International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA)

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Abstract

Antineutrophil cytoplasmic antibody (ANCA) tests are used to diagnose and monitor inflammatory activity in the primary systemic small vessel vasculitides. ANCA is best demonstrated in these diseases by using a combination of indirect immunofluorescence (IIF) of normal peripheral blood neutrophils and enzyme-linked immunosorbent assays (ELISAs) that detect ANCA specific for proteinase 3 (PR3) or myeloperoxidase (MPO). For ANCA testing in "new" patients, IIF must be performed on all serum samples. Serum samples containing ANCA, any other cytoplasmic fluorescence, or an antinuclear antibody (ANA) that results in homogeneous or peripheral nuclear fluorescence then should be tested in ELISAs for PR3-ANCA and MPO-ANCA. Optimally, ELISAs for PR3-ANCA and MPO-ANCA should be performed on all serum samples. Inclusion of the most recent positive sample in the IIF or ELISA may help demonstrate a change in antibody level. Reports should use recommended terms. Any report of positive neutrophil fluorescence issued before the ELISA results are available should indicate that positive fluorescence alone is not specific for the diagnosis of Wegener granulomatosis or microscopic polyangiitis and that decisions about treatment should not be based solely on the ANCA results.

Antineutrophil cytoplasmic antibodies (ANCA) have been recognized for more than a decade to be important adjuncts to the diagnosis of the primary systemic small vessel vasculitides.[1–3] However, in some patients, problems with ANCA testing or with the interpretation of the tests have resulted in incorrect diagnoses and subsequent errors in management. This is partly because new diagnostic tests often create interest before full evaluation of their clinical usefulness and specificity and sensitivity. In addition, the reproducibility of such tests is often poor until the critical technical aspects have been identified, the methods standardized, and quality control implemented. Routine laboratories may face clinical pressures to perform new diagnostic tests using commercial assays before these difficulties have been resolved.

The present article represents guidelines for ANCA testing and reporting that have been developed to minimize the technical difficulties and to produce more uniformity in the results issued by different laboratories. It was originally prepared at the request of the Australian Society of Clinical Immunologists and Allergists by immunologists from 8 Australian laboratories. The draft was circulated to each contributor and modified, and the resulting document was distributed to the 120 participants from 21 countries who attended the "ANCA and Vasculitis" symposium held as a satellite to the XIV Congress of Nephrology in Melbourne, Australia, May 30–June 1, 1997. At that meeting, the document was vigorously discussed at a 2-hour plenary session chaired by Allan Wiik. Further changes were made, and a revised version was distributed to the original contributors and to 11 experts from 3 continents who attended the meeting

and participated in the discussion. After further revisions, the final document was returned to these persons for ratification.

Background

ANCA are autoantibodies directed against cytoplasmic constituents of neutrophils and monocytes.[1–3] The most common reasons for requesting an ANCA test are to diagnose and monitor activity in the primary systemic small vessel vasculitides, namely Wegener granulomatosis, microscopic polyangiitis and its renal-limited form, pauciimmune segmental necrotizing glomerulonephritis, and Churg-Strauss syndrome.[1–6]

Table 1 shows the clinical manifestations that suggest the diagnosis of Wegener granulomatosis or microscopic polyangiitis and indicate that ANCA testing is warranted.[7,8] When these clinical features are present, the demonstration of ANCA is probably 95% sensitive and 90% specific for these diseases and has a much higher positive predictive value than in other hospitalized patients.[9] The diagnosis of Churg-Strauss syndrome is suspected when asthma and eosinophilia are present in addition to vasculitis, but series are small, and the diagnostic usefulness of ANCA is less well studied.

The European standardization trials have found that ANCA are most accurately demonstrated in patients with Wegener granulomatosis and microscopic polyangiitis by using a combination of indirect immunofluorescence (IIF) of normal peripheral blood neutrophils and enzyme-linked immunosorbent assays (ELISAs) for proteinase 3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA.[10–12] IIF alone detects 90% to 95% of all ANCA-positive serum samples in patients with Wegener granulomatosis, microscopic polyangiitis, and pauciimmune segmental necrotizing glomerulonephritis, and ELISAs for PR3-ANCA and MPO-ANCA detect about 90%.

Serum samples from patients with the primary systemic small vessel vasculitides produce 2 neutrophil IIF patterns. These are cytoplasmic fluorescence with central interlobular accentuation (C-ANCA), which usually occurs with PR3 specificity,[13,14] and perinuclear fluorescence often with nuclear extension (P-ANCA) that occurs with MPO specificity.[3,15] Most patients with active generalized Wegener granulomatosis have C-ANCA with PR3 specificity, but up to 25% have P-ANCA with MPO specificity.[12] About 60% of patients with microscopic polyangiitis or pauciimmune segmental necrotizing glomerulonephritis have P-ANCA with MPO specificity, and 30% have C-ANCA with PR3 specificity. Occasionally in these patients, C-ANCA with MPO specificity and P-ANCA with PR3 specificity occur. ANCA are less common in patients with Churg-Strauss syndrome, and the patterns and specificities resemble those found in microscopic polyangiitis.[4] Clinical definitions of Wegener granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome do not include the demonstration of ANCA or target antigen specificity.[16,17]

In Wegener granulomatosis, microscopic polyangiitis, and pauciimmune segmental necrotizing glomerulonephritis, ANCA levels usually correlate with disease activity and are high at initial examination, decline with treatment, and increase in about 50% of the patients who experience a relapse.[7,8] In addition, about 50% of the patients in whom antibodies recur will relapse. The results are likely to be similar in Churg-Strauss syndrome. About 10% of patients with Wegener granulomatosis, microscopic polyangiitis, or pauciimmune segmental necrotizing glomerulonephritis also have antiglomerular basement membrane antibodies.[18]

Most of the neutrophil cytoplasmic fluorescence detected in a routine laboratory is not found in patients with the primary systemic small vessel vasculitides.[19] About half the cytoplasmic fluorescence is "flat" and lacks the interlobular accentuation seen with PR3-ANCA.[19–22] This is *C-ANCA (atypical),* in which the corresponding antigen specificities include MPO and bactericidal/permeability–increasing protein but are usually unknown.[21,23,24] Overall, the most common IIF pattern is a P-ANCA that lacks the nuclear fluorescence seen with many MPO-ANCA.[15] These P-ANCA occur in 50% to 70% of patients with ulcerative colitis,[25,26] 20% to 40% of patients with Crohn disease,[25,26] 80% of patients with chronic active hepatitis,[27] 25% to 35% of patients with rheumatoid arthritis,[28] and about 20% of patients with systemic lupus erythematosus.[29] These frequencies differ between laboratories because of cohort variation and differences in detection methods. A less common fluorescence pattern is the granulocyte-specific antinuclear antibody (ANA) seen in patients with rheumatoid arthritis and sometimes with inflammatory bowel disease[30] and that probably represents a form of P-ANCA.

Atypical ANCA is used for fluorescence patterns other than those described as C-ANCA, C-ANCA (atypical), or

Table 1
Clinical Indications for Antineutrophil Cytoplasmic Antibody Testing*

Glomerulonephritis, especially rapidly progressive glomerulonephritis
Pulmonary hemorrhage, especially pulmonary renal syndrome
Cutaneous vasculitis with systemic features
Multiple lung nodules
Chronic destructive disease of the upper airways
Long-standing sinusitis or otitis
Subglottic tracheal stenosis
Mononeuritis multiplex or other peripheral neuropathy
Retro-orbital mass

* When there is no other obvious cause. Modified from DeRemee[7] and Hagen[8] with permission.

P-ANCA and most often is a combination of cytoplasmic and perinuclear fluorescence. Atypical ANCA occur in patients with inflammatory bowel disease, rheumatoid arthritis, and some types of drug-induced vasculitis.[31,32] All forms of P-ANCA and atypical ANCA fluorescence are artifactual and occur only with ethanol-fixed (and acetone-fixed) neutrophils; the same serum samples produce cytoplasmic fluorescence on formalin-fixed cells.[3] Many P-ANCA (including granulocyte-specific P-ANCA) and atypical ANCA have multiple antigen specificities. These include α-enolase,[33] catalase,[34] high-mobility-group nonhistone chromosomal proteins,[35] actin,[36] cathepsin G,[37] elastase,[31] lactoferrin,[38] lysozyme,[39] and other unidentified targets.[40] With ANCA in which the specificities are not PR3 or MPO, antibody levels are variable but often low, and there usually is no clear correlation with disease activity or other clinical variables.

Testing for ANCA

ANCA testing should not be performed if the following minimum requirements cannot be fulfilled. The optimum recommendations ensure the most reproducible and clinically relevant results.

IIF Assay

Minimum

- IIF should be performed on serum samples from all "new" patients, since 10% of ANCA-positive serum samples in patients with Wegener granulomatosis or microscopic polyangiitis can be demonstrated only by IIF.
- Serum samples from patients that were previously ANCA-positive by IIF alone can be tested subsequently only by IIF. However, ANCA specificities occasionally become detectable or change in individual patients with Wegener granulomatosis or microscopic polyangiitis.
- In-house methods should use whole buffy coat preparations prepared according to the guidelines in the First International ANCA Workshop,[41] because these contain neutrophils and lymphocytes. Commercial slides may be used.
- Titration is unnecessary if neutrophils and monocytes are the only fluorescent cells.

Optimum

- The IIF titer is determined if serum samples are positive by IIF but negative in the ELISAs for PR3-ANCA and MPO-ANCA.
- Titration may be useful if an interfering ANA or other cytoplasmic fluorescence (eg, antibodies against Jo-1 or ribosomal nucleoprotein) is present in addition to the ANCA, since 1 pattern may disappear before the other.
- If the serum has been positive previously only by IIF, comparison of the IIF titer in the present sample with the previously positive sample may be useful in demonstrating a change in antibody level.

Comments

- Important details on the technique of neutrophil slide preparation and IIF, together with notes on troubleshooting, have been published.[42]
- Serum samples should not be heat-inactivated, since this will lead to false-positive results.
- The optimal serum dilution for ANCA screening varies according to local conditions (nature of conjugate, microscope, light source), eg, a 1:20 dilution was recommended at the First International ANCA Workshop,[41] but a 1:40 dilution may help to exclude false-positive results.
- The addition of 1% bovine serum albumin to the serum diluent and wash solution (such as phosphate-buffered saline) decreases the background fluorescence.
- Polyclonal anti-IgG should be used since anti-IgM and anti-IgA conjugates may result in false-positive results.[43]
- Evans blue counterstain should be used with care since it can mask low positive fluorescence.
- Almost all patients with a clinically active systemic small vessel vasculitis (including pauciimmune segmental necrotizing glomerulonephritis) have strongly positive fluorescence (and high antibody levels by ELISA) at initial examination.

Serum Samples With Concomitant ANA

Minimum

- All serum samples with P-ANCA or atypical ANCA, together with an ANA that results in homogeneous or peripheral nuclear fluorescence and, thus, that interferes with the reading of neutrophil IIF, should be tested in ELISAs for PR3-ANCA *and* MPO-ANCA. Serum samples with noninterfering ANAs (eg, nucleolar) can be processed as if no ANA were present.

Comments

- P-ANCA and atypical ANCA react only with neutrophil and monocyte nuclei, but an ANA reacts with the nuclei of all cells, including lymphocytes.

- With P-ANCA, some nuclear fluorescence of lymphocytes near an IIF-positive neutrophil is noted occasionally because the fixation process results in the leakage of neutrophil cytoplasmic constituents that subsequently adhere to nearby lymphocyte nuclei.

Reporting of IIF Results

Nomenclature

- C-ANCA: classic granular cytoplasmic fluorescence with central or interlobular accentuation.
- C-ANCA (atypical): diffuse flat cytoplasmic fluorescence without interlobular accentuation.
- P-ANCA: perinuclear fluorescence, with or without nuclear extension; includes granulocyte-specific ANA.
- Atypical ANCA: includes all other neutrophil-specific or monocyte-specific IIF reactivity, most commonly a combination of cytoplasmic and perinuclear fluorescence.

Comments

- The use of the terms C-ANCA and P-ANCA,[44] rather than cANCA and pANCA, to describe the IIF patterns conforms with the style for the descriptions of antigen specificities (eg, PR3-ANCA).
- This classification distinguishes between cytoplasmic, but not perinuclear, fluorescent patterns. This is because IIF results can be released before the ELISAs are performed, and many clinicians still mistakenly believe that all cytoplasmic fluorescence correlates with PR3 specificity and indicates a small vessel vasculitis. While it is usually easy to distinguish between different P-ANCA with and without neutrophil nuclear extension, the correlation of P-ANCA with nuclear extension and MPO specificity, and P-ANCA without nuclear extension with other antigen specificities, is less consistent than the correlation of C-ANCA with PR3 specificity and small vessel vasculitis. In addition, most clinicians are aware that P-ANCA occur in many autoimmune diseases.

Minimum

- The IIF pattern is described as C-ANCA, C-ANCA (atypical), P-ANCA, or atypical ANCA.
- The report indicates that neutrophil fluorescence alone is not specific for the diagnosis of Wegener granulomatosis or microscopic polyangiitis.
- The report indicates that all IIF-positive serum samples have been or will be tested for antigen specificity by ELISAs for PR3-ANCA and MPO-ANCA.
- The report indicates when the laboratory is unable to confirm a P-ANCA because of a concomitant ANA and that ELISAs for PR3-ANCA and MPO-ANCA will be performed.

Optimum

- The fluorescence intensity is graded negative, weakly positive, or strongly positive on the basis of the screening dilution only.
- The IIF results for negative serum samples are not reported until the ELISAs for PR3-ANCA and MPO-ANCA have been performed.
- The report indicates whether ELISAs for ANCA specificities other than PR3 and MPO are appropriate and which specificities are available.

ELISAs

Antigens

Minimum

- Manufacturers supply details of the purification method and the purity of each antigen.
- If PR3 and MPO are purified in house, the purification method that causes the least denaturation should be used.[10]
- Native antigens are preferred to recombinant antigens, whose usefulness still must be verified.[45]
- Assays using new batches of commercially available or in-house purified antigen should be validated against "old" preparations.

Optimum

- The purity of the antigens prepared in house should be verified in an ELISA system, with monoclonal or polyclonal antisera against potentially contaminating ANCA antigens, such as lactoferrin.

Assay

Minimum

- Serum samples positive for ANCA by IIF, with other cytoplasmic fluorescence, or with an interfering ANA, are assayed for PR3-ANCA and MPO-ANCA.
- Serum samples from patients previously positive for PR3-ANCA or MPO-ANCA can be tested subsequently in the appropriate ELISA alone. However, ANCA specificities occasionally change in individual patients with Wegener granulomatosis or microscopic polyangiitis.

- Each plate includes negative and positive standards, an in-house reference serum (preferably compared with the international serum standard), and blanks.
- The results are calculated from a curve plotted from the standards.

Optimum

- All serum samples are assayed in PR3-ANCA and MPO-ANCA ELISAs, since 5% of serum samples are positive only by ELISA.
- The assays are validated in-house or are commercial ELISAs that quantitate PR3-ANCA and MPO-ANCA and have interassay and intra-assay variations of less than 20%.[10,11]
- Serum samples are assayed in duplicate.
- The cutoff point, even for commercial ELISAs, is verified in house. This value is calculated by using samples from hospitalized patients and set high enough to achieve more than 90% specificity for the systemic vasculitides compared with control samples from hospitalized patients. If an earlier serum sample from the patient was positive by ELISA, then antibody levels can be compared with a previously positive serum sample from the same patient by ELISA.

Comments

- PR3 purification techniques do not substantially influence the level of binding of PR3-ANCA by ELISA.[11]
- A serum standard for C-ANCA with PR3 specificity is available from AW, Statens Seruminstitut, Denmark (e-mail: aw@ssi.dk).
- Subtracting the binding to antigen-free or protein-coated plates helps reduce false-positive results caused by the nonspecific binding of "sticky" serum samples.
- Nonspecific binding should be suspected if PR3-ANCA *and* MPO-ANCA are detected in the same serum sample at low levels.
- There are no standard units for the results from PR3-ANCA and MPO-ANCA ELISAs.
- Some laboratories use rapid qualitative techniques for PR3-ANCA and MPO-ANCA, and positive results are confirmed quantitatively later.
- Some laboratories screen serum samples from patients with acute pulmonary-renal syndrome for ANCA and antiglomerular basement membrane antibodies.[46] Wegener granulomatosis and microscopic polyangiitis are more common causes of the pulmonary-renal syndrome than is antiglomerular basement membrane disease,[47] but the conditions coexist in 10% to 30% of patients.[48]

Reporting of ELISA Results

Nomenclature

- Autoantibodies are described, for example, as PR3-ANCA, MPO-ANCA, BPI-ANCA.
- Terms such as antiproteinase 3 antibodies indicate antibodies that have been produced for research purposes, eg, monoclonal antibodies.

Minimum

- Results of the ELISAs for PR3-ANCA and MPO-ANCA should follow the IIF reports as soon as possible.
- Serum results are given as ELISA units, and the values above which serum samples are positive (cutoff points) are stated.
- Comments on and interpretation of individual positive results are provided.
- More extensive information, such as the following, is provided.

1. C-ANCA occur in at least 70% of patients with active generalized Wegener granulomatosis, less frequently in those with limited disease (ie, not involving the kidneys), and in 30% of those with microscopic polyangiitis. Almost all of these are PR3-ANCA.
2. C-ANCA (atypical) has no clinical significance.
3. P-ANCA with MPO specificity occur in 50% to 80% of patients with active microscopic polyangiitis (which may be confined to the kidney as segmental necrotizing glomerulonephritis) and up to 25% of patients with Wegener granulomatosis.
4. In Wegener granulomatosis and microscopic polyangiitis, ANCA levels usually decline with treatment but increase in about 50% of patients who experience a relapse; conversely, about 50% of patients in whom ANCA recur will relapse.
5. P-ANCA (usually with specificities other than MPO) occur in some patients with inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, chronic active hepatitis, and other autoimmune diseases. In these patients, ANCA levels are often low and of uncertain clinical significance.
6. Atypical ANCA (and P-ANCA) are found in some drug-induced vasculitides but are otherwise of uncertain clinical significance.

- The report contains the warning that decisions about treatment should not be made solely on the basis of the results of the ANCA tests.

Optimum

- The IIF and ELISA results are reported at the same time for all serum samples.

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