Review Article

The antigenic significance and methods of detection of the anti-neutrophil cytoplasmic autoantibodies (ANCA)

Xavier Bosch and Ronald A. Asherson¹

Service of General Internal Medicine, Hospital Clinic i Provincial, Barcelona, Spain and ¹Division of Rheumatology and Connective Tissue Diseases, St Luke's/Roosevelt Division of Columbia University, New York, USA

Introduction

Systemic vasculitis is often difficult to diagnose. The recent discovery of a new class of autoantibodies, the anti-neutrophil cytoplasmic autoantibodies (ANCA), provides clinicians with a serological test strongly supportive of the diagnosis of the commonest forms of systemic necrotizing vasculitis. ANCA are IgG autoantibodies specific for different constituents of neutrophil azurophilic granules and monocyte lysosomes. These antibodies are the first serological markers for several forms of systemic vasculitis, particularly Wegener's granulomatosis and polyarteritis nodosa, as well as for the commoner types of necrotizing and crescentic glomerulonephritis.¹ In addition to their recognized use in the diagnosis and follow-up of these conditions, it appears that ANCA may be a pathogenic factor inducing vascular injury in patients with ANCA-associated disease. This suspicion arises from *in vitro* evidence that ANCA are capable of activating neutrophils causing the release of lytic proteases and toxic oxygen radicals.²

The existence of autoantibodies against neutrophils has been known for almost 30 years. A granulocyte-specific antinuclear factor was reported in 1964.³ Eight years later, Wiik and Munthe⁴ described a standardized method for the detection of granulocyte-specific anti-nuclear antibodies (GS-ANA). These investigators performed an indirect immunofluorescence (IIF) procedure on ethanol-fixed neutrophils, which remains the standard technique for ANCA detection today. GS-ANA were found to be specific for neutrophil and monocyte nuclei, did not react with lymphocytes, and were mainly detected in patients with rheumatic diseases, especially rheumatoid arthritis and Felty's syndrome, as well as in some patients with ulcerative colitis.

Cytoplasmic immunostaining pattern of granulocytes (c-ANCA) was reported in 1982 in eight patients with segmental necrotizing glomerulonephritis.⁵ Later, four further patients with vasculitis, glomerulonephritis and the serum presence of c-ANCA were reported.⁶ It was only in 1985 when van der Woude *et al.*⁷ noticed that c-ANCA mainly occurred in patients with Wegener's granulomatosis, that interest in these antibodies became intense. In addition, Falk and Jennette⁸ recognized in 1988 the clinical importance of the perinuclear immunostaining of neutrophils (p-ANCA) as well as its association with myeloperoxidase (MPO). They demonstrated that this was an artifactual pattern of cell fixation with ethanol and found these antibodies mainly in patients with idiopathic necrotizing and crescentic glomerulonephritis.

It is now well accepted that ANCA have two main antigenic specificities each one of them associating with two main clinical disorders. Thus, the c-ANCA mainly represent anti-proteinase 3 (PR3) antibodies which mostly identify patients with biopsy-proven Wegener's granulomatosis. The p-ANCA represent anti-MPO antibodies which are mainly seen in patients with pauci-immune necrotizing and crescentic glomerulonephritis showing little or no evidence of extra-renal involvement.^{1,9–11}

Methods for the detection of ANCA

Several methods have been used to detect ANCA. These include IIF, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), Western blotting, dot blotting and immunoprecipitation. The first method to detect ANCA was IIF. As mentioned above, this procedure was originally used for the detection of GS-ANA,¹² and today is still used in the same way. At the First International ANCA Workshop held in Copenhagen in 1988,

Correspondence: Xavier Bosch, M.D., Servicio de Medicina Interna General, Unidad 1, Hospital Clinic i Provincial, Villarroel, 170, 08036 Barcelona, Spain.
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this method was definitively adapted as the reference for future studies of ANCA.¹³ Some investigators have also performed HL-60 cell cultures for detection of ANCA by IIF and for distinguishing these from serum autoantibodies other than ANCA.¹⁴

The potential problem with the subjective interpretation of the two IIF patterns prompted the rapid development of antigen-specific, solid-phase assays. To assess the validity of these assays, it is important to be aware of the nature of the antigen preparation used as a substrate as well as the specificity and label of the secondary antibody used. The substrates employed in solid-phase assays are crude extracts of cells, extracts of granules, or sandwich techniques using monoclonal antibodies or purified proteins. The secondary antibodies commonly used are directed against human IgG. However, since the presence of IgM ANCA early in disease has been reported,¹⁵ some laboratories use anti-human Ig polyclonal antibodies as the secondary antibody.

The first solid-phase assay described was a RIA using an acid extract of neutrophils.¹⁶ This resulted in a sensitivity of 96% and a specificity of 80%. The low specificity was due to false-positive reactions among systemic lupus erythematosus (SLE) patients. This may be expected, since a whole cellular extract is used which also contains nuclear antigens. Likewise, the acid extract of whole cells has also been used to develop an ELISA with the same drawbacks, that is, a high background and a high proportion of false-positive reactions.¹⁷ However, the solid-phase assays were greatly improved when it was shown that the c-ANCA antigen localized in the primary granules,¹⁸ and assays were developed using purified extracts of these granules with a response at least 20 times better as compared with the whole cell extract.^{19,20} This also resulted in a high specificity (greater than 95%), a sensitivity of 80–90% and there were no significant problems with false-positive reactions from SLE patients. Although this method mainly detects PR3, high serum concentrations of MPO or other azurophilic antigens may unfortunately also give a positive result. Another approach was taken by Lüdemann *et al.*²¹ who used an affinity column with immunoglobulin from a patient with Wegener's granulomatosis. An ELISA using this method seems to be highly specific for c-ANCA and avoids the problem with false-positivity from other autoimmune sera. However, the antigen is difficult to purify. At the First International ANCA Workshop, this assay was found to correlate very well with the assay using purified granules.²² Finally, monoclonal antibodies against the c-ANCA antigen have also been used to capture the antigen in a solid-phase assay.²³ This has the advantage of selecting monoclonal antibodies to the antigen of choice. How-

ever, a major problem in this type of assay is that many sera containing rheumatoid factors will react with these monoclonal antibodies and give a false-positive result.

Since it is now well known that the major c-ANCA antigen is PR3, this protein is being used in many laboratories for detecting anti-PR3 antibodies in solid-phase assays.^{9,10} Likewise, after the discovery that the chief p-ANCA antigen was MPO,⁸ this well-characterized protein is also being used in solid-phase assays for MPO-ANCA detection in many centres. These assays are highly specific and sensitive for c-ANCA and p-ANCA, respectively. A small percentage of patients with p-ANCA will have anti-elastase or anti-lactoferrin antibodies which may also now be detected by means of specific ELISAs using either purified elastase or lactoferrin as a substrate.

The IIF procedure correlates well with the solid-phase assays in that the number of positive samples is roughly the same, but the antibody titres show a low correlation with the solid-phase readings. A high IIF titre may thus be low in the ELISA and vice versa. The ELISAs are, as expected, more sensitive than IIF, but the IIF will detect a few sera having specificities other than for the antigens used for coating in the solid-phase assays. Normally, between 80% and 90% of samples which are positive by IIF will be positive in ELISA, and about 90% of samples that are positive in ELISA will be positive in IIF.²⁴

The strategy used in many laboratories today is to screen for ANCA by IIF. Due to problems with interpretation of the IIF pattern, an ELISA is now often also needed to confirm the specificity. This is especially true when a p-ANCA pattern is seen. The p-ANCA pattern can mask a c-ANCA pattern or could actually be a true GS-ANA pattern or perhaps a regular ANA pattern seen in patients not suffering from systemic vasculitis. At all, one may then check for lymphocytes, which are negative with p-ANCA, and again perform the IIF with formaldehyde fixation. The pattern for MPO-ANCA with formaldehyde fixation will change from p-ANCA to granular cytoplasmic. If the pattern now remains nuclear, this probably represents the presence of ANA or a true GS-ANA.^{1,24} The strategy used in our centre is first to screen for ANCA by standard IIF. When c-ANCA are seen, we then perform an ELISA using purified PR3. When a p-ANCA pattern is seen, an ELISA with purified MPO is then performed. When the results are doubtful by IIF, we perform an ELISA using purified extract of primary granules as substrate (Figure 1).

Figure 1 Strategy for ANCA detection.

The antigenic basis of ANCA

Since the Leaker and Cambridge leading article in this journal,²⁵ efforts for a fine identification of the ANCA antigens have experienced unexpected progress. After the initial report of the association of ANCA with Wegener's granulomatosis,⁷ several groups began to isolate the antigen involved. Using the elegant method of Borregaard *et al.*²⁶ for subcellular fractionation, Rasmussen *et al.*¹⁸ found that the antigen localized in the primary granules and isolated a protein with an estimated molecular weight of 29 kd. The N-terminal sequence of the 29 kd protein is now available in several laboratories.^{27–29} In 1989, Goldschmeding *et al.*²³ and Niles *et al.*²⁸ found that the major c-ANCA antigen was a diisopropylfluorophosphate-binding serine protease contained in the azurophilic granules. At the Second International ANCA Workshop (The Netherlands, 1989), Lüdemann *et al.* proposed that the c-ANCA antigen was PR3, a constituent of neutrophil primary granules characterized by Kao *et al.* in 1988.³⁰ The identity between PR3 and the target of Wegener's granulomatosis was later established by Jenne *et al.*²⁷ and confirmed by Jennette *et al.*³¹ using purified proteinase 3 (PR3). In addition, the revised sequence of PR3 suggested that this serine protease is identical to AGP7^{27,32} and to p29.^{28,33} During the Third International ANCA Workshop (Washington, 1990), Lüdemann *et al.*³⁴ and Gupta *et al.*³⁵ proposed that PR3, p29, and AGP7 were identical to myeloblastin present in HL-60 human leukaemic cells involved in the control of growth and differentiation of leukaemic cells.³⁶ Moreover, van del Wiel *et al.*³⁷ established that the major plasma inhibitor of PR3 is α 1-antitrypsin (α 1-AT), the molecular weight of the PR3- α 1-AT complex being 83 kd. This may perfectly correspond to the 91 kd

antigen recognized by several c-ANCA positive sera described by van der Woude *et al.*³⁸ in sputum or in degranulation supernatant of leucocytes being in contact with plasma protein, including α 1-AT.

The original report by Falk and Jennette⁸ showed that the majority of the p-ANCA detected by IIF corresponded to antibodies against MPO. Falk *et al.*³⁹ and Roberts *et al.*⁴⁰ have demonstrated that the MPO epitope recognized by patient's antibodies is expressed by the native molecule (130 kd), but not by the isolated chains after denaturation and reduction. The inhibitory effect of anti-MPO IgG on MPO enzymatic activity seems to be marginal, suggesting that the epitope recognized by patient's antibodies and the enzymatic site are distinct. In addition, Höier-Madsen *et al.*⁴¹ have shown that anti-thyroid peroxidase antibodies do not cross-react with MPO or reciprocally. In our laboratory in Barcelona, we have developed a simple method to decide, in positive cases, if ANCA are directed against MPO.^{42,43} Briefly, when ANCA (c-ANCA or p-ANCA) are positive by IIF, the same IIF assay is performed again using as a substrate both normal neutrophils as well as neutrophils from a voluntary donor known to have a complete and selective inherited deficiency of MPO. This rare and otherwise commonly asymptomatic condition consists of a lack of MPO in the azurophilic granules of the polymorphonuclear leucocytes and monocytes. This disorder may be easily detected in routine haematological analyses by automated flow cytochemistry with the Technicon H-1 analyser (Technicon Inc., Tarrytown, New York) that is used to determine the white blood cell differential count according to the peroxidase activity of myeloid cells. Thus, sera giving a positive IIF result with normal neutrophils at the minimal dilution (1:20) but a negative result with neutrophils devoid of MPO are considered as possessing MPO-ANCA. Conversely, when no differences appear at progressive dilutions, we conclude that ANCA have no specificity for MPO. The results obtained by this method have shown a perfect correlation with the ELISA using purified MPO.

Although the two major ANCA antigens, PR3 and MPO, correspond to c-ANCA and p-ANCA, respectively, there may be some exceptions. Both patterns may be associated with other rare antigenic specificities and some anti-MPO antibodies may exhibit a c-ANCA pattern. Whether this latter fact is due to non-reproducibility in the artifactual redistribution of MPO after ethanol fixation or to a true variability in anti-MPO specificities is as yet unclear.⁴⁴ Using the standard IIF procedure for ANCA detection, we have found a typical immunostaining pattern for MPO-ANCA positive sera.^{42,43} This consists of a diffuse nuclear

immunostaining pattern with additional dense endonuclear bands (Figure 2). This type of immunostaining appears to be specific for MPO-ANCA and, when carefully examined, differs from other p-ANCA patterns that correspond to antibodies recognizing antigens other than MPO (Figure 3).

There seems to be a functional analogy between the two major ANCA antigens, PR3 and MPO. Thus, both are co-localized in the azurophilic granules of granulocytes, and both are translocated to the cell surface during neutrophil activation.⁴⁴ This has been recently established in human.⁴⁵ The two major ANCA antigens are able to interact directly with ANCA after neutrophil preactivation. Because of their cationic charge, both PR3 and MPO interact with cell membranes and glomerular basement membrane.^{46,47} The enzymatic activity of PR3 and MPO may be involved in the vascular lesions after ANCA amplified release. Indeed, PR3 has a capacity to degrade elastin and to induce emphysema in the hamster, exceeding that of elastase of cathepsin G.³⁰ Moreover, the MPO-hydrogen peroxide-halide system is also directly involved in tissue injury through the generation of hypochlorous acid and chloramine radicals.^{47,48} MPO may also play an indirect role, since hypochlorous acid induces proenzyme activation of collagenase and gelatinase.⁴⁹

Minor ANCA antigens

In addition to the two major ANCA antigens, a number of neutrophil cytoplasmic antigens recognized by ANCA have been also identified (Table I). However, the small proportion of patients in whom

Figure 2 Diffuse staining of neutrophil nuclei with dense endonuclear bands (arrows), typical of anti-myeloperoxidase autoantibodies. (Immunostaining of neutrophils on immunofluorescence microscopy, × 100.)

Figure 3 Perinuclear immunostaining pattern of ANCA. In this case, these antibodies were not directed against myeloperoxidase. A clearly perinuclear band is seen (arrows). (Immunostaining of neutrophils on immunofluorescence microscopy, × 100.)

Table I Rare ANCA antigenic specificities

ANCA antigen	Immunostaining pattern
CAP57	c-ANCA
Elastase	p-ANCA
Lactoferrin	p-ANCA
Cathepsin G	Homogeneous non-c-ANCA
Eosinophil peroxidase	p-ANCA

c-ANCA = cytoplasmic pattern; p-ANCA = perinuclear pattern.

autoantibodies with rare ANCA specificities are observed (probably less than 5%) prevents definitive conclusions as to their diagnostic and pathogenetic values.⁴⁴ One of these rare ANCA antigens is CAP57, and antimicrobial cationic protein contained in a subpopulation of the azurophilic granules in normal neutrophils.⁵⁰ ANCA with specificity for CAP57 have been demonstrated by Falk *et al.*³⁹ CAP57-ANCA produce a c-ANCA immunostaining by IIF which is blocked by monoclonal anti-CAP57. The exact frequency of this particular ANCA and its co-occurrence with anti-PR3 antibodies is not fully established.

Autoantibodies to human leucocyte elastase (HLE), another constituent of the azurophilic granules, have been described independently by Goldschmeding *et al.*²³ and by Nässberger *et al.*⁵¹ In general, these anti-HLE antibodies produce a

perinuclear IIF staining pattern. They usually occur in the absence of anti-MPO or anti-PR3 antibodies in less than 0.5% of all p-ANCA positive sera. Two exceptional patients, treated for hyperthyroidism with propylthiouracil, were found to have antibodies to HLE and PR3.⁵² On the other hand, the actual frequency of anti-HLE antibodies in SLE originally observed by Nässberger *et al.*⁵¹ was debated during the Third International ANCA Workshop.⁵³ Some positive anti-HLE (as well as anti-MPO) results may, in fact, be due to interaction of HLE (or MPO) with DNA and should be detected after serum treatment by DNase.

Antibodies to lactoferrin were first reported in vasculitis by Thomson and Lee.⁵⁴ It is the experience of some that anti-lactoferrin antibodies may occur as a single specificity in rare vasculitic patients and are associated with a p-ANCA staining pattern.⁴⁴ Thus, Pozzi *et al.*⁵⁵ have observed anti-lactoferrin antibodies in less than 5% of ANCA positive patients with necrotizing glomerulonephritis as well as in some cases of lupus nephritis. In their experience, anti-lactoferrin antibodies were not associated with either anti-MPO or anti-PR3 antibodies. Although rare, the anti-lactoferrin ANCA are of theoretical importance with regard to the mechanisms of vasculitis, since lactoferrin is the only known ANCA antigen contained in the specific (β , secondary) granules.

ANCA directed against cathepsin G, a 25 kd neutral protease of the azurophilic granules, are very unusual, but have been observed by Flesch *et al.*⁵⁶ in Wegener's granulomatosis patients' sera selected on the basis of atypical IIF ANCA staining (homogeneous non-c-ANCA pattern). The presence of anti-cathepsin G antibodies was demonstrated by ELISA and usually occurred in combination with anti-elastase and anti-MPO antibodies. Finally, Dolman *et al.*⁵² found antibodies directed against eosinophil peroxidase (EPO) reacting only with eosinophils by IIF and precipitating the two bands at 58 kd and 14 kd, identical to those precipitated by polyclonal rabbit anti-EPO antibodies.

The vast majority of ANCA positive sera are monospecific for a single ANCA antigen. It is striking that anti-PR3 and anti-MPO antibodies almost never occur in one given sera. With regard to the rare specificities, anti-HLE and anti-lactoferrin antibodies usually occur as a single specificity, but are occasionally associated with anti-PR3 and anti-MPO antibodies, respectively.

In addition, anti-CAP57 antibodies may be isolated or associated with PR3-ANCA.⁴⁴ The limited number of patients with multispecific ANCA does not allow firm conclusions, but may favour the hypothesis that ANCA are mainly directed against PR3 or MPO and that multiple ANCA specificities in a single serum probably reflect a secondary response to antigens that are released from neutrophils as the vasculitis progresses.

Other ANCA antigens remain yet to be identified. In particular, the fine specificity of GS-ANA described by Wiik¹² occurring in rheumatoid arthritis and ulcerative colitis has not yet been recognized. During the Third International ANCA Workshop, Peter *et al.*⁵⁷ and Jørgensen *et al.*⁵⁸ established that GS-ANA were indeed heterogeneous with defined specificities to MPO, lactoferrin, or nuclear antigen(s) in 60% of the sera studied. The remaining 40% of sera contained antibodies to undefined cytoplasmic neutrophil antigen(s) and thus belong to the ANCA family.

Finally, it is the experience of many investigators that approximately 5% of the c-ANCA positive sera do not bind to β or α -granules, PR3, MPO, nor to the other rare ANCA antigens.⁴⁴

Conclusions

It seems to be clear that autoimmune mechanisms involving the neutrophil antigens are of growing importance. The role of the two major ANCA antigens, PR3 and MPO, in the vasculitic process, including Wegener's granulomatosis, is now well established. On-going studies will elucidate the role and specificity of ANCA in other conditions such as rheumatoid arthritis and ulcerative colitis. The variety of clinical conditions associated with ANCA is reflected by the large number of ANCA antigens described to date and, in future, no doubt the ANCA family will have other unexpected ramifications and implications.

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