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Frequency of anti-bactericidal/permeability-increasing protein (BPI) and anti-azurocidin in patients with renal disease

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SUMMARY

The major subtypes of anti-neutrophil cytoplasmic antibodies (ANCA) detected by indirect immunofluorescence assay (IFA) are P-ANCA and C-ANCA. In patients with vasculitis, myeloperoxidase (MPO) is the major P-ANCA antigen and proteinase 3 (PR3) is the major C-ANCA antigen. BPI and azurocidin, which are also called 57-kD cationic antimicrobial protein (CAP 57) and 37-kD cationic antimicrobial protein (CAP 37), respectively, have been proposed as less frequent target antigens for C-ANCA and P-ANCA. In patients with renal disease, we determined the frequency of antibodies against BPI and azurocidin. By IFA on alcohol-fixed neutrophils, monoclonal and polyclonal anti-BPI antibodies produced a C-ANCA pattern, whereas rabbit anti-azurocidin antibody produced a P-ANCA pattern. By ELISA, sera from 229 P-ANCA-positive patients, 99 C-ANCA-positive patients and 48 ANCA-negative (by IFA) patients with renal biopsies were tested for reactivity with recombinant human BPI and purified human azurocidin. Of these sera, 17·5% of P-ANCA, 30·3% of C-ANCA and 20·8% of IFA-ANCA-negative sera were positive for anti-BPI; and 8·3% of P-ANCA, 3·0% of C-ANCA and 8·3% of IFA-ANCA-negative sera were positive for anti-azurocidin. There was no statistical difference in frequency of anti-BPI between pauci-immune necrotizing and crescentic glomerulonephritis (NCGN) and other glomerular disease (OGD), and there was a lower frequency of anti-azurocidin in NCGN samples than in OGD samples. By Western blot, anti-BPI-positive sera reacted with a 57-kD BPI band and anti-azurocidin-positive sera with a 29-kD azurocidin band. In conclusion, there is a low frequency of anti-BPI and anti-azurocidin antibodies in ANCA-positive patient sera; however, this does not correlate with NCGN, which is a marker for ANCA-associated small vessel vasculitis, and a similar positivity is found in IFA-ANCA-negative patients with renal disease. Therefore, serologic detection of anti-BPI and anti-azurocidin is not diagnostically specific in patients with renal disease.

Keywords bactericidal/permeability-increasing protein azurocidin anti-neutrophil cytoplasmic autoantibodies vasculitis glomerulonephritis

INTRODUCTION

The serologic association of anti-neutrophil cytoplasmic antibodies (ANCA) with systemic small vessel vasculitis, including Wegener's granulomatosis (WG), microscopic polyangiitis and Churg–Strauss syndrome, and with pauci-immune necrotizing and crescentic glomerulonephritis (NCGN) is well established [1–5]. The two major subtypes of ANCA that can be distinguished by indirect immunofluorescence assay (IFA) are C-ANCA that produce a cytoplasmic staining pattern and P-ANCA that produce a perinuclear staining pattern of alcohol-fixed neutrophils [3]. Several antigen specificities for ANCA have been elucidated by more specific immunochemical assays, such as Western blot and enzyme immunoassays. In patients with vasculitis, P-ANCA most often have specificity for myeloperoxidase (MPO) [3–5], although minor specificities, such as for elastase [5], have been described. The major specificity for C-ANCA is proteinase 3 (PR3) [5–9].

It was recently reported that BPI, also named 57-kD cationic antimicrobial protein (CAP 57) [10], and azurocidin, also termed 37-kD cationic antimicrobial protein (CAP 37) [10], are ANCA antigens in vasculitis [11,12].

In this study we report the frequency of anti-BPI and anti-azurocidin in patients with renal disease, and find that anti-BPI and anti-azurocidin, unlike anti-MPO and anti-PR3, do not correlate with the presence of pauci-immune NCGN, which is a marker for small vessel vasculitis. A similar frequency of positivity was found in patients with renal disease who were ANCA-positive or ANCA-negative by IFA.

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PATIENTS AND METHODS

Patients

P-ANCA-positive serum samples in 229 patients, C-ANCA-positive serum samples in 99 patients, and IFA-ANCA-negative serum samples in 48 patients were obtained from the University of North Carolina Nephropathology Laboratory. ANCA pattern was defined by indirect IFA on alcohol-fixed normal human neutrophils as substrate [3]. All 376 patients had a renal biopsy and the morphologic diagnosis was defined by examination of renal biopsy specimens with light, immunofluorescence, and electron microscopy. NCGN was defined as glomerulonephritis with necrosis and crescent formation by light microscopy, less than 2 (on a scale of 0–4) glomerular staining for immunoglobulin by immunofluorescence microscopy, and no glomerular immune complex-type electron dense deposits by electron microscopy. All other patterns of glomerular disease were designated other glomerular disease (OGD). Of the 229 P-ANCA patients, 90 had NCGN (with or without systemic small vessel vasculitis) and the other 139 had OGD. Of the 99 C-ANCA patients, 78 had NCGN and the other 21 had OGD. All IFA-ANCA-negative patients had OGD.

Purification of BPI and azurocidin

Human polymorphonuclear leucocytes (PMN) were obtained from leukaemic human donors [13]. PMN were suspended in cavitation buffer with phenylmethanesulfonyl fluoride (PMSF), cavitated and centrifuged [11]. The pellet containing the granule fraction was extracted and sonicated in 0·2 M sodium acetate buffer pH 4·0 and then centrifuged [13]. The supernatant fluid contained granule protein.

The granule protein was chromatographed on fast performance liquid chromatography (FPLC) Mono S cation exchange column, HR 5/5 (Pharmacia, Uppsala, Sweden). The bound proteins were eluted with a gradient from 0·15 M to 1·8 M NaCl in 0·05 M sodium acetate buffer pH 4·0 at a flow rate of 1 ml/min. A single peak between 0·8 M and 1·0 M NaCl contained BPI and azurocidin as determined by anti-BPI and anti-azurocidin ELISA. The fractions of this peak were pooled, concentrated and applied to a FPLC Superose 12 gel filtration column, HR 16/50 at a volume of 1 ml. The protein was eluted with 0·2 M sodium acetate buffer pH 4·0 at a flow rate 0·3 ml/min [14]. The first peak was BPI and the second peak was azurocidin documented by SDS–PAGE electrophoresis, ELISA, Western blot and amino acid sequence.

Recombinant BPI, anti-BPI antibodies and anti-azurocidin antibodies

Recombinant BPI from Chinese hamster ovary cells and rabbit anti-BPI antibody were a generous gift from Dr Randy Scott and Dr Craig G. Wilde (INCYTE Pharmaceuticals, Inc., Palo Alto, CA). Anti-BPI MoAbs that were thoroughly characterized [14] were kindly provided by Dr H. Anne Pereira and Dr John K. Spitzangel (Department of Microbiology and Immunology, Emory University, Atlanta, GA). Rabbit anti-azurocidin sera were obtained from New Zealand White rabbit immunized with 150 μg purified human azurocidin emulsified with TiTerMax (CytRx, Inc., Norcross, GA). The anti-azurocidin antibody was detectable by ELISA and Western blot.

Indirect immunofluorescence microscopy assay

The reaction patterns of patient serum samples, monoclonal and polyclonal anti-BPI antibodies, and rabbit anti-azurocidin antibody were detected by indirect IFA using commercially prepared slides coated with alcohol-fixed human PMN (INOVA Diagnostics Inc., San Diego, CA) and 1:320 diluted dichlorotriazinylamino fluorescein (DTAF)-conjugated goat anti-human IgG (H + L), 1:200 diluted DTAF-conjugated goat anti-mouse IgG + IgM (H + L) or 1:100 diluted DTAF-conjugated goat anti-rabbit IgG (H + L) (Jackson Immunoresearch Labs Inc., West Grove, PA) as the secondary antibody.

The ELISA procedure

The anti-BPI ELISA procedure. Microtitre plates (Costar, Cambridge, MA) were coated overnight with 100 μl recombinant BPI per well at a protein concentration of 0·4 μg/ml in 0·1 M sodium carbonate buffer pH 9·6, or coated with 1·0 μg/ml purified human BPI in PBS 100 μl/well. Wells were coated with 0·5% heat-inactivated goat serum (Sigma, St Louis, MO) in PBS acting as antigen-free negative control. The plates were blocked with 0·5% heat-inactivated goat serum in PBS containing 0·05% Tween 20 and then incubated with patient sera diluted 1:100 in blocking buffer. Bound antibodies were detected with an alkaline phosphatase-conjugated affinity-purified goat anti-human IgA + IgG + IgM (H + L) (Jackson) in blocking buffer and developed with phosphatase substrate tablets (Sigma) in diethanolamine buffer. Optical density (OD) was read at 405 nm and the results were calculated from the OD value on antigen wells minus antigen-free wells. Assay results were expressed as a percentage of a standard positive control value. Fifty healthy control sera from blood bank donors were used to establish a reference range for the anti-BPI assay. A positive result on ELISA was defined as a value greater than the mean + 3 s.d. of a healthy control population.

The anti-azurocidin ELISA procedure. Plates were coated with purified human azurocidin at a protein concentration of 1 μg/ml in PBS 100 μl/well. All other steps were as described above.

The anti-PR3 ELISA procedure. Anti-PR3 antibodies were measured by anti-PR3 ELISA Kit (Progen Biotechnik GmbH, Heidelberg, Germany). The activity of anti-PR3 antibodies in patient sera was obtained from a standard curve and expressed as U/ml.

The anti-MPO ELISA procedure. Plates were coated with commercial MPO (Calbiochem, La Jolla, CA) at a protein concentration of 10 μg/ml in 0·05 M sodium acetate, 0·1 M NaCl buffer pH 6·0. All other steps were the same as anti-BPI ELISA.

Western blot analysis

In order to confirm the results of ELISA according to a different method, anti-BPI or anti-azurocidin-positive serum samples were analysed by Western blot. Purified or recombinant BPI, or purified azurocidin were subjected to SDS gel electrophoresis and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membrane strips were blocked with 5% non-fat dry milk, incubated in a 1:40 dilution of patients' sera. A 1:10 000 dilution of alkaline phosphatase-conjugated goat anti-human IgG + IgM + IgA (H + L) (Jackson) was added and the reaction was developed with an alkaline phosphatase substrate kit (BioRad, Hercules, CA).

Statistical analysis

Statistical analyses were performed using a continuity adjusted χ^2 test and a P value of $\leqslant 0{\cdot}05$ was considered significant.

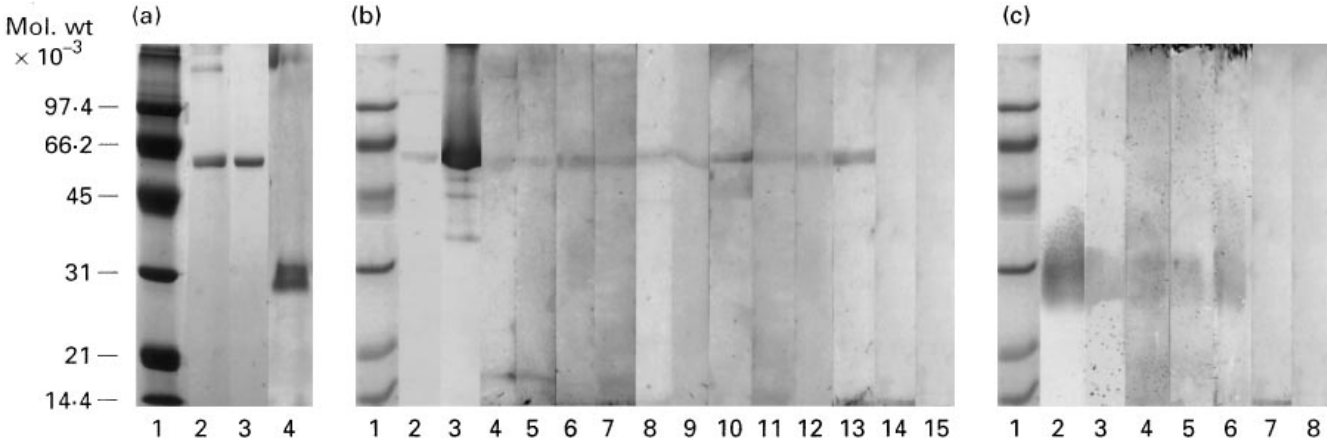


Fig. 1. In a, b and c, standard molecular markers are shown in lane 1. (a) SDS–PAGE. Purified BPI (lane 2) and recombinant BPI (lane 3) produced a 57-kD band, and purified azurocidin (lane 4) a 29-kD band in SDS–PAGE stained by coomassie. (b) Western blot. Regardless of whether using purified BPI (lanes 4 and 6) or recombinant BPI (lanes 5 and 7) as antigen, a 57-kD band is observed with anti-BPI-positive sera (lanes 4 and 5, patient 1; lanes 6 and 7, patient 2). Using purified BPI as antigen, monoclonal (lane 2) and polyclonal (lane 3) anti-BPI antibody, two anti-BPI-positive and IFA-ANCA-negative sera (lanes 8 and 9) and four anti-BPI-positive and IFA-ANCA-positive sera (lanes 10–13) react with a 57-kD band, whereas anti-PR3-positive serum (lane 14) and normal human serum (lane 15) do not. (c) Western blot. Using purified azurocidin as antigen, rabbit anti-azurocidin serum (lane 2), two anti-azurocidin-positive and IFA-ANCA-negative sera (lanes 3 and 4) and two anti-azurocidin-positive and IFA-ANCA-positive sera (lanes 5 and 6) react with a 29-kD band, whereas normal human serum (lanes 7 and 8) does not.

RESULTS

Purity of BPI and azurocidin

The purity of BPI and azurocidin were documented as follows. Purified BPI produced a 57-kD band and purified azurocidin produced a 29-kD band on SDS–PAGE under non-reducing conditions (Fig. 1a). By Western blot analysis using purified BPI as antigen, a 57-kD band was observed with monoclonal or polyclonal anti-BPI antibody (Fig. 1b). Using purified azurocidin as antigen, a 29-kD band was observed with rabbit anti-azurocidin antibody (Fig. 1c). As demonstrated by ELISA, purified BPI reacted with monoclonal or polyclonal anti-BPI antibody, purified azurocidin reacted with rabbit anti-azurocidin-positive serum, and neither BPI nor azurocidin contained MPO, PR3, elastase, cathepsin G, lactoferrin or lysozyme based on reactivity with specific monoclonal or polyclonal antibodies. The first 20 N-terminal amino acid residues sequenced from the purified BPI and purified azurocidin were identical to the published sequences [16,17]: purified BPI sequence: VNPGVVVRISQKGL-DYASQQ; purified azurocidin sequence: IVGGRKARPRQFP-FLASIQN.

Indirect immunofluorescence microscopy

By indirect IFA, the monoclonal and polyclonal antibodies against human BPI produced diffuse granular cytoplasmic staining of alcohol-fixed human neutrophils (CG-ANCA) (Fig. 2a). This CG-ANCA pattern was more granular and more diffuse throughout the cytoplasm than the typical C-ANCA staining caused by anti-PR3. The rabbit serum against human azurocidin produced perinuclear staining of alcohol-fixed human neutrophils (P-ANCA) (Fig. 2b).

As shown in Fig. 3, anti-BPI-positive human serum samples produced CG-ANCA staining pattern of neutrophils (Fig. 3a) that differed from the typical C-ANCA pattern with central accentuation produced by PR3-ANCA sera (Fig. 3b).

ELISA

Sera from 99 C-ANCA, 229 P-ANCA and 48 IFA-ANCA-negative renal biopsy patients were tested for reactivity with recombinant BPI, purified azurocidin, PR3 and MPO by ELISA (Table 1). The C-ANCA sera were also tested with purified BPI. Of the 99 C-ANCA sera, 20 sera (20·2%) were positive for both anti-BPI and anti-PR3, 10 sera (10·1%) positive for anti-BPI alone, 51 sera (51·5%) positive for anti-PR3 alone and 18 sera (18·2%) negative for both anti-BPI and anti-PR3. Among the 229 P-ANCA sera, 21 sera (9·2%) were positive for both anti-BPI and anti-MPO, 19 sera (8·3%) positive for anti-BPI alone, 107 sera (46·7%) positive for anti-MPO alone and 82 sera (35·8%) negative for both anti-BPI and anti-MPO. The relatively low frequencies of anti-PR3 positivity in C-ANCA sera and of anti-MPO positivity in P-ANCA sera resulted from selection of a disproportionate number of anti-PR3 and anti-MPO-negative samples in hopes of identifying anti-BPI and anti-azurocidin-positive sera.

Comparison of recombinant BPI with purified BPI. Regardless of whether recombinant BPI or purified BPI was used as antigen in the anti-BPI ELISA, there was a similar frequency of anti-BPI-positive sera in C-ANCA patients (30·3% *versus* 31·3%).

Comparison of C-ANCA with P-ANCA patient sera. C-ANCA patient sera were 1·7 times more likely (95% confidence interval (CI) 1·2, 2·6) than P-ANCA patient sera to have anti-BPI antibodies ($P = 0{\cdot}009$). There was a higher frequency of anti-azurocidin antibodies in P-ANCA than in C-ANCA sera, but this was not statistically significant ($P = 0{\cdot}06$).

Comparison of NCGN with OGD ANCA-positive patient sera. In C-ANCA patient sera, NCGN patients were 2·1 times more likely (95% CI 1·2, 3·7) than OGD patients to have anti-PR3 antibodies ($P = 0{\cdot}0003$). In P-ANCA patient sera, NCGN patients were 2·1 times more likely (95% CI 1·7, 2·7) than OGD patients to have anti-MPO antibodies ($P < 0{\cdot}0001$). Of 168 IFA-ANCA-positive patients with NCGN, 24·4% (41/168) had anti-BPI and 1·8% (3/168) had anti-azurocidin. Of 160 IFA-ANCA-positive patients with OGD, 18·1% (29/160) had anti-BPI and 11·9% (19/

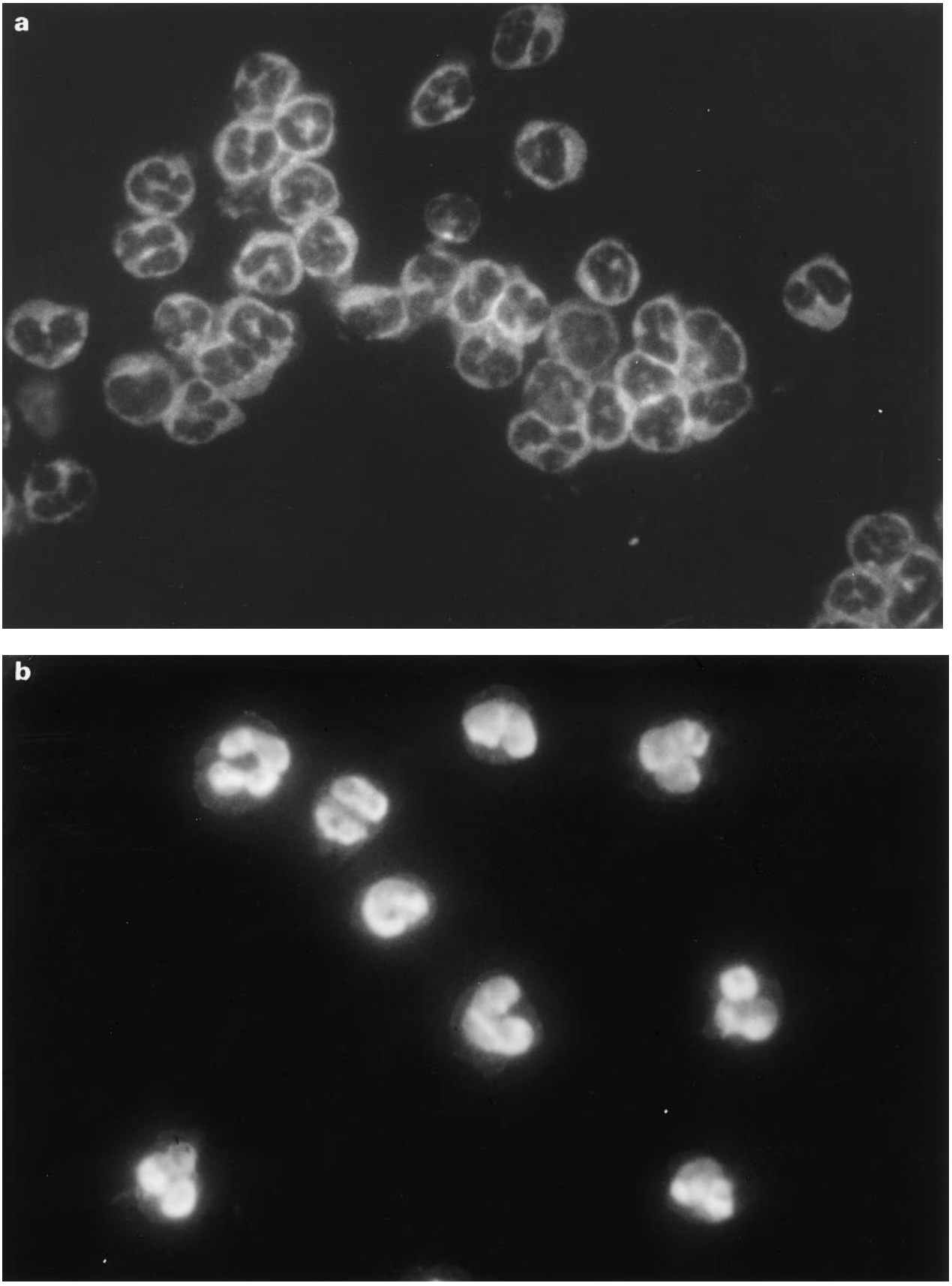


Fig. 2. With alcohol-fixed neutrophils, indirect immunofluorescence microscopy reaction pattern of anti-BPI MoAbs demonstrating diffuse granular cytoplasmic staining (a) and of rabbit anti-azurocidin serum showing perinuclear staining (b).

Table 1. Anti-BPI, anti-azurocidin, anti-proteinase 3 (PR3), and anti-myeloperoxidase (MPO) antibodies in serum samples measured by ELISA array

	Percentage of antibody-positive sera			
Serum	Anti-MPO	Anti-PR3	Anti-BPI	Anti-azurocidin
C-ANCA	7·1 (7/99)	71·7 (71/99)	30·3 (30/99)	3·0 (3/99)
NCGN	5·1 (4/78)	*80·8 (63/78)	32·1 (25/78)	1·3 (1/78)
OGD	1·4 (3/21)	38·1 (8/21)	23·8 (5/21)	9·5 (2/21)
P-ANCA	55·9 (128/229)	0·9 (2/229)	17·5 (40/229)	8·3 (19/229)
NCGN	*82·2 (74/90)	1·1 (1/90)	17·8 (16/90)	*2·2 (2/90)
OGD	38·8 (54/139)	0·7 (1/139)	17·3 (24/139)	12·2 (17/139)
IFA-negative ANCA				
OGD	0·0 (0/48)	0·0 (0/48)	20·8 (10/48)	8·3 (4/48)

*Necrotizing and crescentic glomerulonephritis (NCGN) *versus* other glomerular disease (OGD), $P < 0{\cdot}05$.

160) had anti-azurocidin. Unlike anti-PR3 and anti-MPO, there was no statistically significant difference in frequency of anti-BPI antibodies in patients with NCGN compared with patients with OGD (in C-ANCA, $P = 0{\cdot}6$; in P-ANCA, $P = 0{\cdot}9$). However, NCGN patient sera were 5·5 times less likely (95% CI 0·1, 0·8) than OGD patient sera to have anti-azurocidin antibodies, and this was statistically different ($P = 0{\cdot}005$) in P-ANCA but not in C-ANCA patients ($P = 0{\cdot}1$).

Comparison of IFA-ANCA-positive and IFA-ANCA-negative patient sera. The percentages of anti-BPI-positive sera in C-ANCA, P-ANCA and IFA-ANCA-negative sera were 30·3%, 17·5% and 20·8%, and the percentages of anti-azurocidin-positive sera were 3·0%, 8·3% and 8·3%, respectively. There was no statistically significant difference between IFA-ANCA-positive and IFA-ANCA-negative sera in frequency of anti-BPI and anti-azurocidin antibodies.

Renal pathology in OGD patients with anti-BPI or anti-azurocidin

OGD patients who were positive for anti-BPI had the following heterogeneous types of renal injury: lupus glomerulonephritis ($n = 6$), IgA nephropathy ($n = 4$), membranoproliferative glomerulonephritis ($n = 2$), other proliferative glomerulonephritis ($n = 6$), membranous glomerulopathy ($n = 3$), minimal change glomerulopathy ($n = 1$), focal segmental glomerulosclerosis ($n = 8$), thrombotic microangiopathy ($n = 1$), and miscellaneous other ($n = 8$). Likewise, OGD patients who were positive for anti-azurocidin had the following heterogeneous renal diseases: lupus glomerulonephritis ($n = 3$), IgA nephropathy ($n = 5$), membranoproliferative glomerulonephritis ($n = 1$), other proliferative glomerulonephritis ($n = 2$), membranous glomerulopathy ($n = 3$), minimal change glomerulopathy ($n = 2$), focal segmental glomerulosclerosis ($n = 2$), and miscellaneous other ($n = 5$). Therefore, among the OGD patients, there was no specific pattern of renal disease that was associated with anti-BPI or anti-azurocidin.

Western blot

By Western blot analysis, regardless of whether purified BPI or recombinant BPI was used as antigen, a 57-kD band was observed with both IFA-ANCA-positive and IFA-ANCA-negative anti-BPI-positive sera, whereas anti-PR3-positive sera and normal control sera were negative (Fig. 1b). When purified azurocidin was used as antigen, a 29-kD band was shown with both IFA-ANCA-positive and IFA-ANCA-negative anti-azurocidin-positive sera, whereas normal control sera were negative (Fig. 1c).

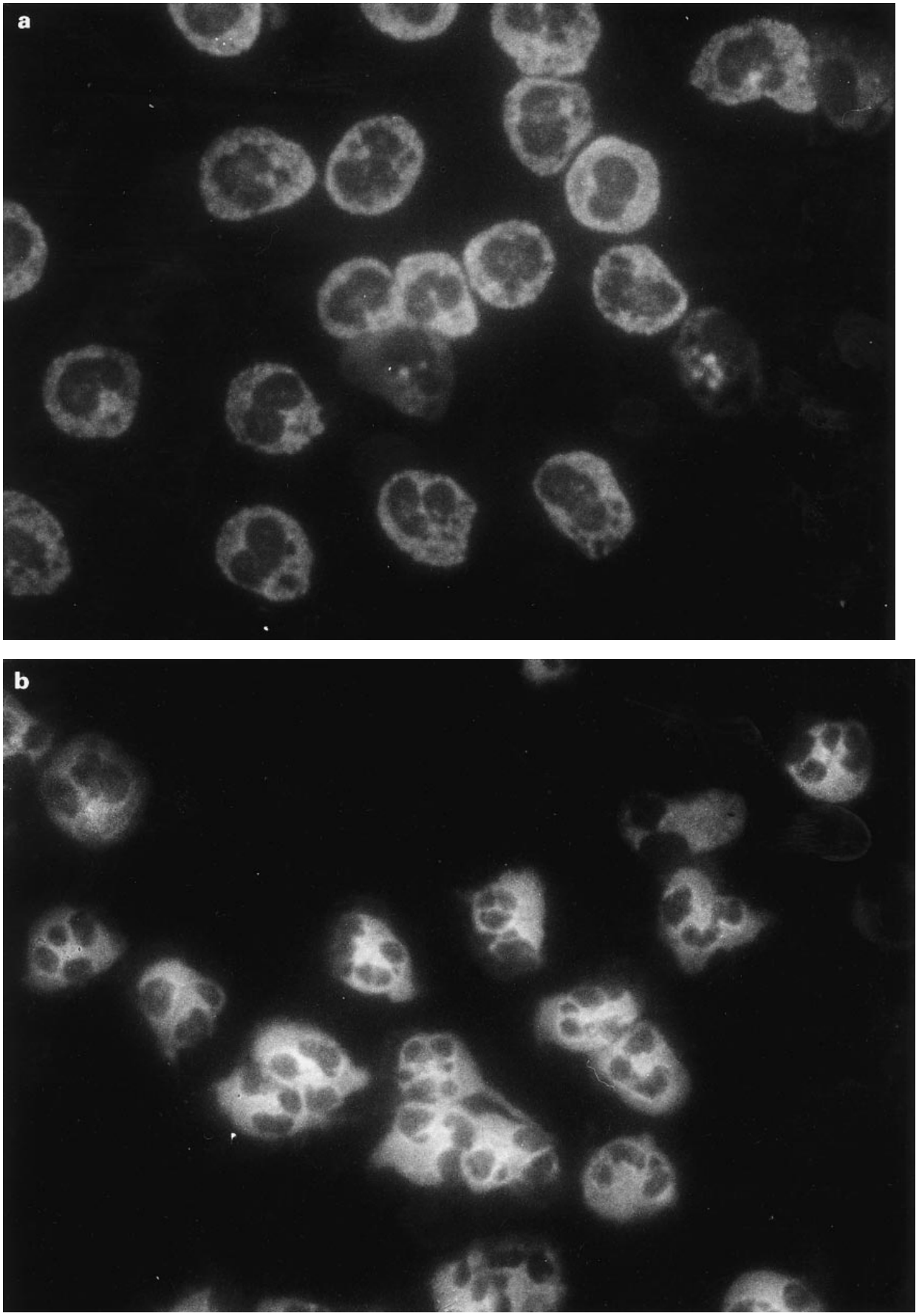
DISCUSSION

Several specificities for ANCA have been elucidated. In patients with pauci-immune necrotizing glomerulonephritis and/or systemic small vessel vasculitis, ≈90% of P-ANCA are specific for MPO as determined by ELISA and Western blot analysis using purified antigens [4]. A few P-ANCA also react with other neutrophil constituents, such as elastase and lactoferrin [5,18]. Most C-ANCA sera are specific for PR3 [5–9]. Immunochemical assays, such as ELISA, that use purified neutrophil antigens are more definitive than IFA for detecting ANCA with specificity for particular antigens.

Recently, Zhao and associates have reported that BPI is an important ANCA antigen. They found that 45 of 100 samples that were IFA-ANCA-positive, yet recognized neither PR3 nor MPO (double-negative samples), and 44 of 400 new routine samples recognized BPI by ELISA [11]. Zhao & Lockwood have also reported that azurocidin is an ANCA antigen in vasculitis. They found that 10 of 50 anti-MPO-positive samples, and five of 37 double-negative samples were positive for azurocidin by ELISA, whilst none of the 33 anti-PR3 positive samples was positive for azurocidin [12].

In our study, we found that 30 of 99 C-ANCA serum samples recognized recombinant BPI and 31 of 99 C-ANCA serum samples recognized purified BPI by ELISA. We also found that 19 of 229

Fig. 3. (see page 130) Indirect immunofluorescence microscopy reaction pattern with alcohol-fixed neutrophils of anti-BPI-positive patient serum producing diffuse granular cytoplasmic staining (a) and of PR3-ANCA patient serum producing typical cytoplasmic staining with central accentuation (b).


a
b

P-ANCA serum samples recognized purified azurocidin by ELISA. The reactivity of anti-BPI-positive sera to the 57-kD band of either purified or recombinant BPI, and anti-azurocidin-positive sera to the 29-kD band of purified azurocidin were confirmed by Western blot analysis. Monoclonal or polyclonal anti-BPI antibodies produced a CG-ANCA pattern, and rabbit anti-azurocidin antibody produced a P-ANCA pattern on alcohol-fixed human neutrophils by IFA. These results suggest that BPI can be a C-ANCA antigen and azurocidin a P-ANCA antigen.

When we divided 328 IFA-ANCA-positive samples into NCGN and OGD groups according to renal biopsy diagnoses, there was no difference in frequency of anti-BPI antibodies between the two groups, and there was a higher frequency of anti-azurocidin antibodies in OGD samples. These results indicate that anti-BPI and anti-azurocidin do not correlate with pauci-immune NCGN, which is a marker for ANCA-associated small vessel vasculitis. Furthermore, a similar anti-BPI and anti-azurocidin frequency was found in IFA-ANCA-positive and IFA-ANCA-negative renal disease patient sera. This result indicates that anti-BPI and anti-azurocidin ELISA positivity does not always correlate with IFA-ANCA positivity. This may be because of greater sensitivity of ELISA for anti-BPI and anti-azurocidin.

In conclusion, anti-BPI and anti-azurocidin antibodies occur in a minority of patients with renal disease, but they do not correlate with type of renal disease or IFA ANCA result.

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