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Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders

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SUMMARY

It was our purpose to determine the immunodiagnostic value of ANCA directed against BPI in diseases known to be associated with ANCA, such as ANCA-associated vasculitides, inflammatory bowel disease (IBD) and the associated condition primary sclerosing cholangitis. The immunoreactivity of recombinant BPI (rBPI) was established in order to develop an ELISA specific for rBPI. By means of this assay, BPI-ANCA were assessed in sera of 178 patients with IBD or the associated disorder primary sclerosing cholangitis, 112 patients with ANCA-associated vasculitides, and in sera of 182 disease and 140 healthy controls. BPI-ANCA were found to be closely associated with IBD and primary sclerosing cholangitis (34% and 44% of ANCA-positive sera, respectively). By contrast, BPI-ANCA positivity was low (<10%) in the double-negative sera of patients with ANCA-associated vasculitides and in disease and healthy controls. BPI-ANCA appear to constitute an important marker for IBD and primary sclerosing cholangitis, but not for the ANCA-associated vasculitides.

Keywords anti-neutrophil cytoplasmic antibodies (ANCA) bactericidal/permeability increasing protein inflammatory bowel disease

INTRODUCTION

ANCA are diagnostic hallmarks of ANCA-associated vasculitides [1]. ANCA directed against proteinase 3 (PR3-ANCA) [2,3] and ANCA against myeloperoxidase (MPO-ANCA) [4,5] are seromarkers for Wegener's granulomatosis (WG) and microscopic polyangiitis, respectively [6]. ANCA have not attained comparable immunodiagnostic value in other idiopathic inflammatory disorders, such as inflammatory bowel disease (IBD), primarily because the corresponding target antigens have not been elucidated. Recently, ANCA directed against BPI, an endotoxin-binding protein of polymorphonuclear granulocytes, were described. This prompted us to examine the immunodiagnostic potential of BPI-ANCA as a seromarker for IBD and the associated disorder primary sclerosing cholangitis. In addition, to ascertain whether BPI-ANCA can further specify sera negative for both PR3-ANCA and MPO-ANCA, we studied a series of 'double-negative' sera from patients with ANCA-associated vasculitides and compared them with sera from disease and healthy controls.

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ANCA have been detected in IBD and associated disorders, in particular primary sclerosing cholangitis. In contrast to the staining patterns revealed by immunofluorescence techniques in ANCA-associated vasculitides, both P-ANCA and A-ANCA are commonly found in these disorders [7]. The dominant target antigen(s) generating ANCA in these diseases are not yet known, but they are obviously not PR3 or MPO. Cathepsin G [8], lactoferrin [9], lysozyme [10], and other as yet undefined proteins have been described as candidate autoantigens. However, these antigens were found in only a minor portion (<20%) of patients with IBD, and in a similarly low percentage of patients with vasculitis [6].

Since microorganisms are thought to play a role in the immunopathogenesis of IBD and related disorders [11], we speculated that BPI could be the target of the ANCA seen in these diseases. We therefore examined sera of 102 patients with IBD and primary sclerosing cholangitis. For comparison, 182 disease controls with various chronic inflammatory disorders and 140 healthy blood donors were screened by means of an ANCA-ELISA using recombinant BPI (rBPI) as antigen. Immunoreactivity of the rBPI was proven and Western blotting techniques were employed to confirm the antigen recognized by BPI-ANCA.

More recently, BPI-ANCA were detected by ELISA using chromatographically purified native BPI as antigen in 45% of

Table 1. BPI-ANCA in ANCA-associatd vasculitides according to the main target antigens

	Wegener's granulomatosis		Microscopic polyangiitis		Churg–Strauss syndrome	
	+/Σ	%	+/Σ	%	+/Σ	%
IFT/ANCA (+)	35/45	78	13/22	59	30/45	67
PR3 (+)	18/35	51*	0/13	0	3/30	10
MPO (+)	0/35	0	8/13	62	5/30	17
BPI (+)	3†/35	9	1‡/13	8	0/30	0
Unknown	14/35	40	4/13	31	22/30	73
IFT/ANCA (−)	10/45	22	9/22	41	15/45	33
BPI (+)	0/10	0	0/9	0	3/15	20

*Low due to selection method.
†No proteinase 3 (PR3) specificity.
‡No myeloperoxidase (MPO) specificity.

selected vasculitis patients [12]. It was found that BPI-ANCA can narrow the immunodiagnostic window in patients with ANCA-associated vasculitis who are negative for PR3-ANCA and MPO-ANCA ('double-negative' sera). These two findings prompted us to investigate the frequency of BPI-ANCA in sera from 112 patients with ANCA-associated vasculitides (histologically confirmed, fulfilling ACR criteria), focusing on 'double-negative' sera (Table 1).

PATIENTS AND METHODS

Patients

Serum samples were obtained from 112 patients with ANCA-associated vasculitides and 178 patients with IBD or primary sclerosing cholangitis. None of the patients with primary sclerosing cholangitis had concomitant ulcerative colitis. One hundred and eighty-two patients with various other non-ANCA-related primary vasculitides, with collagen vascular diseases, or with rheumatoid arthritis (RA), served as disease controls (Table 2). Both ANCA-positive (i.e. by immunofluorescence technique; see below) and ANCA-negative patients were present in all three groups. Patients with ANCA-associated vasculitis without target antigens were preferentially selected to enable evaluation of the importance of BPI-ANCA. Sera of 140 blood donors served as healthy controls. All patients with ANCA-associated vasculitis [13], IBD [14], primary sclerosing cholangitis [15], as well as the disease controls fulfilled established classification criteria, as described elsewhere [7]. The patients with ANCA-associated vasculitides were explicitly selected to facilitate analysis of double-negative sera, which accounts for the low frequency of classical target antigens found in this study (Table 1).

Serological analysis

Detection of ANCA by immunofluorescence technique [16] and testing of ANCA target antigens by ELISA [17] were performed according to standardized European guidelines.

To develop an ELISA for anti-rBPI antibodies, a rBPI holoprotein of $\approx$ 55 kD was used as antigen. rBPI was produced in transfected chinese hamster ovary (CHO) cells and purified essentially as described for recombinant lipopolysaccharide binding protein (rLBP) [18]. The final product was >98% pure, as judged by SDS–PAGE, and exhibited a molecular mass of $\approx$ 55 kD. The identity of the protein was confirmed by N-terminal sequencing. The antigenicity of rBPI was ascertained by ELISA and immunoblotting using the MoAbs P_1G_8 and P_2A_5 (developed and kindly provided by Dr J.K. Spitznagel, Atlanta, GA) directed against native BPI (kind gift of Dr M.H. Zhao and Dr C.M. Lockwood, Cambridge, UK; for further details see [12]). All rBPI-positive sera were analysed for reactivity against native BPI (75% of rBPI-positive samples also tested positive on native BPI). P_1G_8 and P_2A_5 recognized rBPI as determined by ELISA and Western blot analysis, proving the antigenicity of the rBPI holoprotein (Fig. 1). Coating was done with 2 μg/ml (100 μl) rBPI/PBS, blocking with 200 μl of 0·1% bovine serum albumin (BSA)/PBS, and all washing steps were performed with 0·05% Tween 20/PBS. Samples (diluted 1:50 with 1% BSA, 0·05% Tween 20/PBS) were incubated with alkaline phosphatase-conjugated goat anti-human IgG (1% BSA, 0·05% Tween 20/PBS). Following another washing, the substrate *p*-nitrophenylphosphate was added. Optical density (OD) was read by an ELISA processor at 405 nm (reference wavelength 650 nm). On every plate we included BPI-ANCA-negative samples representative of a pool of 140 healthy blood donors and a selection of rheumatoid factor-positive sera (IgM subclass). The OD mean value $+3 \times$ s.d. of 140 healthy donors served as the cut-off point. BPI-ANCA-positive samples were also examined for reactivity with a recombinant 193 amino acid N-terminal fragment of BPI ($rBPI_{21}$), the biologically active N-terminal fragment of $rBPI_{50}$.

Competitive inhibition ELISA using rBPI was performed to determine the specificity of anti-rBPI activity. rBPI (10 μg/ml) was incubated with an equal volume of varying dilutions of BPI-ANCA-positive sera (1:50–1:6400) overnight at 4°C. Controls consisted of aliquots of the same sera preincubated with BSA.

The inhibition was considered significant at a reduction of OD values of at least 30% compared with controls. Tests for interassay variability were performed on 5 consecutive days with three different sera. Intra-assay variability was assessed using one serum in five different positions on one plate. The coefficient of variability (CV) was calculated using the formula: CV (%) = (s.d. $\times$ 100)/mean.

Western blot analysis was carried out to evaluate rBPI immunoreactivity and to confirm the positive ELISA results. Briefly, BPI was electrophoresed (1 μg/lane) under non-reducing conditions on 12·5% SDS–PAGE. After electrophoresis the samples were

Table 2. Immunodiagnostic value of BPI-ANCA; BPI-ANCA label inflammatory bowel disease (IBD) and primary sclerosing cholangitis but occur only rarely in ANCA-associated vasculitides

	BPI-ANCA^{+}*					
	Total		ANCA^{+}†		ANCA^{-}†	
Diseases	n/Σ	%	n/Σ	%	n/Σ	%
ANCA-associated vasculitides						
Wegener's granulomatosis	3/45	7				
Churg–Strauss syndrome	3/45	7		<10%†		
Microscopic polyangiitis	1/22	5				
Chronic IBD						
Crohn's disease	10/44	23	5/20	25	5/24	21
Ulcerative colitis	20/54	37	15/39	38	5/15	33
Primary sclerosing cholangitis	13/36	36	11/25	44	2/11	18
Disease controls						
'Classical' polyarteritis nodosa	0/14	0				
Kawasaki's disease	0/9	0				
Takayasu's disease	1/10	10		<10%‡		
Henoch–Schönlein purpura	1/16	6				
Temporal arteritis	4/42	9				
Systemic lupus erythematosus	2/28	7				
Systemic sclerosis	0/13	0		<7%‡		
Polymyositis	0/12	0				
Rheumatoid arthritis	3/38	8		<10%‡		
Healthy controls	1/140	<0.1		<0.1%‡		

*ELISA.
†Indirect immunofluorescence test screening.
‡Considered not significant.

transferred to a polyvinylidene difluoride membrane and blocked with 3% BSA/PBS for 1 h at room temperature. The strips were then incubated with control sera or MoAb (diluted 1:100 with 2% BSA/PBS) overnight at 4°C. After washing, incubation with the second antibody (diluted 1:20 000 with 2% BSA/PBS) for 1 h at room temperature was performed. Gold affinity-purified goat anti-human IgG and goat anti-mouse IgG were added, followed by silver enhancement (Fig. 1).

The specificity of the rBPI-ANCA-ELISA was demonstrated using competitive inhibition assays and Western blot techniques.

Analysis of polyspecific sera

Sera specific for at least one ANCA other than BPI-ANCA were assessed by competitive inhibition ELISA using the corresponding antigen as inhibitor according to the procedure for competitive inhibition assays outlined above.

RESULTS

BPI-ANCA were detected in 36% (13/36) of patients with primary sclerosing cholangitis (44% of ANCA-positive patients), in 37% (20/54) of ulcerative colitis (UC) patients (38% of ANCA-positive patients) and in 23% (10/44) of Crohn's disease (CD) patients (25% of ANCA-positive patients), compared with less than 10% of patients with ANCA-associated vasculitides and disease controls. Only six of the BPI-ANCA-positive sera (12%) reacted with $rBPI_{21}$.

Among the patients with ANCA-associated vasculitides (selected according to ANCA status), 32% (36/112) were C-ANCA-positive, 33·1% (37/112) P-ANCA-positive, and 35% (39/112) were ANCA-negative (Table 1). In patients with WG, only 7% (3/45) of sera were BPI-ANCA-positive, in microscopic polyangiitis only 5% (1/22), and in Churg–Strauss syndrome only 7% (3/45). All three patients with BPI-ANCA-positive Churg–Strauss syndrome belonged to the ANCA-negative subset (Table 1).

In IBD and primary sclerosing cholangitis, 52% (70/134) of patients were P-ANCA- and 12% (16/134) were A-ANCA-positive, while 36% (48/134) were ANCA-negative (Table 2). BPI-ANCA were present in 37% (31/84) of patients with ANCA-positive (by immunofluorescence) IBD and primary sclerosing cholangitis. Primary sclerosing cholangitis showed the highest proportion of BPI-ANCA positivity (44%), followed by UC (38%) and CD (25%). In contrast to ANCA-associated vasculitides, where BPI-ANCA did not further specify ANCA-positive and ANCA-negative sera, BPI-ANCA were detected in 33% (UC), 21% (CD), and 18% (primary sclerosing cholangitis) of ANCA-negative patients as assessed by immunofluorescence.

In the disease controls, none of the patients was C-ANCA-positive, 31% (56/182) were P-ANCA- and 10% (18/182) were A-ANCA-positive, while 59% (107/182) were ANCA-negative.

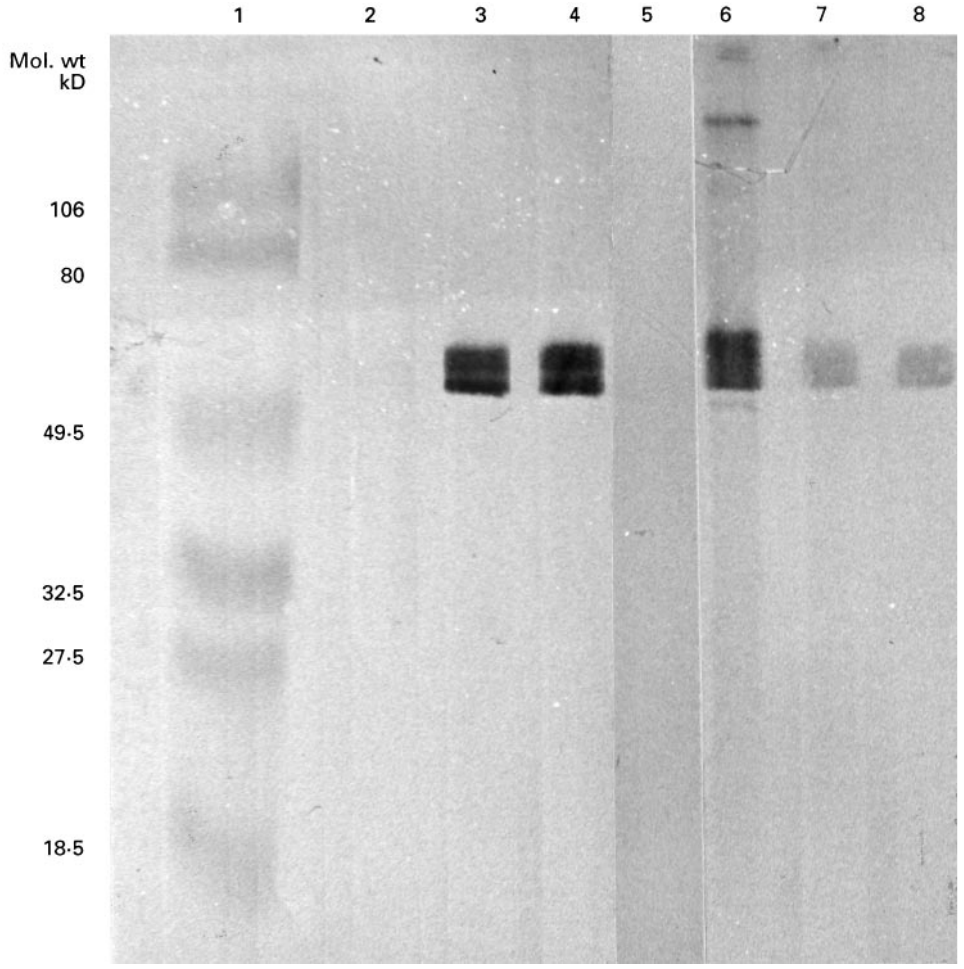

Fig. 1. Immunoblotting of rBPI-ANCA-positive sera. BPI was separated in SDS–PAGE under non-reducing conditions and incubated with BPI-ANCA-positive sera (lanes 6–8) and the MoAb P_1G_8 (lane 3) and MoAb P_2A_5 (lane 4). Lane 2 constitutes a negative control (mouse IgG isotype) and lane 5 a BPI-ANCA-negative serum. Relative molecular masses of the marker proteins separated under reducing conditions (lane 1) are shown on the left in kD.

The sera from these patients did not show BPI-ANCA in significant numbers, either in the non-ANCA-associated vasculitis group (represented by 'classic' polyarteritis nodosa, Kawasaki's disease, Takayasu's and temporal arteritis, and Henoch–Schönlein purpura) or in collagen vascular diseases (systemic lupus erythematosus, systemic sclerosis, polymyositis) and RA. The frequency of BPI-ANCA in the diseases studied is shown according to their ANCA status (by immunofluorescence) in Table 2.

All 140 healthy blood donors were ANCA-negative. Only one donor was positive for BPI-ANCA as demonstrated by ELISA, but exhibited no apparent clinical disease.

DISCUSSION

In this study BPI-ANCA were found to be closely associated with IBD and the related condition primary sclerosing cholangitis, but with neither ANCA-associated vasculitides nor with other idiopathic rheumatic disorders. They were virtually absent in healthy donors.

We first addressed the question of whether BPI is a second target antigen of C-ANCA and whether it can narrow the diagnostic window in ANCA-positive patients with ANCA-associated vasculitides lacking defined target antigens. We could not confirm earlier findings of a high incidence of BPI in vasculitides [12] or of their close association with the C-ANCA staining pattern [19]: only 7% of all the ANCA-associated vasculitides and only 6% of the C-ANCA-positive sera (WG, Churg–Strauss syndrome) we tested were BPI-ANCA-positive. By contrast, 23% of P-ANCA-positive samples from various chronic inflammatory disorders showed BPI-ANCA positivity. The discrepancy between our findings and those of Zhao *et al*. [12] could be due to (i) possible impurity of the native antigen compared with the recombinant antigen (a problem encountered by the European Task Force for standardization of the PR3-/MPO-ELISA); (ii) subtle conformational or carbohydrate differences between the two antigens; or (iii) the nature of the assays used and the patient samples examined. Additional studies are necessary to resolve these differences.

We next investigated the role of BPI-ANCA in IBD and primary sclerosing cholangitis to test the contagion hypothesis regarding the immunopathogenesis and maintenance of IBD. Based on the relatively high incidence of rBPI reactivity in primary

sclerosing cholangitis and, to a lesser extent, in IBD, rBPI appears to be the major target antigen of ANCA in primary sclerosing cholangitis (44%) and a predominant ANCA subspecificity in IBD (27%). In disease controls and in patients with ANCA-associated vasculitides, the prevalence of BPI-ANCA was <10%.

Conflicting evidence has been found on the frequency of ANCA and its main target antigen(s) in IBD and associated conditions. In primary sclerosing cholangitis, immunofluorescence revealed ANCA in up to 82% of cases [20]. Mulder and coworkers identified three different target antigens, lactoferrin, a 67/66-kD doublet, and a 40-kD doublet in Western blotting technique in 38% of patients with primary sclerosing cholangitis they investigated. ANCA rates oscillating between 49% and 88% in UC and 10% and 40% in CD were reported by separate authors [21,22]. Two other groups [8,23] found ANCA to be directed against cathepsin G, but this could be confirmed by neither Duerr *et al.* [20] nor by us [6]. Furthermore, Duerr *et al.* [20] reported that ANCA in IBD are not directed against PR3, MPO, or elastase. Instead, P-ANCA in UC are directed primarily against lactoferrin, as identified by Mulder *et al.* [22] in 33% of cases. Skogh & Peen [9] even detected ANCA against lactoferrin in 50% of UC sera irrespective of the ANCA status in immunofluorescence. This, however, could not be confirmed by our group [6]. By contrast, ANCA against lactoferrin have been reported in higher frequencies in a variety of inflammatory disorders, including systemic lupus erythematosus [24] and vasculitis [6].

Because BPI is susceptible to elastase cleavage [25], its antigenicity could be destroyed by elastase and other serine proteases (e.g. PR3, cathepsin G). This would explain our findings that anti-native-BPI MoAbs did not react with ethanol-fixed human neutrophil granulocytes and that no definite ANCA immunofluorescence pattern could be ascribed to BPI-ANCA. The two MoAbs we applied recognize conformational epitopes on intact BPI. Ethanol fixation of polymorphonuclear granulocytes releases proteins from granules within the cytoplasm by altering membrane structures [5]. Consequently, some of these proteins could be subjected to protease cleavage (e.g. by elastase), or denaturation, thus decreasing the antigenicity of BPI.

It remains to establish the pathophysiological nature of ANCA directed against BPI in IBD and primary sclerosing cholangitis. The low detection rate of $rBPI_{21}$ by ANCA suggests that ANCA-reactive epitopes reside predominantly within the C-terminal 263 amino acids of BPI. It can be hypothesized that BPI-ANCA attenuate the endotoxin-neutralizing capacities of BPI, leading to overexpression of bacterial antigenic stimuli. The involvement of superantigens in IBD was only recently highlighted [26], adding further support to the hypothesis implicating contagion in the immunopathogenesis of this disorder. Our results show that further studies into the clinical and pathophysiologic implications of BPI-ANCA are clearly warranted.

In conclusion, we identified BPI as a main target antigen of ANCA in IBD and primary sclerosing cholangitis. BPI thus represents the third ANCA antigen to be recognized as a seromarker for a distinct disease entity, along with PR3-ANCA in Wegener's granulomatosis and MPO-ANCA in microscopic polyangiitis.

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