Anti-neutrophil cytoplasmic antibodies (ANCA) in serum and bronchoalveolar lavage fluids of cystic fibrosis patients and patients with idiopathic bronchiectasis

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SUMMARY: Çobanoğlu N, Özçelik U, Çetin İ, Yalçın E, Doğru D, Kiper N, Bakkaloğlu A. Anti-neutrophil cytoplasmic antibodies (ANCA) in serum and bronchoalveolar lavage fluids of cystic fibrosis patients and patients with idiopathic bronchiectasis. Turk J Pediatr 2010; 52: 343-347.

We investigated the presence of anti-neutrophil cytoplasmic antibodies (ANCA) in the serum and bronchoalveolar lavage fluid (BALF) of 21 cystic fibrosis (CF), 7 idiopathic bronchiectasis (IBR), and 11 control children and the relation between ANCA and any bacteria grown in BALF. Six of the CFs, but none of the IBRs or controls had positive serum cytoplasmic or perinuclear-ANCA (c-ANCA, p-ANCA). Serum autoantibodies against bactericidal/permeability increasing protein (BPI-ANCA) were positive in 2 CFs, 1 IBR and 1 control. While none of the CFs, IBRs or controls had positive BALF (c- or p-ANCA), 1 CF, 1 IBR and none of the controls had positive BALF BPI-ANCA. *Pseudomonas aeruginosa* **was not grown in the specimens of any of the subjects. As the number of the patients in our study was very limited, further longitudinal and well-designed studies are necessary to show whether or not the presence of ANCA in serum or BALF relates to the presence of** *P. aeruginosa* **infection in the airways of CF and IBR patients.**

Key words: bactericidal/permeability increasing protein-ANCA, cystic fibrosis, bronchiectasis, bronchoalveolar lavage fluid.

Anti-neutrophil cytoplasmic antibodies (ANCA) form a heterogeneous group of antibodies. In the 1980s, vasculitis was identified as a common sign of the diseases associated with ANCA[1]. After the typical connection between ANCA and Wegener's granulomatosis was established, ANCA have become a part of routine diagnostic procedures applied in clinical laboratories[2,3].

Anti-neutrophil cytoplasmic antibodies are directed against antigens located in the cytoplasmic space of polymorphonuclear leukocytes (PMN) and monocytes. The cytoplasmic type (c-ANCA) is mainly associated with the reactivity of an enzyme, proteinase 3 (PR3), whereas the perinuclear type (p-ANCA) is mainly associated with the reactivity of myeloperoxidase (MPO). However, an increasing number of cytoplasmic proteins have been identified to be ANCA target antigens, including bactericidal/permeability-increasing protein (BPI), lactoferrin, cathepsin G, human elastase, or lysozyme[1].

Bactericidal/permeability-increasing protein (BPI) is a 55-kD membrane-associated protein that is an important host defense against Gram-negative bacteria and lipopolysaccharide (4). Although there is a typical association of PR3-ANCA with Wegener's granulomatosis, MPO-ANCA with microscopic polyangiitis, and BPI-ANCA with cystic fibrosis (CF), ANCA against BPI have been recognized in the serum of patients with bronchiectasis, diffuse panbronchiolitis, and transporter associated with antigen presentation (TAP) deficiency as a result of airway infection with Gram-negative bacteria[5-8].

In this study, we aimed to study the presence of ANCA in the serum and bronchoalveolar lavage fluid (BALF) of patients with CF and

with idiopathic bronchiectasis (IBR) and to identify the relation between ANCA and any bacteria grown in BALF.

Material and Methods

In this cross-sectional study, we analyzed the serum and BALF of 21 children with CF, aged 4.5 to 18 years (12 females), seven children with IBR, aged 5 to 16 years (3 females), in whom CF, congenital and acquired immune deficiency disorders, primary ciliary dyskinesia, tuberculosis, and foreign body aspiration were ruled out, and 11 control children, aged 0.5 to 6.5 years (4 females) in whom flexible fiberoptic bronchoscopy was applied due to a problem other than infection (the list and number of diagnoses of control patients are summarized in Table I). Patients with CF and IBR were regularly followed up at Hacettepe University Pediatric Pulmonary Diseases Unit in Ankara. The diagnosis of CF was established by typical clinical features, increased sweat chloride concentrations over 60 mEq/L and detection of DNA mutations.

None of the subjects had acute respiratory tract infection or vasculitis, and were in stable health status. None of the subjects was on systemic or inhaled corticosteroid treatment.

BAL was done by wedging the bronchoscope into the selected bronchus and 1 ml/kg aliquot of sterile saline was instilled three times. Fluid was aspirated into a sterile suction catheter for each aliquot and the first sample was withdrawn because of the risk of contamination from the upper respiratory tract. Specimens were immediately processed after bronchoscopy. After using a small part of all BALF samples for culturing, the rest were centrifuged to separate supernatant of mucus debris. All serum and BALF samples were stored at -70°C until analysis. We studied ANCA using indirect immunofluorescence (IIF) pan-isotype, and BPI-ANCA using direct enzyme-linked immunosorbent assay (ELISA) (immunoglobulin (Ig)G isotype). ANCA determination by IIF was performed according to the recommendations of the First International ANCA Workshop, using 1/20-diluted sera on ethanol-fixed and formalin-fixed neutrophils[9]. ELISA was performed using commercial kit (BPI protein IgG antibodies ELISA kit, Genesis Diagnostics, Ely, UK). BALF cultures were accepted as positive if the bacterial pathogens were detected at $\geq 10^5$ colony-forming units (cfu)/ml.

Research ethics approval and informed consent from the parents of the subjects were obtained.

Table I. The Diagnoses in Control Patients

Diagnosis	Number of patients
Aberrant subclavian artery	1
Atelectasis	1
Bronchial asthma	1
Congenital lobar emphysema	2
Foreign body aspiration	1
Hemoptysis	2
Sarcoidosis	1
Wheezy infant	2
Total	11

Results

Among 21 CF patients, 6 (28.5%) had positive serum c-ANCA and p-ANCA, but none of the IBR patients or controls had positive serum c- or p-ANCA. However, serum BPI-ANCA was positive in 2 (9.5%) CF, 1 (14.2%) IBR, and 1 (9%) control subjects (Table II). While none of the CF, IBR or control subjects had positive BALF (c- or p-ANCA), 1 (4.7%) CF, 1 (14.2%) IBR and 0 control subjects had positive BALF BPI-ANCA (Table II).

The names of the bacteria grown after cultural studies of BALF in patients whose serum c-ANCA, p-ANCA, serum BPI-ANCA and BALF BPI-ANCA were positive are listed in Table II. *Haemophilus influenzae, Staphylococcus aureus, H. parahaemolyticus, H. haemolyticus*, and *Streptococcus pneumoniae* were grown in BALF. *Pseudomonas aeruginosa* (PA) was not grown in the specimens of any subject.

Discussion

In the present study, we assessed the presence of ANCA in the serum and BALF of CF and IBR patients. We also assessed the relation between ANCA and microorganisms that were grown after the culture of BALF.

Since Zhao et al.[5] in 1996 and Sediva et al.[10] in 1998 reported that ANCA were frequently detected in CF patients (either adults, 91% or children, 71%), several studies have been

Table II. Results of BALF Cultures of Subjects Positive for Serum c-ANCA, Serum p-ANCA, Serum BPI-ANCA, and BALF BPI-ANCA

	Subject and no	n (%)	BALF culture result
Serum c-ANCA positive	CF 2	6 (28.5%)	*Haemophilus influenzae*
	CF 5		*Staphylococcus aureus*
	CF 6		*Haemophilus parahaemolyticus*
	CF 16		*None*
	CF 17		*Staphylococcus aureus*
	CF 21		*None*
Serum p-ANCA positive	CF 2	6 (28.5%)	*Haemophilus influenzae*
	CF 5		*Staphylococcus aureus*
	CF 6		*Haemophilus parahaemolyticus*
	CF 16		*None*
	CF 17		*Staphylococcus aureus*
	CF 21		*None*
Serum BPI-ANCA positive	CF 2	2 (9.5%)	*Haemophilus influenzae*
	CF 16		*None*
	Bronchiectasis 2	1 (14.2%)	*Streptococcus pneumoniae*
	Control (Sarcoidosis)	1 (9%)	*Haemophilus hemolyticus*
BALF BPI-ANCA positive	CF 15	1 (4.7%)	*None*
	Bronchiectasis 6	1 (14.2%)	*Haemophilus hemolyticus*

BALF: Bronchoalveolar lavage fluid. BPI: Bactericidal/permeability increasing protein. c-ANCA: Cytoplasmic anti-neutrophil cytoplasmic antibodies. CF: Cystic fibrosis. p-ANCA: Perinuclear anti-neutrophil cytoplasmic antibodies.

performed by different authors. Carmona et al.[11] analyzed 105 patients with CF (56 adults, 49 children) for ANCA. In adults, 29 of the 56 patients (51.8%) exhibited ANCA in their serum samples while only seven of the 49 children (14.3%) were positive for this autoantibody. Tacetti et al.[12] studied ANCA in the serum of 65 CF patients without CF vasculitis, aged 5-45 years. ANCA were detected in the sera of 19 of the 65 patients (29.2%). Compatible with Tacetti et al.'s study[12], we detected ANCA in the serum of 28.5% of CF children. None of the patients with IBR and none of the control subjects had positive serum ANCA in our study. These results support the association of ANCA with CF.

We found an equal positivity of serum c- and p-ANCA in CF patients. There have been some reports of an association of c-ANCA with CF[1]. However, according to the current English literature, our study is the first one showing an association of p-ANCA with CF. In studies comprising 24-148 CF patients, serum BPI-ANCA have been found in 33.8-91% of the tested patients[5,10-16]. In our study, serum BPI-ANCA was positive in 9.5% of CF patients. Anti-neutrophil cytoplasmic antibodies against BPI have been recognized also in the serum of patients with bronchiectasis, diffuse panbronchiolitis and TAP deficiency[6-8]. While the reports about bronchiectasis and diffuse panbronchiolitis were case reports[6,7], Schultz et al.[8] showed that five of six TAP-deficient patients had positive serum BPI-ANCA. Although the number of patients with IBR was very limited in our study, we showed the presence of BPI-ANCA in the serum of 14.2% of them.

It was surprising that one (9%) of our control patients had BPI-ANCA in his serum although no bacteria were grown in the BALF culture. However, this is not the first case reported in the current literature. In their studies, Rotschild et al.[17] found BPI-ANCA in the serum of 67% of CF patients and in 22% of healthy subjects.

Aichele et al.[18] analyzed the expression of BPI in the airways of 51 patients with CF. In their sputum samples or BAL specimens, nearly all patients (43 of 51) expressed BPI mRNA and protein, which were mainly products of neutrophil granulocytes as revealed by intracellular staining and subsequent flow cytometry. Once BPI-ANCA are generated and circulate in the bloodstream, plasma exudation allows transition of immunoglobulins in the

alveolar space and makes possible binding of BPI-ANCA to BPI. Simultaneous binding of BPI-ANCA to BPI results in release of granule content and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 or IL-6[19]. Although the quantitative role of BPI in the alveolar space is not yet determined, the antibiotic and anti-endotoxin activities of the BPI can be neutralized by patients' BPI-ANCA locally, and reduce host-defense responses to Gram-negative bacteria and endotoxin and create a chronically inflamed alveolar environment that facilitates lung damage[8]. While none of the CF patients, patients with IBR or control subjects had positive BALF ANCA, one (4.7%) of the CF patients and one (14.2%) of the patients with IBR had positive BALF BPI-ANCA, despite not having positive serum BPI-ANCA. To our knowledge, this is the first study in the current English literature showing the presence of BPI-ANCA in BALF of CF patients and patients with IBR.

In CF, BPI-ANCA have not been detected in patients with Gram-positive colonization only. Gram-negative bacteria other than PA, such as *Burkholderia cepacia*, are too rare in the populations studied[20]. Mahadeva et al.[13] suggested that certain epitopes on PA may trigger ANCA production by acting as a molecular mimic of BPI, as seen in some viral infections[21], and helper T cells specific for PA would then provoke BPI-dedicated B cells into autoantibody production. Epitope mapping of patients' BPI-ANCA has revealed, in a subset of these individuals, strong similarity of certain BPI epitopes to specific outer membrane proteins of *Escherichia coli* and PA[22]. Thus, molecular mimicry could be one mechanism for initiation of BPI-autoantibody development. In the great majority of the studies comprising CF patients, the airways of the patients who had BPI-ANCA in their serum were chronically colonized by PA[8,12,13,16,17,23]. Interestingly, one study found no correlation between BPI-ANCA antibodies and infection with Pseudomonas species and has described the presence of anti-BPI autoantibodies before the development of clinical disease in CF [10]. The youngest patients in the study of Dorlochter et al.[16] were six and seven years and both were positive for BPI-ANCA without PA colonization. Similarly, PA was not grown in the BALF cultures in any of the subjects in our study, and none of them was chronically colonized with PA. This raises the possibility that it may be linked to the early inflammation in the absence of infection described in CF[24].

Some studies[13,16,17,20,23] have assessed the relationship of BPI-ANCA to pulmonary disease severity in CF subjects. BPI-ANCA were independently associated with more severe lung disease as assessed by chest radiograph or high resolution computerized tomography (HRCT) score and a significantly lower forced expiratory volume in 1 second %. In our study, we did not assess the severity of lung disease and its correlation with BPI-ANCA.

In conclusion, to show whether the presence of ANCA in the serum and BALF may or may not relate to the presence of PA infection in the airways of CF patients and patients with IBR, further longitudinal and well-designed studies are necessary with an efficient number of patients.

REFERENCES

1. Bartunkova J, Tesar V, Sediva A. Diagnostic and pathogenetic role of antineutrophil cytoplasmic autoantibodies. Clin Immunol 2003; 106: 73-82.
2. van der Woude FJ, Rasmussen N, Lobatto S, et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985; 1: 425-429.
3. Gross WL, Schmitt WH, Csernok E. ANCA and associated diseases: immunodiagnostic and pathogenetic aspects. Clin Exp Immunol 1993; 91: 1-12.
4. Weiss J, Olsson I. Cellular and subcellular localization of the bactericidal/permeability-increasing protein of neutrophils. Blood 1987; 69: 652-659.
5. Zhao MH, Jayne DR, Ardiles LG, Culley F, Hodson ME, Lockwood CM. Autoantibodies against bactericidal/permeability-increasing protein in patients with cystic fibrosis. Q J Med 1996; 98: 259-265.
6. Kobayashi O. Clinical role of autoantibody against bactericidal/permeability-increasing protein in chronic airway infection. J Infect Chemother 1998; 4: 83-93.
7. Matsuyama W, Wahimoto J, Watanabe A, et al. Bronchiectasis with myeloperoxidase antineutrophil cytoplasmic antibody and bactericidal/permeability-increasing protein antineutrophil cytoplasmic antibody. Intern Med 1999; 38: 813-816.
8. Schultz H, Schinke S, Weiss J, Cerundolo V, Gross WL, Gadola S. BPI-ANCA in transporter associated with antigen presentation (TAP) deficiency: possible role in susceptibility to Gram-negative bacterial infections. Clin Exp Immunol 2003; 133: 252-259.
9. Wiik A. Delineation of a standard procedure for indirect immunofluorescence detection of ANCA. Acta Pathol

Microbiol Immunol Scand 1989; 97: 12-13.

10. Sediva A, Bartunkova J, Kolarova I, et al. Antineutrophil cytoplasmic autoantibodies (ANCA) in children with cystic fibrosis. J Autoimmun 1998; 11: 185-190.
11. Carmona E, Perez-Aguilar F, Lopez JA, Ferrer-Calvete J, Sanchez-Cuenca JM. Anti-neutrophil-cytoplasmic auto-antibodies (ANCA) in 105 patients (56 adults and 49 children) suffering from cystic fibrosis, attending a Spanish hospital. J Intern Med 2002; 252: 281-282.
12. Tacetti G, Campana S, Marianelli L, Procapio E. Autoantibodies against bactericidal/permeability-increasing protein in cystic fibrosis patients: comment on the article by Hoffmen and Specks. Arthritis Rheum 1999; 42: 1304-1308.
13. Mahadeva R, Dunn AC, Westerbeek RC, et al. Anti-neutrophil cytoplasmic antibodies (ANCA) against bactericidal/permeability-increasing protein (BPI) and cystic fibrosis lung disease. Clin Exp Immunol 1999; 117: 561-567.
14. Cooper T, Savige J, Nassis L, et al. Clinical associations and characterisation of antineutrophil cytoplasmic antibodies directed against bactericidal/permeability-increasing protein and azurocidin. Rheumatol Int 2000; 19: 129-136.
15. Schultz H, Csernak E, Schuster A, Schmitz TS, Ernst M, Gross WL. Anti-neutrophil cytoplasmic antibodies (ANCA) against bactericidal/permeability-increasing protein (BPI) in pediatric cystic fibrosis patients do not recognize N-terminal regions important for the anti-microbial and lipopolysaccharide binding activity of BPI. Pediatr Allergy Immunol 2000; 11: 64-70.
16. Dorlochter L, Carlsson M, Olafsdottir EJ, Roksund OD, Rosendahl K, Fluge G. Anti-neutrophil cytoplasmic antibodies and lung disease in cystic fibrosis. J Cyst Fibros 2004; 3: 179-183.
17. Rotschild M, Elias N, Berkowitz D, et al. Autoantibodies against bactericidal/permeability-increasing protein (BPI-ANCA) in cystic fibrosis treated with azithromycin. Clin Exp Med 2005; 5: 80-85.
18. Aichele D, Schnare M, Saake M, Rollinghoff M, Gessner A. Expression and antimicrobial function of bactericidal permeability-increasing protein in cystic fibrosis patients. Infect Immun 2006; 74: 4708-4714.
19. Schultz H, Weiss J, Carroll SF, Gross WL. The endotoxin-binding bactericidal/permeability-increasing protein (BPI): a target antigen of autoantibodies. J Leukoc Biol 2001; 69: 505-512.
20. Carlsson M, Eriksson L, Pressler T, et al. Autoantibody response to BPI predicts disease severity and outcome in cystic fibrosis. J Cyst Fibros 2007; 6: 228-233.
21. Davies JM. Molecular mimicry: can epitope mimicry induce autoimmune disease? Immunol Cell Biol 1997; 75: 113-126.
22. Schultz H. From infection to autoimmunity: a new model for induction of ANCA against the bactericidal/permeability increasing protein (BPI). Autoimmun Rev 2007; 6: 223-227.
23. Carlsson M, Eriksson L, Erwander I, Wieslander J, Segelmark M. Pseudomonas-induced lung damage in cystic fibrosis correlates to bactericidal-permeability increasing protein (BPI)-autoantibodies. Clin Exp Rheumatol 2003; 21: S95-S100.
24. Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Richers DW. Early pulmonary inflammation in infants with cystic fibrosis. Am J Resp Crit Care Med 1995; 151: 939-941.