

Antineutrophil cytoplasmic antibodies (ANCA) in idiopathic pulmonary hemosiderosis

Blanco A, Solis P, Gomez S, Valbuena C, Telleria JJ. Antineutrophil cytoplasmic antibodies (ANCA) in idiopathic pulmonary hemosiderosis. *Pediatr Allergy Immunol* 1994; 5: 235–239. © Munksgaard 1994.

Four children were diagnosed with idiopathic pulmonary hemosiderosis (IPH), over a period of 4 years. Retrospectively, antineutrophil cytoplasmic antibodies (ANCA) were studied by indirect immunofluorescence (IIF) and ELISA in 18 sera from these patients, stored at -20°C . ANCA-positive sera, from 1/20 to 1/1, 200 dilution, were found in 3/4 of the patients, by IIF. The patient with the highest titre of ANCA died 3 months later during an acute crisis, the other two patients need a minimal dose of steroids. In one case only, a patient who is still without treatment, had no ANCA. The antibodies anti-myeloperoxidase and anti-proteinase-3 were negative or at border line levels. Rheumatoid factor, anti-nuclear (Hep-2), anti-endomysial, anti-reticulin and antibasement membrane antibodies were negative in all sera. The surviving patients were followed-up for more than 10 years with no systemic or renal disease appearances. The presence of serum ANCA may help to classify children with pulmonary haemorrhage and may have a prognostic value.

**A. Blanco, P. Solis, S. Gomez,
C. Valbuena, J. J. Telleria**

Department of Pediatrics, Medical School, Hospital Clinico, University of Valladolid, Spain

A. Blanco Quirós, Facultad de Medicina, c/Ramón Cajal 5, 47005-Valladolid, Spain

Key words: Idiopathic pulmonary hemosiderosis; anti-neutrophil cytoplasmic antibodies; ANCA

Received December 29, 1993

Accepted May 20, 1994

Idiopathic pulmonary hemosiderosis (IPH) is a rare disorder of unknown etiology, characterized by recurrent episodes of pulmonary haemorrhage, diffuse infiltrates in chest roentgenogram and secondary iron deficiency anaemia. Most of the reported cases are diagnosed during childhood. The term "pulmonary hemosiderosis" indicates an abnormal accumulation of hemosiderin in lung macrophages; it results from a diffuse alveolar haemorrhage. The pathogenesis of PHI has been discussed. It could be due to an increased pulmonary venous pressure, immunologic mechanisms or perhaps intrinsic alveolar defects (1), but a clear and common mechanism has not been established.

Antineutrophil cytoplasmic antibodies (ANCA) have been found in the sera of almost all patients with a biopsy positive for Wegener's granulomatosis (WG). The target antigen of these autoantibodies is a protein located in azurophilic granules of neutrophils and monocytes that is called proteinase-3. Furthermore, several other antineutrophil antibodies have been described. Through the indirect immunofluorescence (IIF) technique different patterns are found, in particular cytoplasmic or perinuclear staining. Articles published in the last decade describe the characteristics of ANCA and suggest a main role in tissue damage (2, 3).

We decided to study serum ANCA in patients diagnosed of IPH because a pulmonary vasculitis could be implicated in this alveolar haemorrhage and because patients with Wegener's granulomatosis can show a pulmonary haemorrhage as the only manifestation.

Methods

Indirect immunofluorescence assay

Slides with ethanol-fixed neutrophils (Inova Diagnostic Inc. CA, USA) were used. The sera were initially tested at 1/10 dilution in phosphate-buffered saline (PRS) pH 7.4, and incubated for 30 min at room temperature. After three washes in PBS, pH 7.4, the slides were stained for 30 min in a humidified chamber with fluorescein-conjugated antihuman IgG antibody mixed with Evan's blue, as counterstain (Inova). The slides were washed three times, mounted with one drop of glycerol buffered in PBS, pH 7.4 and examined at a relative high magnification (x400) by a fluorescence epi-illumination microscopy (Zeiss). The patient results were compared to ANCA+ international standards (Inova) with perinuclear and cytoplasmic patterns. The sera that were positive at 1/10 dilution for IgG-ANCA were studied again with a labelled anti-IgA antibody searching

IgA-ANCA and diluted in progressive titration. The presence of antinuclear antibodies in ANCA+ sera were ruled out by indirect immunofluorescence with Hep-2 cell.

Elisa assay

Microtiter plates with proteinase-3 and myeloperoxidase coated to the wells were used (IBL, Hamburg, Germany). Samples were diluted to 1:50 and incubated for 2 h at room temperature. The washing process with PBS-tween was repeated 4 times in an automatic device. The conjugate incubation step was done with 100 µl of anti-human-IgG labelled with peroxidase. After another was 100 µl of a solution containing tetramethylbenzidine was added to the wells. The reaction was stopped with sulphuric acid about 15 min later and the intensity of colour read in a photometer at 450 nm. The sample levels were calculated on a calibration curve plotted from the absorbency values obtained with 5 standards (IBL); the results were multiplied by the dilution factor.

Patients

Four children, 1 boy and 3 girls, were diagnosed with IPH in our hospital over 4 years. The diagnosis was based on clinical symptoms, chest radiography and hemosiderin-laden macrophages in sputum or gastric juice. The histological confirmation was obtained in Case 1. In all cases, other causes of secondary pulmonary haemorrhage, such as cardiac defects, systemic or rheumatic diseases, were excluded. Clinical or analytical urinary anomalies were not seen in any patient, neither at diagnosis nor during the subsequent outcome. Several sera were collected during acute crises and relapse periods and stored at -20°C and they were used for the ANCA screening. The surviving patients were followed-up, after the diagnosis date, during a period of no less than 10 years.

We have also included in this study 10 normal children from 1 to 5 years of age; 18 patients with Henoch-Schonlein purpura and 6 with cystic fibrosis and chronic pulmonary disease. Only 1 patient suffering Henoch-Schonlein purpura and 1 case with Kartagener's syndrome showed positive serum ANCA at 1/10 dilution. At the present, we are undergoing further studies to find some special feature, although during 4 years follow-up they did not show any peculiarity.

Case 1 – An 8 year-old boy; he was hospitalized complaining of hypotension, pallor, dyspnea and cough. Cardiac enlargement and bilateral pulmonary infiltrates were found in the chest roentgenogram. Iron deficient anaemia, elevated anti-streptolysin

titre and a negative rheumatoid factor were the more significant laboratory data. The lung biopsy showed dilated alveoli containing hemosiderin-laden macrophages, a mild interstitial fibrosis and alveolar degeneration. Basement membrane thickening with hemosiderin deposits was seen by electron microscopy. Direct immunofluorescence staining did not identify immunoglobulins nor complement deposits. The patient had suffered at least 3 similar crises since he was 5 years old. He was treated with steroids and a significant relief was achieved, nevertheless he died 3 months later during his 5th crisis. The necropsy study was not available.

Case 2 – An 11-year-old girl. She had suffered 6 typical crises 3 years before. In all these episodes, she was admitted to the hospital with cough, pallor, dyspnea, abdominal and chest pain. The anemia was occasionally very severe, needing several red cell transfusions. The diagnosis was based on the finding of hemosiderin-laden macrophages in the sputum. The roentgenogram of the chest demonstrated widespread reticulonodular infiltrates. She suffered 4 more crises in the last 2 years and all attempts of decreasing the steroid dose have failed.

Case 3 – A 10-year-old girl. She had recurrent respiratory infections during her first two years of life. She suffered a single acute episode of paroxysmal cough and hemoptysis. The hemoglobin level decreased to 11 g/dl. The chest roentgenogram showed characteristic bilateral infiltrates. The diagnosis was supported by the clinical picture, anemia and chiefly hemosiderin-laden macrophages found in gastric juice and sputum. She has been in complete relapse without treatment for more than 4 years.

Case 4 – A 2-year-old girl. She was mentally retarded due to unknown causes. Her 7-year-old sister has a histological proven glomerulonephritis with >90% of crescents and moderate renal failure. Our patient was admitted with respiratory failure, cough, severe pallor and vomiting. The hemoglobin level was 5.7 g/dl. The roentgenogram showed diffuse pulmonary infiltrates. The diagnosis was made by this clinical picture and the finding of hemosiderin-laden macrophages in gastric juice. The girl suffered a new episode 3 months later. She receives a minimal dose of steroids, twice a week. Over the last 2 years the patient has no acute crises, although she is in a steroid-dependent situation.

Screening of kidney function was made in all patients in order to rule out a renal affectation. Serum creatinine and urinalysis were normal without signs of microscopic hematuria. Blood pressure was normal after the acute crises and no patient showed hypertension at any moment.

Table 1. Patients with idiopathic pulmonary hemosiderosis

Case No.	Sex	Age at diagnosis	Follow-up	Acute crisis	Prednisone treatment	Outcome	ANCA (IIF)		ANCA (ELISA**)		
							IgG-ANCA	IgA-ANCA	PR-3 U/ml	MPO U/ml	Other Auto-Ab*
1	M	8 y.	0 day 1 mo.	Yes Yes	No 1 mg/k	Dead	1.200 200	20 10	<5	6.3	(-) (-)
2	F	11 y.	0 day 1 y. 10 mo. 5 y. 5 mo. 11 y. 1 mo.	Yes No Yes Yes	No 0.3 mg/k 1 mg/k 1 mg/k	Continous treatment (>10 y.)	10 10 <10 20	<10 <10 <10	<5 23 6.5	<5 8 7.6	 (-)
3	F	10 y.	0 day 1 w. 2 w. 4 w.	Yes No No No	No No No No	Complete remission (>4 y.)	<10 <10 <10 <10	<10	<5	13	(-)
4	F	2 y.	0 day 8 mo. 1 y. 3 mo. 2 y. 8 mo. 5 y. 2 mo.	Yes Yes Yes No Yes	No 0.5 mg/k 1 mg/k 0.3 mg/k 0.3 mg/k	Continous treatment (> 12 y.)	10 20 10 <10 <10	<10	<5	<5	 (-) (-)

* Antinuclear (Hep-2); anti-basement membrane; anti-reticulin and anti-endomysial antibodies

** PR-3: anti-pronase-3; MPO: anti-myeloperoxidase

Other immunological studies

Except for no. 4, we found circulating C1q-binding immune complexes (CIC) in all patients, but mainly during the acute crisis. Patient 2 showed elevated levels of CIC in 4 consecutive crises, but they disappeared within the following 36 h. In summary, increased CIC levels were present in 6/7 sera taken during acute crises and only in 1/12 sera taken during relapses (4). Antinuclear (Hep-2), antiendomysial (monkey oesophagus), antireticulin and anti-basement membrane antibodies (monkey and rat tissue), revealed by IIF (Byosistems. Spain) were always negative in all patients. Other immunological

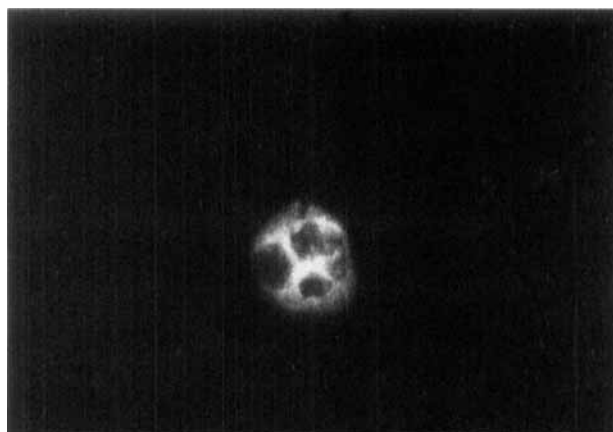


Fig. 1. Indirect immunofluorescence microscopy using alcohol-fixed neutrophils as substrate to show the cytoplasmic pattern of IgG-ANCA in Case 2 ($\times 40$). The fluorescence has a fine granular staining with accentuation between the nuclear lobes. The ANCA pattern was similar in all patients.

tests were also negative or in normal ranges, such as rheumatoid factor, complement factors and CH100, antibodies against lactoglobulin, gliadin and casein. All patients were negative for hepatitis B screening. Serum IgA was frequently elevated in all children, especially in Patient 4, the only one who showed increased IgE.

Three of four patients showed ANCA-positive sera, from 1/20 to 1/1,200 dilution, using the IIF technique (Table). Case 3 did not have any serum with a positivity rating higher than 1/5. The antibodies were chiefly of IgG-class. IgA-ANCA were also found in the sera with the highest IgG-ANCA levels, but IgM-ANCA were always negative. The cell immunofluorescence showed a granular cytoplasmic staining similar to the classic cANCA pattern, with a stronger positivity around the nucleus (Fig. 1). The antibodies against myeloperoxidase and proteinase-3, tested by ELISA solid phase technique, were negative in all sera, even in Case 1 who showed a very high positivity by the IIF test. Only sera from Case 2 had border line levels, higher than normal children. All patients sera were negative for antibodies against monkey kidney and oesophagus tissue. Tests for anti-endomysial, antireticulin, anti-nuclear and anti-basement membrane were always negative.

Discussion

The diagnosis of IPH is frequently made by exclusion of other diseases. This is because many disorders with pulmonary haemorrhages have more specific clinical, analytical and histological features could be also found in many other disorders with

pulmonary haemorrhages. Nevertheless, this exclusion could be misleading in the first stage of the disease (5).

The relationship between ANCA levels and the severity of the renal or vascular disease is a controversial matter, but some data suggest that these antibodies are a major pathogenetic factor causing the vascular damage (6). The ANCA may activate primed neutrophils to release oxygen radicals and lysosomal enzymes, resulting in tissue necrosis. Primed neutrophils, in contrast to normal neutrophils temporarily express on their surface the antigens bound to these autoantibodies (6, 7). Evidence exists that both the autoantigen and ANCA participate in the pathogenesis of ANCA-associated vasculitides (8) and steroids normalize the abnormal generation of superoxide anion (9).

The pathogenesis of IPH is unclear, but some data suggest an immunological mechanism: the clinical picture could be similar to other immunological diseases such as SLE or Goodpasture's syndrome and some severe patients improve with immunosuppressive drugs. Cow's milk precipitins, elevated serum levels of IgA and IgE and antireticulin antibodies, are some of the immunological findings reported in IPH. We found circulating C1q-binding immune complexes (CIC) in 3/4 patients, during the acute phase, although they were never found deposited on tissue (4). Although the harmful role of ANCA remains a controversial question. They could be involved in the pathogenesis of IHP diagnosed in at least some patients.

In patients with suspected IPH, the serum ANCA could be useful in identifying those patients prior to development of detectable nephropathy or Wegener granulomatosis. Nevertheless our patients were followed-up for more than 10 years, without any systemic or renal disturbances.

Bosch et al. (10) reported 3 adult patients in whom diffuse pulmonary haemorrhage without extrapulmonary involvement has been developed. The biopsy revealed a necrotizing alveolar capillaritis in these patients. One patient died and the other two cases needed cyclophosphamide maintenance therapy. IPH in children is a poor defined illness and some cases could be related to the adult patients reported by Bosch et al. (10)

Our findings suggest a poor prognosis in IPH patients with serum ANCA. Case 1 with very high levels of ANCA died, 3 months after diagnosis. The other 2 cases with low levels, are steroid-dependent. Only Case 3 without ANCA is in permanent remission without treatment. No clear relationship was found between ANCA levels and acute crisis. Kerr et al. (11) suggest that a rise in ANCA level alone is not sufficient evidence of a clinical exacerbation

and, therefore, does not justify initiating or increasing immunosuppressive therapy.

In our patients a positive IIF test was not accompanied with a clear positive ELISA test result for anti-myeloperoxidase or proteinase-3. Only one serum (Case 2) was positive for these antibodies whereas other sera had negative or insignificant values, with poor correlation with IIF antibodies. In earlier studies, ANCA in sera from patients with vasculitis were described as reacting with proteins present in granules from human neutrophils, chiefly myeloperoxidase and proteinase-3 (12). In more recent studies, many other autoantigens have been identified, including both granular and cytosolic proteins, such as lactoferrin, cathepsin, elastase, enolase, beta-glucuronidase (13–16). This explains why, the ANCA IIF test may be positive and the ELISA solid test for myeloperoxidase and proteinase-3 negative, as observed in our patients.

The antibody response in ANCA-positive vasculitis is not restricted to neutrophil granule proteins (14). This discordance was particularly demonstrated in chronic inflammatory bowel diseases, in which the ANCA bound to cathepsin-G, distinct from proteinase-3 and myeloperoxidase (8, 13).

In conclusion, the measurement of serum ANCA appears to be of clinical value in patients diagnosed with IPH. Since IPH is a heterogeneous disease which is generally diagnosed by exclusion, serum ANCA measurements may be useful for classifying children with pulmonary hemorrhage. It is also possible that serum ANCA levels may have a prognostic value, although studies with larger numbers of patients are needed.

Acknowledgement

This work was partially supported by a grant of Consejería de Sanidad, Junta de Castilla y León (No. 90/1103).

References

1. DONALD KJ, EDWARDS RL, MCEVOY JDS. Alveolar capillary basement membrane lesions in Goodpasture syndrome and idiopathic pulmonary hemosiderosis. *Am J Med* 1975; 59: 642–49.
2. DREISIN RB. New perspectives in Wegener's granulomatosis. *Thorax* 1993; 48: 97–99.
3. BEER DJ. ANCAs weigh in. *Am Rev Respir Dis* 1992; 146: 1128–30.
4. BLANCO A, SOLIS P, GÓMEZ S, LINARES P, SÁNCHEZ WILARES E. C1q binding immune complexes and other immunological studies in children with pulmonary hemosiderosis. *Allergol Immunopathol* 1984; 12: 37–44.
5. KATZ SM, FOSTER E, MILLER AS, KREVOLIN L, SCHWARTZ AB. Goodpasture's syndrome mimicking idiopathic pulmonary hemosiderosis. *Ann Clin Lab Sci* 1989; 19: 280–86.
6. JENNETTE JC, FALK RJ. Antineutrophil cytoplasmic autoantibodies and associated diseases: a review. *Am J Kidney Dis* 1990; 15: 517–29.
7. KALLENBERG CGM, MULDER AHL, TERVAERT JWC. Anti-neutrophil cytoplasmic antibodies: a still-growing class of

- autoantibodies in inflammatory disorders. *Am J Med* 1992; 93: 675–82.
8. GROSS WL, SCHMIT WHT, CSERNOK E. ANCA and associated diseases: immunodiagnostic and pathogenetic aspects. *Clin Exp Immunol* 1993; 91: 1–12.
 9. MACCONI D, ZANOLI AF, ORISIO S, et al. Methylprednisolone normalizes superoxide anion production by polymorphs from patients with ANCA-Positive vasculitides. *Kidney Int* 1993; 44: 215–20.
 10. BOSCH X, FONT J, MIRAPEIX E, REVERT LL, INGELMO M, URBANO-MARQUEZ A. Antimieloperoxidase autoantibody-associated necrotizing alveolar capillaritis. *Am Rev Resp Dis* 1992; 146: 1326–29.
 11. KERR GS, FLEISHER TA, HALLAHAN CW, LEAVITT RV, FAUCI AS, HOFFMAN GS. Limited prognostic value of changes in antineutrophil cytoplasmic antibody titer in patients with Wegener's granulomatosis. *Arthritis Rheum* 1993; 36: 365–71.
 12. HAGEN EC, BALLIEUX BEBP, VANES LA, DAHA MR, VAN DERWOUDE FJ. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. *Blood* 1993; 81: 1996–2002.
 13. HALBWACHSMECARELLI L, NUSBAUM P, NOEL LH, ET. AL. Antineutrophil cytoplasmic antibodies (ANCA) directed against cathepsin-G in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis. *Clinical Experiment Immunol* 1992; 90: 79–84.
 14. MOODIE FDL, LEAKER B, CAMBRIDGE G, TOTTY NF, SEGAL AW. Alpha-enolase : a novel cytosolic autoantigen in ANCA positive vasculitis. *Kidney Int* 1993; 43: 675–81.
 15. COREMANS IEM, HAGEN EC, DAHA MR, et al. Antilactoferrin antibodies in patients with rheumatoid arthritis are associated with vasculitis. *Arthritis Rheum* 1992; 35: 1466–75.
 16. KANEKO K, SUZUKI Y, YAMASHIRO Y, YABUTA K. Is p-ANCA in ulcerative colitis directed against beta-glucuronidase? *Lancet* 1993; 341: 320.