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Bactericidal/permeability-increasing protein (BPI) is an important antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in vasculitis

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SUMMARY

Indirect immunofluorescence (IIF) techniques have shown that ANCA are useful serological markers for some small vessel vasculitides, and ELISA assays, using purified molecules as solid-phase ligand, have helped to identify proteinase 3 (PR3) and myeloperoxidase (MPO) as two of the major ANCA antigens. There remain a substantial number of serum samples, which are positive by IIF, yet recognize neither PR3 nor MPO (double-negative samples). We found, by Western blot analysis of soluble neutrophil granule proteins, that certain of these double-negative samples recognized a 55-kD doublet of which the first eight residues shared N-terminal amino acid sequence homology with BPI, a potent antibiotic towards Gram-negative bacteria. We developed a simple, quick and robust two-step immunobiochemical method to purify BPI. This was then employed to detect anti-BPI autoantibodies by ELISA and Western blot analysis. We tested 100 double-negative samples and 400 consecutive new samples sent for routine ANCA testing in the anti-BPI ELISA. We found that 45 of the 100 double-negative and 44 of the 400 new routine samples recognized BPI. By Western blot analysis 20/20 positive anti-BPI samples blotted the 55-kD protein. Inhibition assays confirmed the specificity of binding. Review of the 89 anti-BPI-positive patients showed a male dominance (M:F ratio 55:34), a mean age of 60·4 years and clinical diagnoses ranging from organ limited vasculitis to widespread systemic vasculitis.

Keywords ANCA antigen vasculitis bactericidal/permeability-increasing protein

INTRODUCTION

ANCA are serological markers for a range of primary vasculitic diseases, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MP) [1,2]. They can be identified using indirect immunofluorescence (IIF) techniques, by overlaying serum from a patient with suspected vasculitis on to alcohol-fixed human polymorphonuclear leucocytes (PMN). This procedure produces two staining patterns: a cytoplasmic pattern (C-ANCA) and a perinuclear pattern (P-ANCA). Proteinase 3 (PR3) and myeloperoxidase (MPO) are the major C-ANCA [3–6] and P-ANCA [7] antigens, respectively, although other neutrophil granule constituents such as elastase [3,8–10], cathepsin G [9,10], lactoferrin [10,11], and lysozyme [12] have also been associated, more rarely, as autoantigens in vasculitis. In our study we identified a substantial number of serum samples, which produce IIF of either C or P-ANCA type, but did not show specificity for any of these antigens. To pursue their identification we used an acid extract of neutrophil granules in Western blot analysis, and found that certain sera recognized a 55-kD doublet. After further purification of the 55-kD protein, N-terminal amino acid sequence analysis of the first eight residues established that this was a previously characterized entity, termed bactericidal/permeability-increasing protein (BPI).

BPI, a constituent of the azurophilic granules of neutrophils, is a highly cationic, 55-kD membrane-associated, cytotoxic protein found only in cells of the myeloid series [13] which directs its potent toxicity exclusively towards Gram-negative bacteria [13,14]. The particular target cell specificity is attributable to the strong affinity of BPI for lipopolysaccharides (LPS) [13–15].

In this study we have developed a simple, rapid and robust immunobiochemical method to purify human BPI from neutrophil granules. The purified BPI has been used to establish that autoantibodies to this antigen may be important serological markers for vasculitis.

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MATERIALS AND METHODS

Purification of BPI

Preparation of granule proteins. Twenty fresh 'buffy coat' preparations were directly diluted with lysing buffer (0·83%

NH_4Cl, 10 mM $KHCO_3$ and 0·1 mM EDTA) for 10 min and then centrifuged at 250 *g* for 10 min (the lysing procedure was repeated if the pellets remained red) and the leucocytes were then washed twice with PBS. Neutrophil granules were isolated according to the method of Wright [16] with some modification. In brief, the neutrophils were suspended in cavitation buffer (10 mM KCl, 3 mM NaCl, 1 mM ATP (disodium salt; Sigma, Poole, UK), 3·5 mM $MgCl_2$ and 10 mM PIPES (Sigma), pH 7·4) with 1 mM phenylmethanesulfonyl fluoride (PMSF) and cavitated at 350 psi for 20 min at 4°C. EGTA (Sigma) was added to the cavitate at a final concentration of 1·25 mM and the mixture was centrifuged for 10 min at 1400 *g*, after which the supernatant was collected and centrifuged for 20 min at 8700 *g*. The pellet, which contained the azurophilic and specific granules, was resuspended within 20 ml 0·08 M citric phosphate buffer (CPB) pH 3·0 with 1 mM PMSF. The granules were disrupted by three freeze/thaw cycles, then clarified by centrifugation (10 000 *g* for 20 min) and the supernatant was decanted and used immediately for the purification of BPI by column chromatography.

Chromatographic separation of BPI. A Mono-S prepacked HR10/10 column (Pharmacia, Uppsala, Sweden), a strong cation exchanger, was equilibrated with 50 ml 0·08 M CPB pH 3·0. The acid extract 150 mg was applied to the column and washed with 50 ml 0·08 M CPB pH 3·0. The bound proteins were first eluted with 30 ml 0·75 mM NaCl in 0·08 M CPB and then eluted with a gradient from 0·75 M to 1·2 M NaCl in CPB pH 3·0. The gradient eluate was collected in thirty-three 3-ml fractions, at a flow rate 1·5 ml/min. The fractions were assayed for their antigenic content of BPI, MPO, PR3, elastase, cathepsin-G and lactoferrin by ELISA as described below. The fractions containing BPI were pooled and dialysed against 0·08 M CPB pH 3·0 and then the pH adjusted to 8·0 with 3 M Tris/HCl pH 8·0 before reapplication to the same Mono-S column, which had been pre-equilibrated with 0·1 M Tris/HCl pH 8·0. After washing, the bound proteins were eluted with 0·5 M NaCl in 0·1 M Tris/HCl pH 8·0 and then eluted with a gradient from 0·5 M to 1·0 M NaCl in Tris/HCl pH 8·0. The eluate was again collected in thirty-three 3-ml fractions, at a flow rate of 1·5 ml/min. The fractions were tested as above and those positive for BPI were pooled and dialysed against 0·08 M CPB pH 3·0 and concentrated with an ultrafiltration unit (Sartorius GmbH, Göttingen, Germany; Membranfilter: SM_{145-49}, mol. wt cutoff 20 000) to 4 ml. The protein concentration of the concentrated BPI was measured before lyophilization for storage at −20°C.

ELISA assay to detect antigenic activities. Fractions, prepared as above, were diluted 1:20 with coating buffer (0·05 M bicarbonate buffer pH 9·6) and 100 μl were incubated in the wells of a 96-well microtitre plate (Dynatech Laboratory, Inc.) in duplicate at 37°C for 1 h. The primary antibodies, diluted in PBS buffer containing 1·0% gelatine and 0·1% Tween 20 ($PBSGT_{20}$), were a known anti-BPI-positive serum 1:200 (characterized initially by its ability to selectively blot this 55-kD protein by Western blot analysis of neutrophil granule acid extract, subsequently shown to be BPI by amino acid sequence analysis; see below), mouse monoclonal anti-PR3 1:2000 (HZ1F12; courtesy of Dr Zhen Huang, Department of Medicine, University of Cambridge, Cambridge, UK), mouse monoclonal anti-human neutrophil elastase 1:250 (Dakopatts, Glostrup, Denmark), mouse monoclonal anti-human MPO 1:250 (Dakopatts), rabbit polyclonal anti-human lactoferrin 1:2000 (Dakopatts) or sheep polyclonal anti-human cathepsin-G 1:1000 (ICN ImmunoBiochemicals, Lisle, IL). The bound primary antibodies were detected with their appropriate specific second antibodies, also diluted in $PBSGT_{20}$ which were respectively alkaline phosphatase-conjugated goat anti-human IgG 1:3000 (Jackson ImmunoResearch Laboratories, Inc.), goat anti-mouse IgG (whole molecule) 1:1000 (Sigma), goat anti-rabbit IgG (whole molecule) 1:1000 (Sigma), and donkey anti-sheep IgG (whole molecule) 1:1000 (Sigma). The temperature and time of the incubations were 37°C and 1 h, respectively. The assay was developed with phosphatase substrate ($Sigma^{104}$) 1·0 mg/ml in substrate buffer (16 mM $NaHCO_3$, 12 mM Na_2CO_3 and 2 mM $MgCl_2$).

Electrophoresis and amino acid sequences of BPI

Purified BPI (5·0 μg/lane) was subjected to 12% SDS–PAGE under non-reducing conditions as described by Laemmli [17] using a mini-gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). Following electrophoresis the gel was stained with 0·1% coomassie blue R-250. To analyse the N-terminal amino acid sequence 20 μg BPI were resolved by 12% SDS–PAGE, and transferred on to polyvinylidene difluoride membranes (PVDF; Millipore, Watford, UK) as described by Matsudaira [18]. The sequence analysis was performed by Dr R. A. Harrison (Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK).

Assays to detect anti-BPI autoantibodies

IIF assay. Standard IIF assays were performed as described [2,19]. To test whether the IIF was caused by anti-BPI autoantibodies a fluid-phase inhibition assay was undertaken, in which purified BPI at a predetermined concentration of 100 μg/ml was preincubated with diluted sera at room temperature for 1 h; the subsequent steps were then the same.

Solid-phase ELISA. Purified BPI was diluted to 1·0 μg/ml with PBS and coated on to the wells of one half of a Dynatech microtitre plate, the wells in the other half being coated with PBS alone and acting as antigen-free wells. The volumes for this step and for subsequent steps were 100 μl, all incubations were carried out at 37°C for 1 h, and plates were washed three times with PBS containing 0·1% Tween 20 (PBS-T) between stages. Test serum samples were diluted 1:50 with $PBSGT_{20}$ and coated in duplicate on both antigen-coated wells and antigen-free wells; every plate contained positive, negative and blank ($PBSGT_{20}$) controls. The binding was detected with alkaline phosphatase-conjugated goat anti-human IgG 1:3000 in $PBSGT_{20}$. The alkaline phosphatase substrate ($Sigma^{104}$) was used at 1·0 mg/ml in substrate buffer. The results were recorded as the net OD_{405nm} (average value on antigen wells minus average value on antigen-free wells) and expressed as percentage of a known positive sample. Samples were considered positive if they exceeded 17% of the reference positive serum (> mean + 3 s.d. from 40 normal blood donors). The specificity of the autoantibodies was determined by inhibition studies, similar to those used for IIF, in which purified BPI, at a concentration of 10 μg/ml in $PBSGT_{20}$, was preincubated with diluted serum samples (1:50–1:400 in $PBSGT_{20}$) at room temperature for 1 h. Normal human haemoglobin, purified PR3 and MPO, at the same concentration as purified BPI (10 μg/ml), were employed as controls. All other steps were as described above.

Western blot analysis. Purified BPI (1·0 μg/lane) was resolved on 12% SDS–polyacrylamide gel under non-reducing conditions. The resolved protein was transferred from the gel on to a nitrocellulose filter (BA 85, 0·45 μm; Schleicher and Schüll, Dassel, Germany) in CAPS buffer (10 mM 3-(cyclohexylamino)-1-propane- sulfonic acid, 10% methanol pH 11·0) for 40 min at 0·5 A [18]. After transfer the nitrocellulose filter was incubated in TBSTM buffer (0·01 M Tris/HCl pH 7·5 containing 0·15 M NaCl, 0·1% Tween 20 and 2% (w/v) non-fat milk (Marvel)) for 30 min at room temperature to block any remaining active sites on the nitrocellulose filter. The filter was subsequently cut into strips which were incubated with test or control sera (diluted 1 : 100 in TBSTM) overnight at 4°C. The strips were then washed (five changes of 5 min each in TBSTM) and incubated with alkaline phosphatase-conjugated, goat anti-human IgG, diluted 1 : 3000 for 1 h at room temperature. Washing was repeated as above and binding was detected by adding alkaline phosphatase substrate nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indoxyl phosphate (p-toluidine salt; Sigma).

Anti-PR3 and anti-MPO ELISA

Purified PR3 and MPO (free of contamination either with each other or BPI, elastase, cathepsin-G and lactoferrin as determined by ELISA) were diluted to 0·5 μg/ml and 1·0 μg/ml, respectively, and coated on to Dynatech microtitre plates. The procedures were the same as described in the anti-BPI ELISA.

Serum samples

Serum samples were frozen until required at −20°C. The anti-BPI ELISA was initially performed on serum samples from 100 vasculitis patients which were positive by IIF but recognized neither PR3 nor MPO (double negatives) and serum samples from 40 normal blood donors. Then 400 consecutive new serum samples, sent to our laboratory for ANCA tests, were also screened by anti-BPI, anti-PR3 and anti-MPO ELISA. From this cohort, samples positive in the ELISAs were additionally tested by IIF. Twenty samples, selected from the monospecific anti-BPI-positive samples, were then further characterized by Western blot analysis using both purified BPI and neutrophil granule acid extract. The specificity of binding was confirmed by inhibition assays (by IIF, $n = 10$; by ELISA, $n = 7$). One positive serum sample was diluted from 1 : 50 to 1 : 12 800 in $PBSGT_{20}$ and tested in the anti-BPI ELISA to establish assay sensitivity.

RESULTS

Purification of BPI

Mono-S chromatography, first phase. The unbound and 0·75 M NaCl eluate contained the majority of the proteins applied. The bound proteins which were eluted from 0·75 M NaCl to 1·2 M NaCl in 0·08 M CPB pH 3·0 were separated into several peaks; most of the BPI was eluted as a single peak at approximately 0·9 M NaCl (Fig. 1a).

Mono-S chromatography, second phase. The unbound and 0·5 M NaCl eluate usually contained about 33–50% protein loaded. The continuous gradient of NaCl (0·5–1·0 M) elution produced two major protein peaks. The BPI antigenic activity was demonstrated in the first peak, eluted at about 0·7 M NaCl

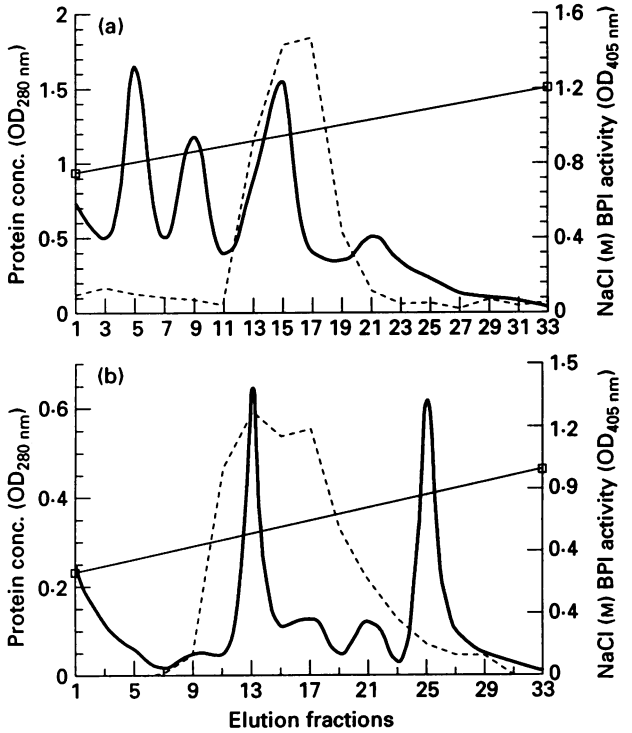

Fig. 1. (a) Mono-S cation exchange chromatography, first phase. BPI was eluted from fraction 13–17 at approximately 0·9 M NaCl. (b) Mono-S cation exchange chromatography, second phase. Further purified BPI was mainly in the first peak eluted from fraction 11–15 at approximately 0·7 M NaCl. ——, Eluted protein; - - - -, BPI activity; ——, NaCl elution.

(Fig. 1b). It was also confirmed that the first peak contained no PR3, MPO, elastase, cathepsin-G and lactoferrin activities, as demonstrated by ELISA. Up to 1·5 mg of purified BPI was produced from 150 mg granule proteins.

SDS–PAGE and N-terminal amino acid sequence

The purified BPI was resolved as a doublet at approximately 55 kD and a trace band at 200 kD (Fig. 2). The first eight N-terminal amino acid residues sequenced from the purified 55-kD band are shown below and compared with the first 40 N-terminal residues of the published BPI amino acid sequence:

Purified 55-kD sequence: VNPGVVVR

Published sequence: ^{1}MRENMARGPC NAPRWVSLMV

LAVIGTAVTA AVNPGVVVRI^{40}

The first eight residues of the purified BPI share sequence homology with the published sequence from residue 32 to 39 (underlined). The first 31 amino acids of the published sequence represent a signal peptide [20].

Characterization of serum specificities

ELISAs. Of 100 double-negative serum samples, 45 were positive in the ELISA incorporating purified BPI as the solid-phase ligand and none of the normal blood donors (Fig. 3a). Of 400 consecutive new samples sent for routine ANCA testing, 44 recognized purified BPI: four of these also recognized other antigens, one PR3 and three MPO; 10 samples recognized PR3 and 14 recognized MPO only. The dilution

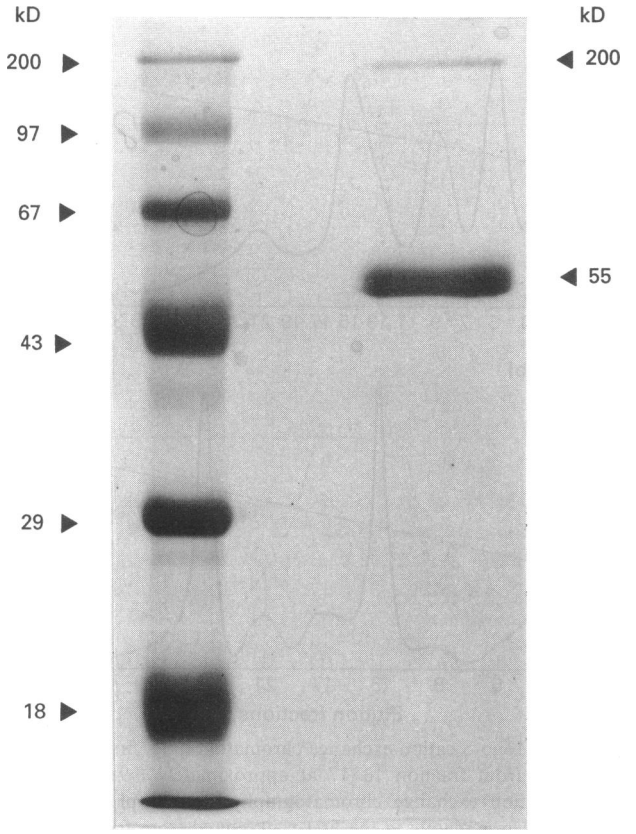

Fig. 2. Coomassie blue-stained SDS–PAGE showing electrophoretic mobility of BPI. Purified BPI (5·0 μg) resolved as a 55-kD doublet and a trace band at 200 kD. The molecular weight standards included: β-lactoglobulin 18 kD, carbonic anhydrase 29 kD, ovalbumin 43 kD, bovine serum albumin (BSA) 67 kD, phosphorylase 97 kD and myosin 200 kD (BRL).

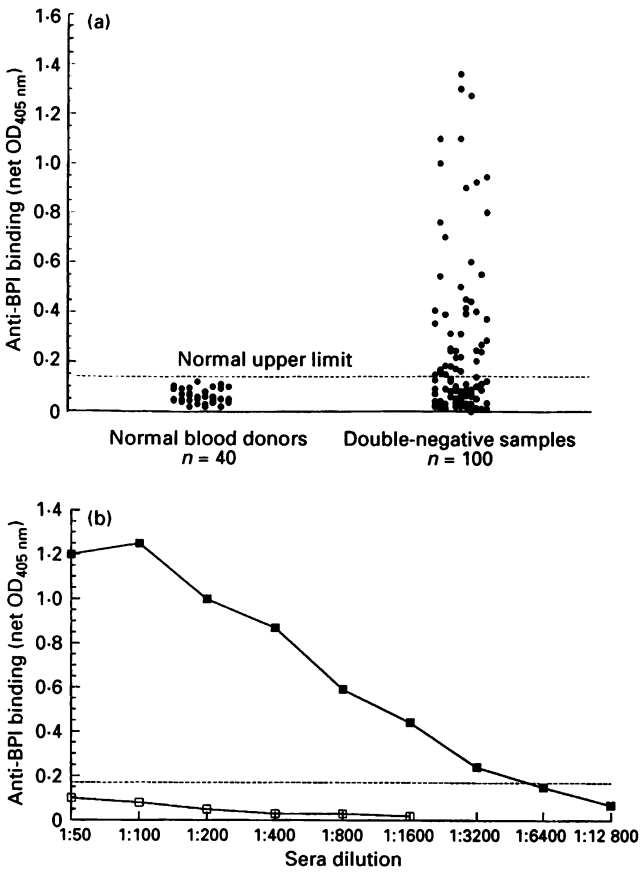

Fig. 3. (a) Anti-BPI ELISA assay performed for 100 double-negative samples and 40 normal blood donors. Forty-five of the 100 double-negative samples were positive but none of the normal blood donors. The normal upper limit was the mean + 3 s.d. from the 40 normal blood donors. (b) Dilution curve of an anti-BPI-positive sample and a negative control. The positive sample was positive up to a dilution of 1:3200. ■, Anti-BPI-positive serum; □, normal control; -----, normal upper limit.

curve of one positive anti-BPI serum sample showed the assay could detect autoantibody activity to a dilution of 1:3200 (Fig. 3b). The major IIF staining pattern for the anti-BPI-positive samples was C-ANCA on ethanol-fixed PMN, but occasional samples also showed a P-ANCA pattern. The distribution of the IIF results and staining pattern observed are given in Table 1. Of the 89 positive patients, 55 were male and 34 were female, with ages ranging from 6 to 88 (mean 60·4) years. Clinical information was obtained by questionnaire for 71 of the 89 positive patients, and the clinical diagnoses are shown in Table 2.

Table 1. The indirect immunofluorescence (IIF) staining pattern of anti-BPI, anti-proteinase 3 (PR3)- and anti-myeloperoxidase (MPO)-positive samples found in the two cohorts of samples: double-negative (n = 100) and recent routine (n = 400)

	Double negative	Routine samples		
Staining patterns	Anti-BPI-positive	Anti-PR3-positive	Anti-MPO-positive	Anti-BPI-positive
C-ANCA	38	8	3	13*
P-ANCA	7	0	8	4†
Borderline	0	2	3	13
Negative	0	0	0	14
Total	45	10	14	44

* One sample also recognized PR3.
† Three samples also recognized MPO.

Table 2. The clinical diagnoses of 71 anti-BPI-positive patients obtained by questionnaire

Diagnoses	No.
Wegener's granulomatosis	8
Microscopic polyangiitis	10
Vasculitis (unclassified)	13
Behçet's disease	4
Churg–Strauss syndrome	1
Organ-limited vasculitis	
Kidney	8
Liver	6
Lung	4
Skin	2
Gut	2
Peripheral nervous system	2
Heart	2
Others*	9
Total	71

* Insufficient proof of vasculitis.

Inhibition assays. Ten samples, positive in the anti-BPI ELISA and giving a C-ANCA pattern on ethanol-fixed PMN, were tested in the IIF inhibition assay: the IIF of all 10 samples could be inhibited by purified BPI (results not shown). Seven of the 10 samples were tested by inhibition assay in the anti-BPI ELISA: the anti-BPI activity of all seven samples could be inhibited (58–100%) by purified BPI but not by purified PR3 or MPO, nor by normal human haemoglobin (Fig. 4).

Western blot analysis. On purified BPI all 20 samples recognized the 55-kD doublet and a 200-kD band. These bands were not recognized by sera from normal subjects, and disease controls, as represented in Fig. 5a. On neutrophil acid extract anti-BPI-positive samples also blotted the 55-kD doublet (Fig. 5b). The presence of the bands at 55 kD and 200 kD raised the possibility that the higher protein band may share the same epitope(s), or that the higher band might be a polymer or a precursor of the 55-kD band. This possibility was tested by acid elution of antibodies [21] bound to the 55-kD and 200-kD band: in reapplication in the Western blot the affinity-purified antibodies could both produce binding to the 200-kD as well as to the 55-kD band (data not shown).

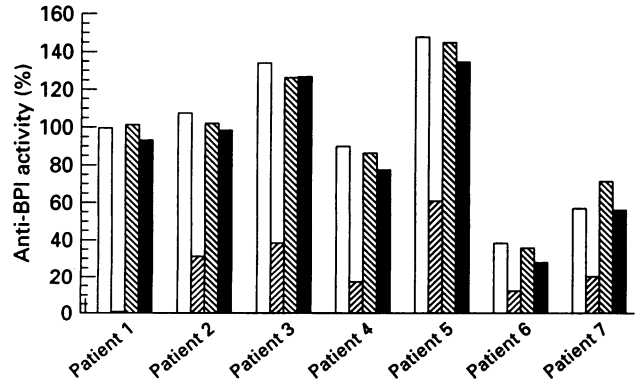

Fig. 4. Competitive inhibition assay by ELISA. The anti-BPI activity of the seven serum samples could be inhibited (58–100%) by purified BPI but not by proteinase 3 (PR3), myeloperoxidase (MPO) or haemoglobin (all inhibitors were added at a concentration of 10 μg/ml). □, Haemoglobin; ▨, BPI; ▧, PR3; ■, MPO.

DISCUSSION

Our laboratory acts as a diagnostic referral centre for sera from patients with suspected vasculitis. We routinely employ two techniques for screening samples, an ELISA modified from an immunoradiometric assay using a crude acid extract of neutrophil cytoplasm antigens as solid-phase ligand [22], and an IIF assay standardized to recently agreed protocols [19]. After screening, confirmation of autoantibody specificity for PR3 and MPO is performed by ELISA using the purified antigens as solid-phase ligands. The presence of a substantial number of double-negative samples led us to characterize BPI as one of the previously unidentified antigens. In this study, we found that 45% of the double-negative samples recognized BPI, and furthermore that 11% of the new routine samples also had this specificity, suggesting that BPI is an important ANCA antigen. The PR3 and MPO specificities in the 400 routine samples were 10/400 and 14/400 respectively. The fact that anti-BPI-positive samples constitute a substantial proportion of the recent routine sera sent to our reference laboratory may be consequent on the use of commercial anti-PR3 and anti-MPO diagnostic kits by other referring hospital laboratories to screen out these specificities.

BPI has previously been considered as an ANCA antigen. In 1991 Falk *et al.* [23] reported that 11 of 51 C-ANCA-positive samples recognized a 57-kD antimicrobial cationic protein (CAP57) by both ELISA and Western blot analysis; in addition, six of the 11 samples reacted with PR3. He also reported that mouse anti-CAP57 MoAbs produced a C-ANCA immunostaining pattern on human neutrophils. CAP57-specific C-ANCA staining was blocked by anti-CAP57 MoAbs but not by MoAbs to MPO or PR3 antibodies [23]. Clinical details of the patients, with respect to whether or not they had vasculitis, and if so, the distribution, as well as any further information about the role of CAP57 as an ANCA antigen, have not been published. Recently it has been shown that CAP57 and the 55-kD BPI share N-terminal amino acid sequence homology, this homology having been further supported in studies demonstrating that a monoclonal raised against CAP57 could also bind BPI [24].

BPI is an antimicrobial protein found in the azurophilic granules of the PMN, and displays a striking cytotoxicity towards many species of Gram-negative bacteria [13–15]. This action may be accounted for by the high affinity of the very basic N-terminal half of the molecule for the negatively charged LPS moieties that are uniquely found in the outer envelope of Gram-negative bacteria [13–15,25], 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 [26]. In 1982 and 1985 Weiss *et al.* found that the bactericidal activity of crude acid extract of human neutrophils toward susceptible *Echerichia coli* corresponded closely to the BPI content, and was completely blocked by a goat anti-BPI IgG-rich fraction, but not by the preimmune IgG-rich fraction [27,28]. These results on the one hand suggested that BPI was an important cytotoxic protein of the PMN, and on the other hand

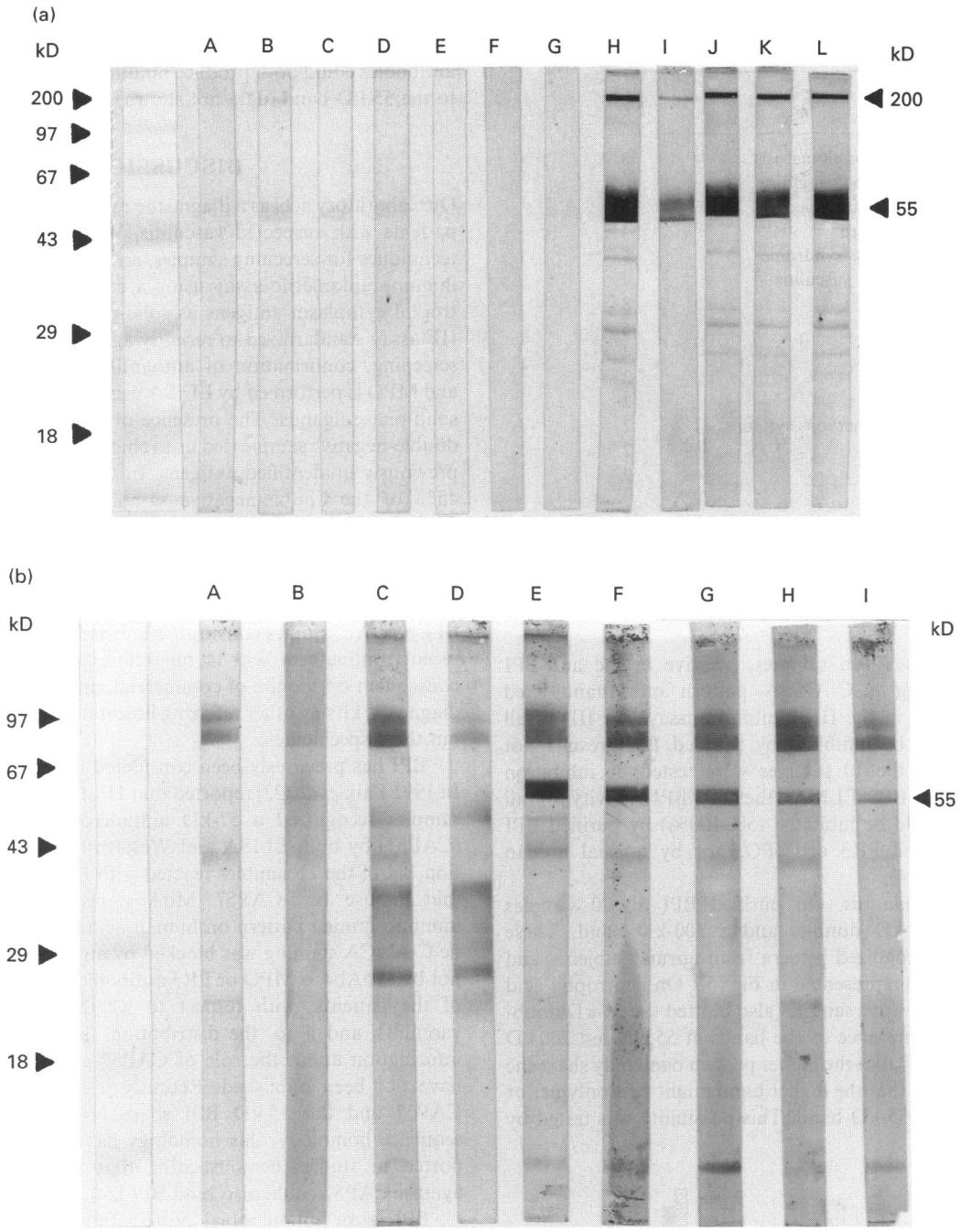

Fig. 5. (a) Western blot analysis. Purified BPI (1·0 μg/lane) was run in SDS–PAGE under non-reducing conditions and transferred on to a nitrocellulose filter. Lanes H–L were blotted with five anti-BPI-positive sera from five different vasculitis patients; both the 55-kD doublet and the 200-kD band could be blotted. Lane A was blotted with normal serum; lanes B and C two anti-glomerular basement membrane (GBM) sera; lanes D and E two anti-proteinase 3 (PR3) sera; and lanes F and G two anti-myeloperoxidase (MPO) sera. (b) Western blot analysis on neutrophil granule acid extract. Fresh granule acid extract (50 μl; pH 3·0) was resuspended in 50 μl non-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 : 50. Lanes E–I were blotted with five anti-BPI-positive sera from five different patients and the 55-kD doublet identified. Lanes C and D were blotted with anti-PR3-positive samples from two different patients with Wegener's granulomatosis: both bound to a broad band with accentuation at 26 and 35 kD and a possible weak band around 47 kD. Lanes A and B were overlaid with two sera from normal blood donors.

that human autoantibodies against BPI might similarly block the bactericidal and LPS-neutralizing activities of BPI, allowing these non-neutralized products to directly cause vascular damage and initiate vasculitis.

BPI is organized into two domains: the N-terminal half contains all known antimicrobial activity, and the C-terminal half contains several potential transmembrane regions which may anchor the holo-protein in the granule membrane [20].

Holo-BPI contains 487 amino acids; from residue 240 to 245 there is a potential cleavage site for elastase [20]. This potential susceptibility to elastase cleavage may explain why BPI has not received appropriate recognition as an ANCA antigen, since failure to acidify buffer used after the extraction of ANCA antigens allows the pH optimum for elastase activity to be reached, resulting in the cleavage of BPI and potential loss of antigenicity (unpublished data).

The isolation and purification of human BPI by different methods has been reported by several authors [14,29–31]. The methods described are time consuming and involve use of bacteria as target cells to identify the existence of BPI. We describe here a simple, robust procedure which we have developed for the isolation of BPI. The 0·08 M CPB pH 3·0 effectively extracts BPI from mixed granules in acid conditions, and the addition of PMSF provides further inhibition of neutral serine proteinase cleavage. Under these conditions the extract can be directly applied to a Mono-S column. After the first cation exchange chromatography the BPI is enriched in several fractions. The dialysed BPI containing fractions can then be adjusted to pH 8·0 and applied to the same Mono-S column again to obtain further purified BPI. The use of antibody probes instead of bacteria and a one-column two-step procedure (the ionic charge capacity of Mono-S is independent of pH over the range 2–12, and can accept a high flow rate) makes the isolation easier, rapid and stable. The yield by this method is about 1% of the protein in the crude granule extract. The purity of the BPI isolated in this investigation was assessed by coomassie blue staining of SDS–PAGE gels: the presence of a broad 55-kD doublet and a trace 200-kD band which on Western blot analysis could both be blotted by affinity-purified antibodies bound to the 55-kD band as well as the 200-kD band indicated the two perhaps contained the same epitope(s), and that the higher one was either a polymer or a precursor of the lower one.

That the anti-BPI ELISA could detect autoantibody activity at a dilution of 1:3200 of a positive serum sample and detect autoantibodies in samples negative or borderline by IIF suggested that the assay was sensitive. The competitive inhibition assays and the Western blot analysis confirmed the assay's specificity. In this study only 17 of the 44 anti-BPI ELISA-positive samples showed typical C- or P-ANCA staining by IIF, and these were the samples with the highest titres; 13 were borderline and 14 negative. These results suggest that an ELISA with purified BPI is more sensitive than the IIF method to detect anti-BPI autoantibodies, and also perhaps that during the preparation of cells for the IIF or during performance of the IIF assay BPI may be cleaved by elastase and thereby undergo epitope loss. All the sera tested were from patients suspected of having different clinical vasculitic disorders. For the 89 patients, whose sera contained anti-BPI antibodies, a variety of different clinical diagnoses were reported, e.g. WG, MP, Churg–Strauss syndrome and Behçet's disease; some patients had organ-limited vasculitis such as in the kidney, lung, liver, gut, peripheral nervous system and heart involvement, and some had multisystem vasculitis (Table 2). What role these autoantibodies play in these diseases remains unclear, and whether these autoantibodies are just serological markers or take part in the pathogenesis needs further investigation. Considering the antibiotic effect of BPI towards Gram-negative bacteria, whether the occurrence of vasculitis can be linked to Gram-negative bacterial infection also requires further study.

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