Autoantibodies against bactericidal/permeability-increasing protein in patients with cystic fibrosis

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Summary

Cystic fibrosis (CF), a genetic disorder, is characterized by chronic pulmonary infection/inflammation which leads to respiratory failure. The presence of anti-neutrophil cytoplasmic autoantibodies (ANCA) has previously been observed in the sera of patients with CF. In view of the known relationship of ANCA with primary vasculitis and of their putative pathogenetic role in these disorders, we studied the presence, specificity and isotype of ANCA and their clinical associations in 66 adult CF patients. None of the 66 CF samples had autoantibodies to the major ANCA antigens, proteinase 3 or myeloperoxidase. However, 60/66 (91%) CF samples contained IgG, and 55/66 (83%) IgA, autoantibodies to bactericidal/permeability-increasing protein (BPI), a recently-characterized ANCA specificity. All the IgA anti-BPI-positive samples were also IgG anti-BPI-positive. The autoantibody specificity was confirmed by inhibition assay and immunoblotting of CF sera against a neutrophil granule preparation. Furthermore, in this cross-sectional study, anti-BPI levels were inversely correlated with the observed reductions in FEV1 and FVC (IgA anti-BPI & FEV1: r = -0.508, p < 0.0001), and both IgG and IgA anti-BPI levels were higher in CF patients with secondary vasculitis (n = 6) than in those without (p < 0.05). ANCA with specificity for BPI were present in the majority of CF sera in this study and autoimmune processes may be associated with the development of pulmonary injury in CF.

Introduction

Cystic fibrosis (CF), the most common lethal inherited disorder among Caucasian populations, is a recessive disorder resulting from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, a member of the ATP binding cassette (ABC) superfamily, located on the long arm of chromosome seven, that is thought to encode a cAMP-regulated chloride ion channel. CF is characterized by chronic pulmonary infection/inflammation with colonization by Gram-negative bacteria and progressive pulmonary damage, as well as pancreatic insufficiency. There is prominent pulmonary neutrophil infiltration, and levels of the neutrophil enzyme elastase found in the sputum of CF patients are so high as to overwhelm the host's elastase inhibitor α1-antitrypsin. In addition, CF is associated with various autoimmune phenomena, including arthropathy, liver disease resembling sclerosing cholangitis, and both cutaneous and systemic vasculitis. Anti-neutrophil cytoplasmic autoantibodies (ANCA) with specificity for human neutrophil proteinase 3 (PR3) and myeloperoxidase (MPO) are serological markers for the primary vasculitic syndromes, Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), in which chronic inflammation and necrosis of predominantly small blood vessels are found. ANCA were previously identified by indirect immunofluorescence (IIF) and
two patterns of binding were recognized: cytoplasmic (c-ANCA) and perinuclear (p-ANCA). Although ANCA are detectable by IIF in certain sera from patients with other chronic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease and autoimmune liver disease, these antibodies do not recognize PR3 or MPO, but react with other cytoplasmic antigens, e.g. cathepsin-G, lactoferrin and elastase.10 We have recently reported a c-ANCA specific for a 55 kDa azurophil protein, bactericidal/permeability-increasing protein (BPI), found in patients with clinical and histological evidence of vasculitis as well as in patients with suggestive symptoms but insufficient proof of vasculitis.11 ANCA positivity in CF, as measured by IIF, has been observed in previous reports.12'13 To identify the ANCA autoantigens recognized by CF sera, and to investigate the serological relationship between CF and vasculitis, we studied the ANCA activity of sera from 66 adult CF patients by IIF, characterized the autoantigens detected by ELISA as well as immunoblotting, and investigated the associations between ANCA activity and the clinical features of the patients with CF.

**Methods**

**Patients and serum samples**

Sera from adult patients with the following disease and normal control groups were studied: CF patients (n = 66); anti-PR3-positive Wegener’s granulomatosis (WG, n = 41) and anti-MPO-positive microscopic polyangiitis (MPA, n = 41); IgA-related disorders: adult Henoch-Schönlein purpura (HSP, n = 22) and IgA nephropathy (IgAN, n = 10); and normal blood donors (NBD, n = 46). The sera were stored at — 20 °C before use.

Of the 66 CF patients, 62 had complete clinical information (42 male and 20 female) with a mean age of 23.2 years (range 13.5-37.1). Most were adult (59/62 were aged 18 years or over) and they had overall poor lung function (43/62 had a predicted FEV1 <50%). Sputum samples taken at the time of sera collection from 59 patients were also studied for microbiology: most of them (55/59) showed *Pseudomonas aeruginosa* and 23 also had *Haemophilus influenzae* and 17 also had *Staphylococcus aureus*. Six of the 62 CF patients had secondary vasculitis.

**ANCA IIF assay**

Standard IIF pan-isotype assays which detected ANCA, whether they were of IgA, IgG or IgM isotype, were performed as previously described.14 Certain IgA and IgG anti-BPI-positive samples, giving high binding in the isotype-specific ELISA (see below), were also tested for specific IgA and IgG IIF respectively, in which the bound IgA or IgG were detected with FITC-conjugated rabbit anti-human IgA or IgG (α-chain or μ-chain specific, Dakopatts). The IIF was scored blind by two observers and classified as + + (strong positive), + (positive), +/− (borderline) or − (negative); and c- or p-ANCA.

**ANCA solid-phase ELISAs**

PR3 and BPI, purified as described,11,15 together with MPO (Calbiochem) and cathepsin-G (ICN) were used as solid-phase ligands. Antigen-specific ELISA for PR3, MPO and BPI was done as previously described,11 with some modification, in that the condition for BPI coating was changed: purified BPI was diluted in phosphate-buffered saline (0.15 M, pH 7.2) at a concentration of 0.5 μg/ml and incubated at 4 °C overnight. Cathepsin-G was coated at 1.0 μg/ml in coating buffer (0.05 M bicarbonate buffer, pH 9.6), and incubated at 37 °C for one hour. All sera were diluted 1:100. For the IgA isotype anti-BPI assay, binding was detected with alkaline-phosphatase-conjugated rabbit anti-human IgA (α-chain-specific, Dakopatts), 1:500. The specificity of the autoantibodies was determined by inhibition assays in which purified BPI, at a concentration of 10 μg/ml in PBSGT, was pre-incubated with diluted serum samples (1:1000) at room temperature for one hour. Normal human haemoglobin, at the same concentration was used as a control. All other steps were as described.11

**Western blot analysis**

Western blot analysis used 150 μg (50 μl) of neutrophil granule acid extract (pH 3.0) and purified BPI (1 μg/lane) as described.11 The sera were diluted 1:500. Binding of sera, positive in IgA anti-BPI ELISA, was detected by alkaline-phosphatase-conjugated rabbit anti-human IgA (α-chain-specific, Dakopatts) 1:500.

**IgA rheumatoid factor ELISA**

Trace amounts of IgA were removed from normal human IgG (Sandoglobulin) by absorption against jacalin (Vector Laboratories) bound to cyanogen bromide-activated sepharose 4B (Sigma). The purified IgG at a concentration of 2 μg/ml in coating buffer was coated on to the wells of one half of a micotitre plate, the wells in the other half being coated with coating buffer only and acting as antigen-free well controls. The plate was incubated for one hour at 37 °C. Normal and CF sera were diluted 1:100 and coated in duplicate on both antigen-coated wells and antigen-free wells; sequential steps were then
the same as in the IgA anti-BPI ELISA. Results were recorded as the net OD$_{405nm}$ (mean value on antigen wells minus mean value on antigen-free wells) and expressed as percentage of a reference positive control. Samples were regarded as positive when they exceeded the mean +3SD of 46 normal control sera.

**Statistics**

The correlations between IgA or IgG anti-BPI levels and clinical parameters were investigated with either Spearman rank correlation or the Mann-Whitney U-test for non-parametric variables.

**Results**

**IIF**

Of the 66 CF samples, 21 (32%) were c-ANCA positive, 20/66 (30%) were borderline and the other 25/66 (38%) were negative.

**ELISA**

None of the 66 CF samples recognized PR3 and MPO; 10 of the 66 (15%) samples recognised cathepsin-G, but at a low level (data not shown). However, 60/66 (91%) of the CF samples contained IgG and 55/66 (83%) IgA anti-BPI antibodies; all the IgA anti-BPI-positive samples were also IgG anti-BPI-positive. None of 46 sera from normal blood donors were positive for IgG anti-BPI and only 1/46 (2%) was IgA anti-BPI-positive (Figure 1). The frequency of IgG and IgA anti-BPI antibodies in disease control groups is listed in Table 1.

The majority of the anti-BPI-positive samples from the disease control groups had low levels of binding (Figure 1, Table 1). Certain serum samples from CF patients were used to establish dilution curves, and in these it was possible to detect IgG and IgA anti-BPI antibodies up to a dilution of 1:12 800.

**Western blot analysis**

Of the sera most strongly positive by BPI ELISA, 4/4 samples from CF patients bound to purified BPI by immunoblotting. The specificity of this anti-neutrophil cytoplasm activity for BPI was confirmed by the binding of both IgG and IgA, which was restricted to the 55 kDa doublet, representing BPI, in a neutrophil granule acid extract preparation (Figure 2).

**Inhibition assays**

Both the IgG and IgA anti-BPI binding of five CF sera, strongly positive by BPI ELISA, could be inhibited by purified BPI (for IgG 40%–60%, for IgA 36%–73%), but not by normal human haemoglobin, used at the same concentration in the same diluent.

**IgA rheumatoid activity**

Of the 66 CF samples, 22 (33%) were outside the normal range (the mean ±3SD of 46 normal blood donors), and the majority had low activity. The presence of IgA rheumatoid factor was not correlated with IgA anti-BPI activities (Table 2).

**Correlations between anti-BPI levels and clinical parameters**

CF patients who had presented with vasculitis ($n=6$) had higher levels of IgG ($p=0.0395$) and IgA ($p=0.0482$) anti-BPI autoantibodies when compared with those without vasculitis. Both the pulmonary function tests, predicted FEV1(%) and FVC(%), were inversely correlated with IgA and IgG anti-BPI levels, especially for the IgA isotype (Figure 3). No correlation of anti-BPI levels with serum alkaline phosphatase levels, as indices of hepatic injury, was observed (Table 2).

**Discussion**

Sera from 60/66 (91%) adult CF patients had autoantibodies to the neutrophil granule protein BPI, and the specificity of this ELISA reactivity was confirmed by the use of antigen-free wells, by fluid-phase inhibition studies, and by immunoblotting. A lower number had ANCA IIF activity; as has already been reported, we have previously observed the discrepancy between ANCA IIF and anti-BPI ELISA results, probably due to cleavage of the BPI molecule by serine proteinases PR3 and/or elastase. Thus BPI is the major ANCA antigen in CF. Furthermore, the anti-BPI autoantibody levels, especially for the IgA isotype, significantly correlated with reductions in pulmonary function and the presence of secondary vasculitis.

Fifteen years ago, Colten and his colleagues described an inverse correlation between immunoglobulin levels and the clinical course in patients with CF which has subsequently led to many studies of the effects of chronic pulmonary inflammation, probably secondary to bacterial infection, on the clinical status of CF patients. These have stimulated clinical studies and large multicentre trials of prednisone as an anti-inflammatory agent in CF, with the conclusion that in selected CF patients steroid therapy may be of some benefit. More recently, high-dose ibuprofen has been used as an anti-inflammatory agent in CF with good effects in selected patients, such as young patients with good
Figure 1. Anti-BPI ELISA results: a) IgA anti-BPI and b) IgG anti-BPI. Of 66 CF samples, 55 were positive for IgA anti-BPI and 60 were positive for IgG anti-BPI autoantibodies. NBD, normal blood donors; WG, Wegener’s granulomatosis; MPA, microscopic polyangiitis; HSP, Henoch-Schönlein purpura; IgAN, IgA nephropathy; CF, cystic fibrosis. Dashed line indicates the upper limit of the normal range (mean + 3SD of the 46 NBD samples).

Table 1 IgG and IgA anti-BPI in disease controls

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IgG (% positive)</th>
<th>IgA (% positive)</th>
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<tbody>
<tr>
<td>NBD</td>
<td>0/46</td>
<td>1/46 (2%)</td>
</tr>
<tr>
<td>HSP</td>
<td>1/22 (5%)*</td>
<td>3/22 (14%)</td>
</tr>
<tr>
<td>IgAN</td>
<td>0/10 (0)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>WG</td>
<td>10/41 (24%)**</td>
<td>5/41 (12%)***</td>
</tr>
<tr>
<td>MPA</td>
<td>8/41 (20%)</td>
<td>4/41 (10%)***</td>
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</tbody>
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* Also positive for IgA.
** 2/5 also positive for IgG.
*** 2/4 also positive for IgG.

We believe that our finding of autoimmunity against BPI might provide some immunological explanation for the findings in the above studies. With advancing age, CF patients are predisposed to extra-pulmonary disorders, such as gastrointestinal and hepatobiliary problems, vasculitis and arthropathy. Recently, ANCA were detected in some gastrointestinal and hepatobiliary disorders such as inflammatory bowel disease (IBD), and primary sclerosing cholangitis. These observations suggest that perhaps the later complications in CF patients might be related to anti-BPI autoantibodies.

BPI is a 55 kDa membrane-associated cationic protein found in azurophilic granules of PMN, which displays a striking cytotoxicity towards many species of Gram-negative bacteria. This is thought to be due
Anti-BPI autoantibodies in cystic fibrosis

Figure 2. Western blot analyses of a neutrophil granule acid extract. The fresh granule acid extract (50 µl) (pH 3.0, 3 mg/ml) was resuspended in 50 µl reducing buffer and boiled for 3 min. After 12% SDS-PAGE, the resolved protein was transferred on to a nitrocellulose filter. Sera were diluted 1:500. a IgG autoantibodies and b IgA autoantibodies. Lanes 5–8 were incubated with four CF sera, and the 55 kDa doublet was identified by both IgG and IgA autoantibodies. Lane 3 and 4 were incubated with two anti-PR3-positive sera from patients with Wegener’s granulomatosis: a broad band between 27 kDa and 35 kDa with accentuation at both ends was blotted by IgG autoantibodies; there was no recognition by IgA. Lanes 1 and 2 were overlaid with two sera from normal blood donors.

to its high affinity for the lipopolysaccharides (LPS) which are uniquely found in the outer envelope of Gram-negative bacteria,27-29 and which constitute an important part of free endotoxin when it is released from the bacterial cell wall: hence the reported action of BPI as an endotoxin-neutralizing protein.30 LPS-binding protein (LBP), is an acute-phase protein which shares 44% amino acid sequence homology with BPI, and is the other major protein in human plasma which binds to LPS.31 The LBP:LPS complex can be bound by CD14 molecules on monocytes, leading to monocyte activation and release of pro-inflammatory factors such as TNFα, as well as IL-1. These in turn may stimulate neutrophils, so contributing to further tissue injury. In contrast, BPI has an opposite, neutralizing effect on LPS and, because of its 75-fold higher affinity for LPS than for LBP, can inhibit the inflammation induced by LPS.32 Recently, it has been shown that BPI ameliorates acute lung injury in porcine endotoxaemia33 and can protect vascular endothelial cells from LPS-induced activation and injury.34 It is thus important to detect whether anti-BPI autoantibodies can interfere with the functions of BPI and so facilitate the development of vasculitic inflammation. Indeed, it was previously reported that a rabbit polyclonal anti-BPI IgG fraction could completely block the bactericidal activity of BPI35 and that one of two monoclonal anti-BPI antibodies could partly inhibit the bactericidal activity of BPI.36

Autoantibodies against PR3 and MPO activate primed PMN in vitro through membrane expressed antigens and FcyRII,37,38 causing PMN degranulation and release of free oxygen radicals, as well as active enzymes, which may lead to tissue injury in vivo.39 BPI is expressed on the PMN cell surface40 and is available for direct binding to anti-BPI antibodies, which may activate PMN and cause tissue damage, as has been demonstrated for anti-PR3 and anti-MPO autoantibodies.

Our finding of IgA ANCA in CF patients prompts two further speculations: first, that IgA autoantibodies may result from pulmonary mucosal immunity, i.e. that IgA as well as IgG anti-BPI autoantibodies might be linked to chronic pulmonary infections, especially the chronic colonization by Pseudomonas aeruginosa; second, that the paradoxical situation of persistent P. aeruginosa despite an abundance of acute inflammatory cells, especially PMN, may be explained by impairment of BPI bactericidal activity brought about by anti-BPI antibodies. It is of note that PMN in bronchoalveolar lavage (BAL) fluid from CF patients expresses increased levels of the IgA Fc receptor (FcαR),41 and TNFα enhances the expression of FcαR, as well as the production of superoxide residues in response to aggregated IgA and the

Table 2  Correlation between IgA and IgG anti-BPI levels and some clinical parameters

<table>
<thead>
<tr>
<th>Clinical tests</th>
<th>IgG anti-BPI levels</th>
<th>IgA anti-BPI levels</th>
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<tr>
<td>%Pred. FEV1</td>
<td>r = −0.376, p = 0.0026</td>
<td>r = −0.508, p &lt; 0.0001</td>
</tr>
<tr>
<td>%Pred. FVC</td>
<td>r = −0.404, p = 0.0011</td>
<td>r = −0.452, p = 0.0002</td>
</tr>
<tr>
<td>Serum AP</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IgA RF</td>
<td>NS</td>
<td>NS</td>
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AP, alkaline phosphatase; IgA RF, IgA rheumatoid factor; NS, not significant; Pred, predicted.
Figure 3. Correlation between IgA anti-BPI levels and pulmonary function test FEV1(%) in 62 adult CF patients.

phagocytosis of IgA aggregates. In this study, IgA as well as IgG anti-BPI autoantibody levels were significantly correlated with the reduction of pulmonary function in CF patients. These data suggest that IgA anti-BPI, like IgG autoantibodies, might be directly involved in the activation of PMN and tissue damage in the lungs.

The relationship between chronic pulmonary infection, anti-BPI autoreactivity and pulmonary injury in patients with CF merits further investigation, while vasculitis in CF appears to be associated with more advanced disease and possibly is consequent upon high circulating levels of anti-BPI.

Acknowledgements

Sera from patients with HSP and IgAN were collected by Dr A. Allen from Leicester General Hospital. DRWJ has a clinical research fellowship from Gonville and Caius college and CML is a Wellcome Reader in Medicine.

References

14. Lockwood CM, Bates D, Jones S, Whitaker KB, Moss DW, Savage COS. Association of alkaline phosphatase with an


