vector (Stratagene, La Jolla, CA, USA). The library, with a complexity in excess of 1×10^6 , was amplified and the phage stored at 4°C at a concentration of 1×10^{10} pfu/ml. The production and characterization of this library has been described in detail elsewhere [10].

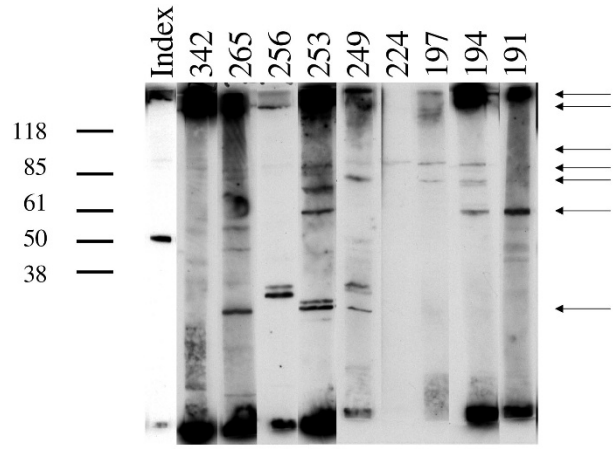
Screening of the library

The index serum was used at a dilution of 1/100. In order to remove any background reactivity to bacterial proteins, antibodies from the serum were absorbed onto an *Escherichia coli* lysate by admixture followed by centrifugation. The process was repeated three times. A total of 5×10^5 plaques were screened using the serum, and positives were identified using an alkaline phosphatase conjugated antihuman IgG (Promega, Madison, WI, USA) and the picoBLUE immunoscreening kit (Stratagene). The positive clones from the primary screen were picked and replated over several rounds until they were monoclonal. *In vivo* excision of the purified plaques as the pBluescript phagemid was carried out using the ExAssist helper phage, as described by the manufacturer (Stratagene). Inserts were sequenced by standard dideoxy chain termination.

Freckle assay

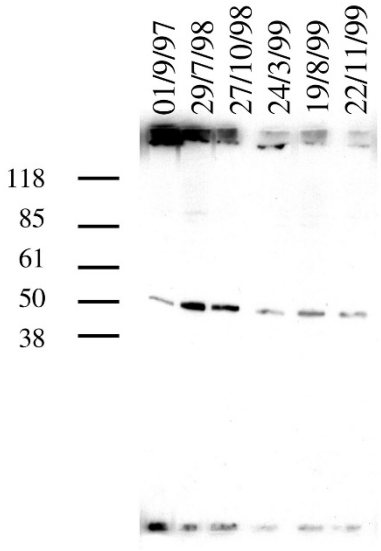
Clonal phage preparations were titrated and applied to a bacterial lawn using a plate replicator to achieve a plaque density of approximately 100/cm^2. Individual filter lifts

Figure 1



Sera from 10 individuals with a confirmed diagnosis of CFA were used to probe a western blot prepared by electrophoretic separation of whole cell (A549) extract. Molecular weight markers (kDa) are shown to the left, and common reactivities are noted (arrows) to the right.

Figure 2



Western blot (as Fig. 1) using serial blood samples from the index patient over a period of 2 years (dates marked at the top). Molecular weight markers (kDa) are shown to the left.

containing plaques from each of the clones of interest were then tested with each of the sera and developed as indicated above. A reaction was scored on a scale from negative to +++ within each filter.

Results

The diversity of the autoantibody repertoire in cryptogenic fibrosing alveolitis

In order to determine the specificity and diversity of autoantibodies in patients with CFA, we used western blots. Patients' sera were applied to blots derived from cell lines that had originated from the lung. We chose to make cell extracts from A549, a lung adenocarcinoma cell line, and from Ju77, a mesothelioma cell line. The 10 sera showed some unique reactivities, but also potential common reactivities to antigens expressed by A549 (Fig. 1). The pattern of reactivity was identical in the two cell lines (data not shown). We could identify seven regions on the blot that might represent serological responses to a common antigen in these patients (Fig. 1). We therefore selected a patient who expressed both common and unique features for further study. This patient demonstrated a remarkable stability in the profile of autoreactivity over a period of 3 years (Fig. 2).

Molecular cloning of antigens associated with cryptogenic fibrosing alveolitis

A λ expression library with a complexity in excess of 1 million was plated at a density of 3000 plaques/cm^2. A total of 5×10^5 plaques were tested for reactivity with the serum of a patient with CFA diluted 1/100. Twenty-two primaries were picked; of these eight retested positive in third-round screening, at which stage they were all clonal. Purified plaques were excised as the pBluescript plasmid and were subjected to sequence analysis. Seven different cDNAs were identified from the sequence information (Table 1). ATS was picked six times from different primary plaques; five of these six were different recombinants. Two of these encoded in-frame fusions that incorporated either 39 or 59 amino acid residues from the large open-reading frame derived from the 5′-untranslated region of the parent cDNA. The others encoded truncated proteins, missing the amino-terminal 38, 54 or 447 residues of the parent 968-residue polypeptide, indicating that at least part of the immune response was directed towards epitopes expressed by the carboxyl half of the protein. As one might expect, ATS is well expressed in most cells. Serial analysis of gene expression (SAGE) libraries [11] indicate that the gene is highly expressed in normal epithelial cells, fibroblasts and a variety of tumour cells.

One recombinant encoded an open-reading frame of 131 amino acids. This cDNA has previously been identified and sequenced from a size-fractionated library derived from human brain. The hypothetical protein shows some similarity to the adapter moiety vav2, which functions in some tyrosine kinase-dependent signalling pathways, but is more highly related to a gene from *Caenorhabditis elegans* named F35A5.8. The function of this gene in the worm is not clear. Analysis of SAGE libraries shows that the gene is highly expressed in normal epithelial cells and fibroblasts. The third recombinant encoded the carboxyl-terminus of the α_4 immunoglobulin-binding protein (IGBP1) gene, which has sequence similarity to the yeast protein (TAP42).

Table 1

Identity of three CFA antigens and the frequency of serological responses to them

ID	Insert size (kb)	Full length (kb)	Identity	Accession	Unigene	CFA
141/2A	3.6	3.6	ATS	D32050	HS75102	1/12
144/1A	3.5					1/12
146/2	3.2					1/12
151/1A	3.2					1/12
147/5A	2.1					1/12
147/5	0.7	1.3	IGBP1	NM_00155	HS136309	1/12
			(A4PP)	1		
147/4B	0.75	1.2	Brain EST	AB007960	HS3631	1/12

A stretch of consensus motifs in the carboxyl-terminus is conserved among the related genes of human, mouse, yeast and rice. IGBP1 is involved in a rapamycin-sensitive signal transduction pathway in B cells. The gene has previously been shown to be expressed as a 1.4 kb mRNA transcript in most lymphoid tissue, but also in heart, brain, placenta, skeletal muscle, kidney and pancreas. An antihuman IGBP1 antibody detects a 45 kDa protein in human lymphoid cell lines [12]. Interestingly, SAGE libraries show that the highest level of expression of this gene is in normal epithelial cells derived from breast.

Serological recognition of the antigens

We then investigated whether the occurrence of antibodies to each of the antigens was associated with individual cases of CFA, or with other respiratory diseases of diverse aetiology.

Purified phage isolates were allowed to form plaques that were induced with isopropyl-β-thiogalactopyranoside, and the recombinant proteins were transferred to nitrocellulose membranes. Replicate membranes were probed with serum (1/50 dilution) and processed as described in Patients and method. Plaques were scored positive by visual inspection and in relation to the each of the other plaques on the membrane. We screened sera from 11 other patients with CFA, nine patients with sarcoidosis, and one patient with SLE. In addition, we screened the sera of 15 normal healthy laboratory volunteers. We found no evidence of antibodies to ATS, IGBP1 or the brain expressed sequence tag (EST) in any of these sera. Convincingly, the index case retained a strong reaction to each of these antigens in sera obtained consecutively over the following 18 months.

Discussion

It is evident that the humoral immune system plays some role in the pathogenesis of CFA. It is less clear whether this role is causative or merely epiphenomenal. Although circulating autoantibodies to lung protein(s) have been mooted as the pathogenic mechanism, relatively few antigens have actually been characterized and none of these is accepted as diagnostic for the disease. An autoantibody reactive to cytokeratin 8 was defined by western immunoblotting and by enzyme-linked immunosorbent assay [5]. Similar data support a role for antibodies that are reactive with cytokeratin 19 in the process of lung injury in pulmonary fibrosis [13]. The majority of autoantibodies appear directed against antigens that are not specifically localized to lung tissue, for example antibodies to DNA topoisomerase IIα, a nuclear antigen that is constitutively expressed by dividing cells. These antibodies are prevalent in the sera of some patients, and analysis of the specific patterns of reactivity with various deletion recombinants revealed the existence of multiple epitopes in the protein [14]. Interestingly, the authors of that study noted a correlation between the type of epitope and the disease duration, supporting the hypothesis that the autoantibody response is an antigen-driven process.

Not many of the antigens associated with CFA actually show a tissue-specific pattern of expression that is limited to the lung. This is surprising because the disease is explicitly manifest as a pulmonary lesion. Lungs may be targeted simply because their high blood flow means that they are more likely to trap immune complexes. Alternatively, the majority of antibodies may be epiphenomenal and associated with tissue damage caused by the primary lesion. Two of the antigens described here show strong association with epithelial cells, although they are clearly expressed in a wider range of tissues. The third antigen, ATS, is expressed in all tissues at high levels. This high level of expression is evident from the finding of six independent clones encoding ATS in the library that we probed. The mRNA source of this library originated from a lung tumour cell line. We chose this library because of its

complexity and because we knew that it contained large inserts. In addition, western blots using patients' sera strongly suggested that there was no bias in the target tissue for the dominant antigens. The SAGE analysis, through its virtual northern programme, showed that each of the antigens that we cloned are widely expressed.

Each of the reactivities that we defined is associated with T-cell recognition of the antigen. This follows simply because we used an isotype-specific second antibody, and class switching to IgG is a T-cell-dependent event. It seems likely then that the spectrum of antigen reactivity in the serological response is dictated by the availability of autoreactive T cells. How these arise, particularly to highly expressed ubiquitous proteins such as ATS, and why subsets of antinuclear antibodies to ubiquitous determinants define particular syndromes remain unresolved issues.

The antisynthetase syndrome is such a syndrome. It typically associates polymyositis and dermatomyositis with complicating ILD, Raynaud's phenomenon and frequently polyarthritis. Autoantibodies against aminoacyl-tRNA synthetases (antisynthetases) define the syndrome. Patients can present with ILD and antisynthetase antibodies, but no clinically evident myositis. Previous studies have shown that anti-PL-12 antibodies (anti-ATS) are the most common in this group, but anti-Jo-1 (antihistidyl-tRNA synthetase), anti-EJ (antiglycyl-tRNA synthetase) and anti-OJ (anti-isoleucyl-tRNA synthetase) also occur [15,16]. Patients usually exhibit reactivity to only one of the synthetases, but different subsets have occasionally been recorded together in the same patient [17]. Of interest, autoantibodies to synthetases have been found in mice with graft-versus-host disease [18], suggesting that they may be a secondary consequence of tissue damage and cell death.

The serum samples were obtained from 3 months to 4 years after the onset of disease in the patients with CFA, and 1–5 years after presentation in the sarcoidosis patients. The SLE patient had been diagnosed with the disease for 3 months. Immunosuppressive therapy, including oral prednisolone, was being taken by five of the CFA patients and by four of the sarcoidosis patients. The index patient was on no treatment when the initial sample was obtained, but he was on immunosuppressive therapy when the later samples were obtained and was improving physiologically. The SLE patient was receiving plaquenil only. Taken together, it seems unlikely that therapy was associated with the negative results because not all patients were on steroids and because steroids made no difference to the results in the index case.

Conclusion

CFA can involve an inappropriate immunological reaction in the lung interstitia. It is not clear what promotes and maintains this reactivity. We anticipated that the targets of the humoral immune response might give us clues to the originating insult. We demonstrated by western blot that there were likely to be common as well as unique antigenic specificities in the patient population studied. However, by molecularly cloning and identifying the antigens that were recognized by our index patient, we were able to demonstrate that they were not recognized by any other patient. CFA may represent the end stage of a number of different disease processes, some of which may be autoantibody mediated. It is also possible that autoantibodies are epiphenomenal to CFA, representing a secondary feature of tissue damage induced by some other mechanism.

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