

ANCA against bactericidal/permeability-increasing protein, azurocidin, calprotectin and defensins in rheumatic and infectious diseases: Prevalence and clinical associations

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ABSTRACT

Objective. To determine the prevalence and clinical associations of ANCA against the antibiotic proteins and peptides: Bactericidal/permeability-increasing protein (BPI), Azurocidin (AZ), Calprotectin (CP) and β -Defensin-1 and -2 (DF).

Methods. Patients with ANCA-associated vasculitides ($n=99$), other vasculitides and rheumatic connective tissue diseases ($n=303$), HIV-infection ($n=66$), other infectious diseases ($n=134$) Crohn's disease ($n=12$) and ulcerative colitis ($n=12$) were tested for BPI-, AZ-, CP-, DF-, PR3-, and MPO-ANCA in indirect immunofluorescence technique (IFT) and ELISA.

Results. In ANCA associated vasculitides BPI-ANCA were detected in 6% of patients. In HIV infection, BPI was the main target antigen of ANCA-IFT positive sera (74%). BPI-ANCA was associated with higher inflammatory activity. In Crohn's disease and ulcerative colitis BPI-ANCA was prominent (34% of patients). AZ-ANCA were found in 5% of patients. No ANCA were detected against defensin and calprotectin.

Conclusion. BPI-ANCA is the main autoantibody in HIV and is associated with higher inflammatory activity. In inflammatory bowel diseases BPI-ANCA is predominant, AZ-ANCA are also present to a lesser extent. Both were not useful to characterize clinical subgroups. No ANCA were detected against calprotectin or defensins.

Introduction

The neutrophil or polymorphonuclear leukocyte (PMN) is a central cellular effector of the innate immune system. Neutrophil-derived antibiotic proteins

and peptides represent a source of innate defence molecules that target the microbial membrane leading to growth arrest and in some instances, neutralization of proinflammatory surface components (e.g. endotoxin). Based on their structure, antimicrobial proteins and peptides can be divided into several families: serprocidins [(human leukocyte elastase (HLE), proteinase 3 (PR3), cathepsin G (CG), and azurocidin (AZ)], cathelicidins (hCAP18), lactoferrin (LF), bactericidal/permeability increasing protein (BPI), lysozyme (LZ), myeloperoxidase (MPO), calprotectin (CP) and defensin (DF) (1).

Interestingly, most of these known antibiotic molecules are target antigens for antineutrophil cytoplasmic autoantibodies (2). For many diseases in which ANCA occur an infectious etiology is discussed. Therefore a connection between PMN-mediated host defence and ANCA-induction is conceivable. Recent observations in a subgroup of patients with chronic onchocerca volvulus infection demonstrated a high prevalence of autoantibodies against defensin that was strongly associated with a simultaneous presentation of defensin on the surface of onchocerca volvulus in patient's tissue and a severe disease course (3). The investigation of the presence of autoantibodies against antibiotic proteins and their interactions with target antigens during exposure to infection might help to explain how the autoantibodies against antibiotic proteins may contribute to the development of autoimmune diseases. In the present study we explored the prevalence and clinical associations of ANCA against azurocidin, bactericidal/permeability increasing protein,

calprotectin and defensins and determined whether they help to define subgroups of patients with infectious and rheumatic diseases.

Patients and methods

Patients

Sera were obtained from patients with ANCA-associated vasculitides [n = 99; M/F 49/35; median age 53.4 years; median disease duration 3.3 (0.1 – 19) years, comprising patients with Wegener's granulomatosis (n = 70), microscopic polyangiitis (n = 14) and Churg-Strauss syndrome (n = 15)]

as well as from patients with other vasculitides and rheumatic connective tissue diseases [n = 303; M/F 134/168; median age 46 years. Patients with systemic lupus erythematosus (SLE) (n = 62), rheumatoid arthritis (RA) (n = 36), rheumatoid vasculitis (n = 20), Horton's disease (n = 19), classical panarteritis nodosa (n = 25), psoriatic arthritis (n = 26), ankylosing spondylitis (n = 20), scleroderma (n = 15), sarcoidosis (n = 14), Kawasaki's disease (n = 19), Sjögren's syndrome (n = 20), Still's disease (n = 10), Behçet's disease (n = 7), Reiter's disease (n = 4), Felty's disease (n = 2) and essential cryoglobulinemia (n = 4)]. Sera were also obtained from patients with HIV [n = 66; M/F 60/6; median age 39 years; median disease duration 3.3 (0.1 – 9.2) years] and other infectious diseases [n = 134; M/F 76/58; median age 51.2 years. Patients with: echinococcosis (n = 34), malaria (n = 35), hepatitis C (n = 14), tuberculosis (n = 10), sepsis (n = 16), borreliosis (n = 5), syphilis (n = 20)]. The ANCA-associated inflammatory bowel disease group consisted of patients with Crohn's disease [n = 12; M/F 3/9; median age 31 years; median disease duration 5.3 (0.2 – 15) years] and ulcerative colitis [n = 12; M/F 5/7; median age 39 years; median disease duration 6.7 (0.1 – 13) years].

All patients were diagnosed according to international standards, leading ultimately to the generally accepted classifications used in routine clinical work, by seeking biopsy proven vasculitis, and applying the 1990 classification criteria of the American College of Rheumatology and the definitions of

the 1992 Chapel Hill Consensus Conference (4, 5) in patients with vasculitides or the internationally accepted classification criteria, e.g. for SLE (6) and RA (7) in patients with connective tissue diseases. The diagnoses of ulcerative colitis or Crohn's disease was made according to established criteria using clinical, serologic and histological findings (8, 9). Sera from 140 healthy blood donors served as controls. Informed consent was obtained prior to drawing blood.

Indirect immunofluorescence technique (IFT)

Detection of ANCA by the indirect immunofluorescence technique (IFT) was performed according to European guidelines (10). Briefly, air dried, ethanol-fixed cytospin preparations of pooled granulocytes from healthy donors were incubated with diluted patients' sera. Autoantibody binding was detected with FITC-conjugated Fab₂ fragments of rabbit anti-human IgG (DAKO, Copenhagen, Denmark). Fluorescence patterns were classified as cytoplasmic (cANCA), perinuclear (pANCA) or atypical (aANCA). For differentiation of pANCA from ANA samples were also tested on formalin fixed neutrophils and HEp2-cells.

ANCA ELISA

Detection of ANCA by ELISA for PR3 and MPO was performed according to standardized European guidelines as earlier described (10, 11). For detection of BPI-ANCA an ELISA was performed as previously described (12, 13). An AZ-ANCA ELISA-Kit was kindly provided by Dr. Joerg Wieslander (Wieslab Inc.).

Defensin (DF)- and Calprotectin (CP)-ANCA ELISA

For detection of autoantibodies against defensin and calprotectin two ELISAs were established. In brief, 96-well microtiter plates were coated with either defensin (HBD-1, and HBD-2, Bachem, Heidelberg, Germany) or calprotectin purified as previously described (8) at a concentration of 2 µg/ml. Sera were added at a dilution of 1:50 in PBS and monoclonal antibodies against de-

fensin (anti-human defensin, Bachem Heidelberg, Germany) and calprotectin (anti-calprotectin, MAC-387, DAKO, Denmark) were used as positive controls as well as sera from onchocerciasis patients with anti-defensin antibodies (kind gift of Dr. Gallin, BNI Institute for Tropical Medicine, Hamburg, Germany). Detection of autoantibodies was performed with anti-human IgG ALP labeled conjugate. Extinction was measured at 405 nm (620 nm). The sera of 140 healthy donors were used to standardize the assay. Cut off was defined as the mean OD-value plus 3 SD. Values in healthy donors did not exceed this value. Intraassay variability ranged from 3.5 to 7.7% (n = 5), inter-assay variability was 9.1% (n = 4). Results were considered positive if the OD value exceeded the cut off value and was at least twice as high as the blank control. Positive results in ELISA were confirmed by inhibition assay.

Statistical analysis

Patients' clinical and laboratory data were compared with regard to differences in ANCA positive and ANCA negative sera by t-test for independent samples, Mann-Whitney U test and chi square test and considered significant at p < 0.05.

Results

In ANCA-associated vasculitides besides ANCA against PR3 and MPO, BPI-ANCA were found in 6% of patients in ELISA. AZ-ANCA were not present in any of these patients, nor did they play a significant role in this study (Fig.1). In the group of other vasculitides and rheumatic connective tissue diseases, neither DF nor AZ nor BPI nor PR3 or MPO played a significant role as an ANCA target antigen. In contrast, BPI was the predominant target antigen of ANCA in HIV infection (29% of all patients). 14 out of 19 ANCA-IFT positive HIV patients were BPI-ANCA positive (=74%). Except for one HIV patient who had also a BPI-ANCA, PR3-ANCA or MPO-ANCA were not found outside the ANCA-associated vasculitides group. Clinical data available for HIV-infected patients showed no significant dif-

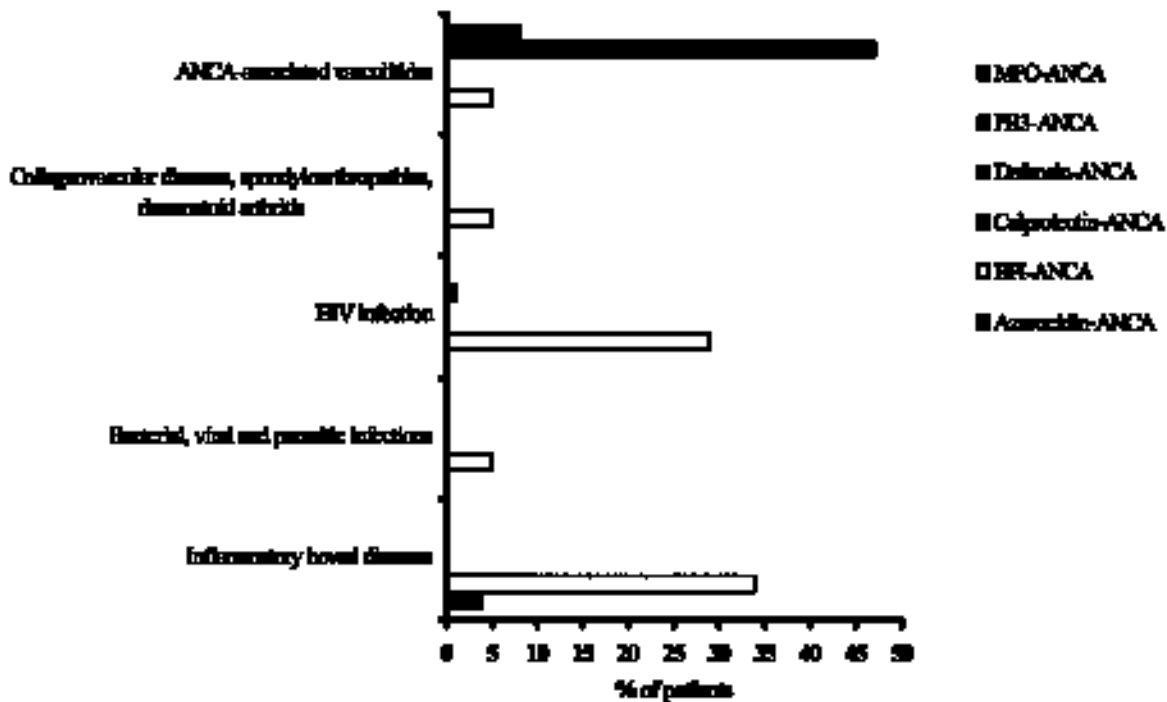


Fig. 1. Prevalence of ANCA against antibiotic proteins and peptides. Sera from patients with ANCA-associated vasculitides, rheumatic and infectious diseases as listed above and healthy controls were tested by ELISA for the presence of ANCA against antibiotic proteins and peptides, as described in *Patients and Methods*. Shown is the percentage of ANCA-ELISA positive patients.

ferences with regard to manifestations of AIDS, opportunistic infections, malignant conditions or normal findings between BPI-ANCA positive and negative patients. However, there was a trend towards higher inflammatory activity and lower lymphocyte counts in BPI-ANCA positive patients (Table I). In the other infectious diseases BPI-ANCA were found in <5%, none of the other ANCA subspecificities was found. In inflammatory bowel diseases BPI-ANCA could be detected in 34% of patients (25% for Crohn's disease and 42% for ulcerative colitis) whereas ANCA against azurocidin occurred only in one patient with ulcerative colitis. No significant prevalence of ANCA was seen in the healthy control group. Finally, no ANCA against either calprotectin or defensins were found in any of the examined sera.

Discussion

The aim of this study was to determine the prevalence and clinical associations of ANCA against the recently discovered ANCA target antigens azurocidin, defensins and BPI and the cytosolic antibiotic calprotectin in rheumatic dis-

eases, inflammatory bowel diseases and infections. In a previous study no autoantibodies against calprotectin were detected in a smaller number of patients with inflammatory rheumatic diseases (15). Striking findings of Gallin *et al.* had shown that ANCA against the antimicrobial neutrophil granule

constituent defensin were generated in all patients with a chronic hyperreactive form of onchocerca volvulus infection (sowda) and disappeared or decreased after microfilaricidal treatment (3). As yet, the prevalence of ANCA in infectious diseases has been addressed but mostly in the differentia-

Table I. Comparison of clinical and laboratory data between BPI-ANCA positive and negative patients with HIV infection (expressed as the mean \pm S.D.).

	BPI-ANCA positive (n=19)	BPI-ANCA negative (n=47)
Sex (m/f)	18/1	43/4
Age	37.8 \pm 10.7	39.8 \pm 9.9
CRP(mg/dl), normal range < 0.5 mg/dl	11.7 \pm 39.1	2.8 \pm 22.6
Lymphocytes (per nl)	810 \pm 691	1058 \pm 701
CD4 positive cells (per nl)	85 \pm 94	109 \pm 104
Neutrophils (per nl)	2074 \pm 1074	1766 \pm 1211
AIDS	12 (63%)	35 (74%)
Kaposi sarcoma	3 (16%)	11 (23%)
Non-Hodgkin's lymphoma	2 (11%)	5 (11%)
CMV infection	4 (21%)	8 (17%)
Normal findings	6 (32%)	14 (30%)
IFT		
aANCA	10	0
cANCA	1	0
pANCA	3	0
negative	5	47

tion of PR3- and MPO-ANCA associated vasculitides (AAV) from infectious diseases (2).

Aside from the classical ANCA target antigens, such as PR3 or MPO that were found in AAV, BPI was the only ANCA target antigen that could be detected in noteworthy amounts in some of the examined diseases such as inflammatory bowel diseases and HIV infection. Neither BPI nor azurocidin, defensin or calprotectin was helpful in distinguishing between granulomatous diseases (Wegener's granulomatosis, tuberculosis, Crohn's disease) and other diseases, localized versus generalized disease or infectious versus rheumatic diseases.

In HIV infection, however, BPI-ANCA positive patients had a higher inflammatory activity as determined by serum CRP and showed a trend towards lower lymphocyte counts. Larger prospective studies might show if this difference is significant. Previous data on the prevalence of ANCA in HIV patients showed positive IFT results in 17-20% with the rare occurrence of PR3- or MPO-ANCA in ELISA but a large number of atypical ANCA (aANCA) in IFT with unknown subspecificities (16-18). An association with autoimmune or opportunistic diseases was not found (16). In the present study BPI-ANCA was the main target antigen of ANCA-IFT positive patients. So far BPI-ANCA have been mostly described in settings where exposure to Gram-negative bacteria and cell-free LPS is appreciable and sometimes chronic such as chronic inflammatory bowel diseases (19), cystic fibrosis (20, 21) or reactive arthritis due to Gram-negative bacteria (22).

In conclusion, firstly ANCA against azurocidin, defensin and calprotectin were not detected in infectious nor in rheumatic diseases. It is remarkable, that certain antibiotic contents of neutrophils become ANCA target antigens, while others seem to be unaffected. It has to be appreciated that the target antigen itself plays a more or less active role in ANCA generation. Details have to be subject of further investigations. Secondly the bactericidal/permeability increasing protein is the main target antigen of ANCA in HIV and

inflammatory bowel diseases. In ANCA-IFT positive HIV patients prevalence of BPI-ANCA may indicate a higher inflammatory activity. Further prospective studies are needed. Finally, azurocidin-ANCA are present in a small number of IBD patients but are not useful to distinguish between and identify subgroups of infectious and rheumatic diseases.

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