

The Appropriate Use of Antineutrophil Cytoplasmic Antibody (ANCA) Testing in Rheumatic Diseases

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Abstract

The antineutrophil cytoplasmic antibody (ANCA) test is now available in most routine diagnostic immunology laboratories. Improvement, simplification and standardisation of the testing methodology have enabled it to become more reliable and accessible to clinicians. ANCA has strong association with and is most useful in the diagnosis and management of the ANCA-associated vasculitides which include Wegener's granulomatosis, microscopic polyarteritis, Churg-Strauss syndrome and primary pauci-immune necrotising and crescentic glomerulonephritis. It is found in lower frequency in the other vasculitides and collagen vascular diseases, in chronic inflammatory bowel disease and autoimmune liver disease, and in miscellaneous infective and neoplastic disorders. While the gold standard for ANCA testing remains the indirect immunofluorescence (IIF) assay, identification of ANCA-specific antigens such as proteinase 3 and myeloperoxidase has enabled the development of antigen-specific tests. The antigen-specific solid-phase assays have comparable sensitivity with IIF assays and improved specificity in some instances. However, appropriate use of the ANCA test requires full knowledge of its capabilities and limitations, and the results should always be correlated with clinical data. In particular, it is important to understand that it is not only test sensitivity and specificity, but patient selection that contributes to the positive predictive value and clinical relevance of the test result.

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Introduction

In 1982, Davies and co-workers¹ in Melbourne described the presence of a serum factor that stained the cytoplasm of neutrophil leukocytes by indirect immunofluorescence in 8 patients with generalised illness associated with segmental necrotising glomerulonephritis. This was followed two years later by a report by Hall and co-workers² of finding antineutrophil cytoplasmic antibody in 4 patients with systemic vasculitis, of whom 3 had necrotising glomerulonephritis. Van der Woude et al³ retrospectively studied 41 patients with biopsy-proven Wegener's granulomatosis (WG) and found antibodies against the extranuclear components of polymorphonuclear granulocytes in 25 of 27 samples from patients with active disease compared to 4 of 32 samples from patients without signs of disease activity.

The numerous studies that subsequently followed these early reports soon confirmed the association of antineutrophil cytoplasmic antibody with WG and expanded the list of diseases associated with these antibodies. As a result, the ANCA test is now available in most hospital laboratories as a diagnostic aid in diverse clinical problems.

Nomenclature and Antigenic Specificities

The original reports of antineutrophil cytoplasmic antibody (ANCA) described the antibodies as giving a centrally accentuated granular staining pattern in the cytoplasm of neutrophils using an indirect immunofluorescence test. Falk and Jennette⁴ subsequently described the presence of an antineutrophil cytoplasmic antibody with a perinuclear to nuclear pattern in patients with idiopathic necrotising and crescentic glomerulonephritis (NCGN). It was then proposed at the Second International Workshop on ANCA to call the first antibody cytoplasmic or C-ANCA and the second perinuclear or P-ANCA.⁵ In addition, a less well-defined pattern of staining with characteristics of both C-ANCA and P-ANCA has been recently referred to as "atypical" ANCA or X-ANCA.^{6,7}

The antigen with which C-ANCA specific to WG reacted was identified as proteinase 3 (PR3), a soluble 29 kD serine protease of the azurophilic granules of normal human granulocytes and monocytes.⁸⁻¹² The specificity of perinuclear staining antibodies to myeloperoxidase (MPO) was also confirmed.^{13,14} Although the presence of C-ANCA generally corresponds with the presence of anti-PR3, patients with anti-MPO

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can produce a C-ANCA pattern at indirect immunofluorescence and anti-PR3 can be associated with a perinuclear pattern.¹⁵⁻¹⁷ Furthermore, many sera that produce a perinuclear or atypical cytoplasmic staining pattern do not show specificity for MPO or PR3. Depending on the population tested, between 11% and 98% of sera with P-ANCA contain anti-MPO.¹⁸⁻²¹ In our own laboratory, we have found only about 20% of P-ANCA positive samples to contain anti-MPO.

Further studies to elucidate the other target antigens have identified autoantibodies to lactoferrin (LF), human leukocyte elastase (HLE), cathepsin G (CG), eosinophil peroxidase (EPO), β -glucuronidase, CAP57 or bactericidal/permeability-increasing protein (BPI), and alpha-enolase. Approximately 5% of C-ANCA positive sera do not bind to B-granules, PR3, MPO or the rarer antigens, and on-going studies continue to characterise the antigens involved.^{14,21-28}

The majority of ANCA-positive sera are specific for only a single ANCA antigen, and anti-PR3 and anti-MPO are almost never found together. Occasionally anti-HLE and anti-BPI have been found with anti-PR3 and anti-LF with anti-MPO, and it is hypothesised that multiple ANCA specificities in a single serum result from a secondary response to antigens released from the neutrophils during the vasculitic process.²⁶

ANCA Testing Methodology

Several methods exist to detect ANCA, including indirect immunofluorescence (IIF), solid phase assays, immunoblotting and immunoprecipitation. The IIF and solid-phase assays are routinely used by diagnostic laboratories for ANCA detection, while immunoblotting and immunoprecipitation are more commonly used as research tools.

Indirect Immunofluorescence

The indirect immunofluorescence (IIF) methodology was standardised and adapted during the First Workshop on ANCA²⁹ as the reference method in studies of ANCA. By using ethanol-fixed leukocytes as the substrate, IIF enables differentiation between the cytoplasmic C-ANCA pattern and the perinuclear P-ANCA pattern of staining.³⁰ The P-ANCA pattern is the result of a fixation artefact caused by the disruption of neutrophil granules during ethanol fixation and redistribution of positively charged basic proteins to the negatively charged nucleus. The use of formaldehyde fixation instead of ethanol fixation prevents this migration and makes it possible to distinguish between a P-ANCA and an antinuclear antibody (ANA). The presence of an ANA can also be excluded by the use of a non-myeloid tissue culture substrate such as Hep-2 cells.

Solid Phase Assays

Because of the problems inherent in the interpretation of IIF assays, solid phase assays were developed to provide a more objective method of ANCA detection. Early solid phase assays using neutrophil extracts as substrates resulted in a high frequency of false positive results due to contamination with nuclear components. Fortunately, the identification of ANCA-associated antigens enabled the use of purified antigens to determine specific ANCA. An international collaborative study has been working on the standardisation and development of standardised methodology of such assays in an effort to overcome major difficulties in these assays involving the purity of the antigen preparations used as substrates, the specificity of the secondary antibodies used and the interpretation of values obtained in the test.³¹ Commercial assays are already readily available for the detection of anti-MPO and anti-PR3, and assays for the detection of antibodies to LF, HLE and CG are expected to be available soon.

In a large clinical study³² evaluating the performance of solid phase ANCA assays, the sensitivity and specificity of the anti-PR3 and anti-MPO assays for histologically proven WG and systemic vasculitis were found to be comparable to, if not better than, results obtained using the IIF test. The combination of both C-ANCA with anti-PR3 and P-ANCA with anti-MPO had a specificity of 99% and are therefore very specific for the diagnosis of WG, microscopic polyarteritis (MPA) and idiopathic rapidly progressive glomerulonephritis.

Clinical Associations

ANCAs have been found to be associated with 3 major groups of diseases:

- 1) primary systemic vasculitides,
- 2) collagen vascular diseases and rheumatoid arthritis, and
- 3) chronic inflammatory bowel diseases and autoimmune hepatic disease.

In addition, ANCAs have been variously described in different infectious and neoplastic disorders.

Primary Systemic Vasculitides

Much of the early difficulties involved in studying the ANCA were due to lack of standardisation of clinical disease definitions and diagnostic criteria in vasculitides. While distinct vasculitic syndromes have been recognised, there was frequent overlap in clinical presentation as well as evolution from one syndrome to another and no single laboratory study was found to have adequate predictive value.³³ Although much of this confusion was resolved with the American College of Rheumatology 1990 criteria and the Chapel Hill Consensus Conference on Nomenclature of Systemic Vasculitis

in 1994,³⁴ differences between MPA and polyarteritis nodosa (PAN) are still a subject of debate.³⁵ Nevertheless, the use of the ANCA test has enabled the categorisation of a subset of vasculitides under the term "ANCA-associated vasculitic syndromes", namely WG, MPA, Churg-Strauss syndrome, and primary NCGN.³⁶

The strongest ANCA associations so far have been between PR3 specific C-ANCA and WG. The specificity of the ANCA testing in WG is greater than 95% while the sensitivity is dependent on the phase of the disease; in active disease, the sensitivity of the test in WG is high (>70%) and significantly lower when the patients are in complete remission (<35%). Anti-PR3 assays exhibit similar sensitivities and specificities for active WG. Nearly all anti-PR3-negative sera in WG contain anti-MPO.^{7,17,18,22,37}

Some studies have reported a positive association between the extent and severity of disease and ANCA, but in at least one prospective study of 106 patients no significant difference was noted between active generalised and active limited WG.³⁸⁻⁴² The majority of studies have also found a correlation between serial C-ANCA titres and disease activity although inter-individual variation was high; in some patients, increased titres preceded clinical relapse. Two studies also reported that ANCA serial titres were able to distinguish between primary disease activity and treatment or infectious complications. However, it must be noted that up to 36% of patients may not show any changes with disease activity and, in several studies, either the C-ANCA titre increase was not accompanied by clinical relapse or disease remission was not associated with a fall in C-ANCA titre in this subset of patients. It is therefore important that if serial C-ANCA titres are to be used in following disease activity it should be correlated with the clinical assessment. It is also important to note that C-ANCA titres can take up to 8 weeks to fall after disease remission, and that interpretation of disease activity based on C-ANCA titres should not be done within 6 to 15 weeks of treatment for vasculitic relapse. The same study also reported poor correlation of P-ANCA titres with disease activity, although a recent study has suggested that serum anti-MPO reflects disease activity in some patients with primary vasculitis.⁴²⁻⁴⁶

Prior to the Chapel Hill consensus conference, PAN and MPA were considered the same entity and this may have contributed to the varying reports of ANCA in association with PAN. The Chapel Hill consensus conference defined MPA as a small vessel vasculitis characterised by a pulmonary-renal syndrome with RPGN and alveolar haemorrhage, while classic polyarteritis nodosa (cPAN) involved medium and small-sized muscular arteries and rarely manifested with RPGN or lung haemorrhage. Using this definition, MPA was found to be strongly associated with the presence of ANCA, particu-

larly P-ANCA. Studies have found 70% to 100% of patients with MPA to be ANCA-positive, with anti-MPO in 40% to 80% and anti-PR3 in 20% (Table I).^{16,17,21,47,48} In contrast, less than 25% of cPAN were anti-MPO-positive and ANCA-positive.⁴⁸⁻⁵⁰

ANCAs have been found in more than two-thirds of patients with primary pauci-immune NCGN compared with less than 5% in patients with other renal disease. The majority of ANCA in these patients have a perinuclear pattern with specificity to MPO.^{4,16} Cohen Tervaert et al²² have reported a 75% sensitivity and 92% specificity for anti-MPO for idiopathic NCGN. Most patients with vasculitis-associated or idiopathic glomerulonephritis have either PR3 or MPO-ANCA (Table I).

TABLE I: PREVALENCE OF ANCA IN SELECTED RHEUMATIC DISEASES

Disease	ANCA %	Anti-PR3 %	Anti-MPO %
Wegener's Granulomatosis (active)	95 to 100	77 to 84	10 to 17
Microscopic polyarteritis	76 to 100	20 to 45	40 to 80
Necrotising and crescentic glomerulonephritis	70 to 80	25	65 to 75
Churg-Strauss syndrome	6 to 60	8 to 30	60 to 64
Classic polyarteritis nodosa	18 to 25	Rare	<25
Giant cell arteritis	Rare	Rare	Rare
Systemic lupus erythematosus	Rare	Rare	Rare
Rheumatoid arthritis	17 to 32	Rare	Rare

ANCA: antineutrophil cytoplasmic antibody; PR3: proteinase 3; MPO: myeloperoxidase

Data are derived from references in the text

Although one group has reported a frequency as high as 66% in Churg-Strauss syndrome, other workers have found lower frequencies of 14% to 30% for C-ANCA and 6% to 21% P-ANCA in their patients. There is a relatively high frequency of anti-MPO, with a lower frequency of anti-PR3 (Table I); some ANCAs of undefined specificity are also seen.^{16,37,49-51}

ANCAs are only infrequently found (<10%) in the vasculitides involving medium-sized and large vessels, i.e. giant cell (temporal) arteritis (GCA)^{52,53} and Takayasu arteritis.^{42,51} Two studies reported a homogenous staining ANCA in one-third of patients with early Kawasaki disease; however, it was also found with the same frequency in febrile control patients^{54,55} and was possibly due to the presence of IgM antibodies directed against CG.⁵⁶ ANCAs have been reported in conjunction with anti-glomerular basement membrane (anti-GBM) antibody in 2% to 9% of patients with anti-GBM disease; these patients have been noted to have systemic vasculitis as well, and the presence of ANCAs has been associated with a better prognosis following therapy compared to patients with anti-GBM alone.^{57,58}

ANCAs are rare in essential cryoglobulinaemic vasculitis and cutaneous leukocytoclastic angiitis.^{18,51,59} Some groups, but not all, have reported finding IgA-ANCA in Henoch-Schonlein purpura.^{51,60-62} It has not been found in Behcet's disease.^{51,63}

Collagen Vascular Diseases and Rheumatoid Arthritis

P-ANCAs are occasionally found in systemic lupus erythematosus (SLE), in association with low levels of anti-MPO, anti-LF, anti-HLE and anti-lysozyme. No correlation has been found between ANCA and the presence of vasculitis or renal disease.^{51,59,64} In one study, a strong association was found between anti-MPO and anti-HLE in hydralazine-induced SLE; following withdrawal of the hydralazine, anti-HLE disappeared but not anti-MPO. It has been suggested that anti-HLE detected in SLE is due to interaction between HLE and DNA.⁶⁵

ANCAs are rare and described occasionally in low frequency in patients with other collagen diseases, such as polymyositis/dermatomyositis, Sjogren's syndrome, mixed connective tissue disease, overlap syndrome, sarcoidosis, and scleroderma.^{47,51} One group studied ANCA in patients with systemic sclerosis and reported correlation between the P-ANCA and anti-MPO titre with rapid deterioration of renal function and development of normotensive renal failure.⁶⁶

ANCAs have been described in 17% to 32% of patients with rheumatoid arthritis. Most of the ANCAs in rheumatoid arthritis are P-ANCAs and have specificity for LF. Several groups have found correlations between anti-LF and vasculitis, and between anti-LF and associated nephropathy, but others have not found any association with extra-articular disease or extent of disease. P-ANCAs have also been described in Felty's syndrome, Still's disease, juvenile chronic arthritis and psoriatic arthritis. It has not been found in Reiter's syndrome, reactive arthritis and ankylosing spondylitis.^{51,67-70}

Chronic Inflammatory Bowel Disease and Autoimmune Liver Disease

While this review has concentrated on rheumatic disorders, it is worthwhile to note that ANCAs, primarily P-ANCAs, have been found in the sera of 50% to 80% of patients with ulcerative colitis (UC), 10% to 40% of patients with Crohn's disease (CD), 75% to 85% of patients with primary sclerosing cholangitis (PSC) and 50% of chronic autoimmune hepatitis (CAH). Recent studies have also shown P-ANCA in low titres in relatives of patients with PSC and UC.⁷¹⁻⁷⁵ Data so far show that some of the ANCAs in patients with UC, CD and PSC are specific for lactoferrin, cathepsin G and BPI rather than MPO and PR3.^{28,74-76}

Miscellaneous Diseases

ANCAs have been described in a variety of malignant

diseases such as lung cancer, myelodysplastic syndrome, monoclonal gammopathies, subacute bacterial endocarditis and atrial myxoma.^{37,77,78} It has also been reported in infectious diseases such as *M. avium* infection, chromomycosis, HIV infection, invasive amoebiasis and acute *Plasmodium falciparum* malaria,^{18,71,79-83} and in patients taking drugs such as propylthiouracil.⁸⁴ In many of the infective disorders, an atypical form of ANCA with a homogenous cytoplasmic staining pattern is seen rather than the typical granular C-ANCA pattern. ANCAs have been found in less than 10% of patients with hepatitis B and hepatitis C, and in 27% of patients with non-A, non-B hepatitis.⁷¹ P-ANCA has also been shown in about 5% of normal blood bank controls.⁷⁴

Appropriate Use of ANCA Testing

It is a common observation that when a newly identified diagnostic test is introduced, the application of the test expands rapidly. Improvements and simplification of testing methodology have enabled ANCA testing to be more reliable and accessible. Unfortunately, due to insufficient understanding about how to use and interpret the test, it is often ordered for patients with little specific indications. "Shotgun testing" may also be practised when there is inadequate knowledge about what tests should be ordered.

Laboratory testing performs best with selected populations and the Positive Predictive Value (PPV) of a test increases with the prevalence of the disease in the population. By careful history taking and examination, the clinician is able to select a population with a high prevalence, thus increasing the PPV of the test. In shotgun testing, the predicted pretest prevalence tends to be low and this results in low PPV.⁸⁵ ANCA testing is most useful in the diagnosis of primary vasculitides, particularly WG, MPA, pauci-immune NCGN and Churg-Strauss syndrome, and less useful in the other rheumatic diseases. It should therefore be used only when patients present with symptoms and signs that are suggestive of these conditions. For example, in a patient presenting with haemoptysis and pulmonary infiltrates, the ANCA test will be helpful in diagnosis and assessment of disease activity. In a patient presenting with vague symptoms, e.g. vague joint pains or fever alone, where the suspicion of ANCA-associated vasculitis is low, the ANCA test will not be useful.

Jennette⁸⁶ has described their experience with approximately 3500 assays, based on a previously determined ANCA test assay sensitivity of 95% and specificity of 95% in ANCA-associated diseases. In patients with clinical and pathological features associated with WG, pulmonary-renal syndrome, systemic vasculitis or RPGN, the PPV was greater than 90% and the ANCA test had good diagnostic utility. However, when all patients undergoing laboratory tests at the hospital were in-

cluded, most patients with positive ANCA assays had false-positive tests and the PPV was less than 5%. Similarly, within the subset of P-ANCA positive patients, only 50% of 900 P-ANCA positive samples ordered in patients with varying degrees of clinical evidence for vasculitis were positive by enzyme immunoassay for anti-MPO activity compared to 90% of P-ANCA-positive samples from patients with biopsy-proven pauci-immune NCGN with typical glomerular manifestations of ANCA-associated vasculitides.⁸⁷

Good correlation between ANCA titres with disease activity has so far been confirmed only between C-ANCA titres and WG. However, because C-ANCA titres do not follow disease activity in a significant proportion of patients, it should always be correlated with clinical assessment and with knowledge of the time lag seen before changes in titre after treatment. If C-ANCA titres are used to determine disease activity too soon after a course of treatment, e.g. at only 4 weeks after completion of treatment, the titres may still be elevated and further treatment initiated unnecessarily.

The results of inappropriate testing can therefore lead to misinformation, confusing the clinician at best and adversely affecting patient management at worst. It is therefore important that ordering of ANCA tests, as with all laboratory tests, be done with knowledge of the test capabilities and limitations and the results considered in the context of the patient's clinical presentation and other laboratory data.

A Systematic Approach to ANCA Testing

The useful application of ANCA testing in the diagnosis and management of patients with suspected vasculitis requires a systematic approach. Clinical assessment of the patient should always be done and strong clinical indications exist before ordering ANCA tests. The approach used by most laboratories in ANCA testing is to screen by IIF using ethanol-fixed neutrophils.

Samples showing the typical granular C-ANCA patterns on IIF should be further tested with a specific assay for anti-PR3. A positive anti-PR3 and C-ANCA result is strongly associated with WG and MPA. The C-ANCA titre in these cases should be done by IIF using ethanol-fixed neutrophils.

Sera with perinuclear or nuclear patterns are tested by IIF using formalin-fixed neutrophils and Hep2 cells to differentiate between P-ANCA and ANA. Positive P-ANCA patterns should be followed with specific assays for anti-MPO and anti-PR3 if ANCA-associated vasculitic disease is suspected. Strong anti-MPO activity is characteristic of primary small vessel vasculitis or a drug-induced syndrome whereas weak or absent anti-MPO activity is more characteristic of another chronic inflammatory disease.³⁰

Some workers also advocate screening sera with requests for an acute assay simultaneously with an ELISA for anti-GBM antibodies because the clinical picture of Goodpasture and WG can be identical;⁸⁸ however, this is not routine practice in most laboratories.

Conclusion

The ANCA test has become a useful tool in the diagnosis of vasculitides, especially in WG, MPA, pauci-immune NCGN and Churg-Strauss syndrome, and has also been found of value in monitoring disease activity in WG. The numerous studies conducted since it was first described have contributed much to the understanding of the diseases associated with ANCA, which have, in turn, contributed to efforts in classification and nomenclature of the vasculitides. As more knowledge is obtained regarding the specific target antigens, the use of specific ANCA testing will no doubt in time replace conventional IIF screening in the clinical laboratory and provide more disease-specific testing. Finally, as investigators learn more and more of the pathogenicity of ANCA, it is entirely possible that this knowledge will be used to formulate new therapeutic interventions in the ANCA-related diseases. With all these possibilities in mind, it is therefore important that it is effectively and knowledgeably used in clinical practice.

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