Addendum to the International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies

Quality Control Guidelines, Comments, and Recommendations for Testing in Other Autoimmune Diseases

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Key Words: Antineutrophil cytoplasmic antibodies; Churg-Strauss syndrome; Microscopic polyangiitis; Quality control; Renal-limited vasculitis; Vasculitis; Wegener granulomatosis

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Abstract

Antineutrophil cytoplasmic antibody (ANCA) tests are used to diagnose and monitor inflammatory activity in Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant (pauci-immune crescentic glomerulonephritis), and Churg-Strauss syndrome. The International Consensus Statement on testing and reporting of ANCA states that ANCA are demonstrated most readily in these conditions 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 or myeloperoxidase. The group that produced the International Consensus Statement has developed guidelines for the corresponding quality control activities, examples of comments for various IIF patterns and ELISA results, and recommendations for ANCA testing when inflammatory bowel disease and other nonvasculitic ANCA-associated autoimmune diseases are suspected.

Antineutrophil cytoplasmic antibody (ANCA) tests are used to diagnose and to monitor inflammatory activity in the small vessel vasculitides, namely Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant (pauci-immune crescentic glomerulonephritis), and Churg-Strauss syndrome.[1-4] The International Consensus Statement on testing and reporting of ANCA[5] states that ANCA are most readily demonstrated in these conditions 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).[3,6,7] The group that produced the International Consensus Statement has developed the following guidelines for ANCA quality control (QC) activities, examples of comments that describe different IIF patterns and ELISA results, and recommendations for ANCA testing when inflammatory bowel disease and other nonvasculitic ANCA-associated autoimmune diseases are suspected.

The following QC guidelines were developed at 2 meetings of 30 Australian scientists from laboratories that test for ANCA. The comments were derived from those submitted by 8 laboratories. The recommendations for testing in nonvasculitic diseases were prepared by the coordinator of this document (J.S.) together with 3 international experts who subsequently attended the Fifth Australasian ANCA Workshop (M.F., J.G., A.W.). A draft of the QC guidelines, the comments, and the recommendations for testing in nonvasculitic diseases were distributed to all 70 participants at the Fifth Australasian ANCA Workshop. At this meeting, the document was debated vigorously, a subsequent draft was

circulated for comments to the participants and 3 other contributors to the original consensus statement, and the final document was prepared and subsequently ratified by all of the participating authors. Their recommendations are summarized in **Table 1**.

QC Guidelines

Indirect Immunofluorescence

Minimum Requirements

- Control serum samples that produce cytoplasmic ANCA (C-ANCA) and perinuclear ANCA (P-ANCA) patterns are tested on each new lot of slides.
- The working concentration of each lot of fluorochrome-labeled antihuman immunoglobulin conjugate is determined for an ANCA substrate by a "checkerboard" titration if not done already by the manufacturer.
- A negative and a positive IgG ANCA reaction are demonstrated in each IIF run.

Optimal Recommendations

- Control serum samples that produce C-ANCA, C-ANCA (atypical), P-ANCA, and atypical ANCA are tested on each new lot of slides to ensure that typical and atypical IIF patterns can be distinguished.
- The working concentration of each lot of fluorochrome-labeled antihuman immunoglobulin conjugate is determined for each lot of ANCA substrate by a checkerboard titration.
- C-ANCA and P-ANCA patterns are demonstrated in each IIF run.
- A borderline positive or a titratable positive control sample is tested in each run.
- A negative and a positive IgG ANCA reaction are demonstrated on each IIF slide.

Enzyme-Linked Immunosorbent Assay

Minimum Requirements

- Production is monitored for each lot of in-house assays, including checking the antigen purity, the efficacy of antigen coating to wells, and the amount of nonspecific binding.
- In-house and commercial assays are evaluated by

Table 1
Minimum Quality Control Requirements for Testing and Reporting Antineutrophil Cytoplasmic Antibodies (ANCA)

Indirect Immunofluorescence (IIF)

- Control serum samples that produce C-ANCA and P-ANCA patterns are tested on each new lot of slides.
- The working concentration of each lot of fluorochrome-labeled antihuman immunoglobulin conjugate is determined for an ANCA substrate by a "checkerboard" titration if not done already by the manufacturer.
- A negative and a positive IgG ANCA reaction are demonstrated in each IIF run.

Enzyme-Linked Immunosorbent Assay (ELISA)

- Production is monitored for each lot of in-house assays, including checking the antigen purity, the efficacy of antigen coating to wells, and the amount of nonspecific binding.
- In-house and commercial assays are evaluated by determining their sensitivity and specificity, precision within and between runs, linearity, and performance at clinically important binding levels. Commercial manufacturers provide these data for each lot of assays. Sensitivity is determined using serum samples from patients with active vasculitis with clinical or pathologic features consistent with the diagnosis of active Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant, or Churg-Strauss syndrome.[8] All ELISAs have interassay and intra-assay coefficients of variation less than 20%, determined ideally with specimens that have clinically significant values that also are within the linear part of the assay's standard curve.
- For in-house assays, the cutoff point for positivity is determined using serum samples from age- and sex-matched patients with inflammatory diseases that usually are not associated with ANCA. The receiver operating characteristic plot analysis is used to determine a cutoff value that provides the best compromise between sensitivity and specificity. This value will reflect the laboratory's referral population.
- In-house and commercial ELISAs test negative and positive control samples, an in-house or nonkit commercial control sample, and additional control samples according to the requirements of local and/or national regulations or accrediting organizations. A well or wells without serum to which all other reagents including diluent are applied ("substrate blank") is also tested. For both in-house and commercial assays, the optical density or derived numeric value of the control samples is recorded and plotted serially to determine whether the run should be accepted or rejected. The values of the control samples should remain within the predetermined levels of the interassay coefficient of variation. For each in-house assay, a dilution series is performed to determine any loss of sensitivity, and the within-run precision is checked.
- Samples are assayed in duplicate, and any serum sample that is positive in only a single well or for which ELISA results for the 2 wells differ by a predetermined amount is tested again by ELISA.
- Otherwise, the manufacturer's guidelines for commercial ELISAs are followed.

Routine Quality Assurance Program

- The testing laboratory should conform to local regulatory requirements with regard to quality assurance guidelines.
- Laboratories must participate in local programs and, if these are not available, in international, external quality assurance programs. The performance of the laboratory in these programs must be reviewed regularly by the laboratory management and the staff performing the assays.

C-ANCA, cytoplasmic ANCA; P-ANCA, perinuclear ANCA.

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determining their sensitivity and specificity, precision within and between runs, linearity, and performance at clinically important binding levels. Commercial manufacturers provide these data for each lot of assays. Sensitivity is determined by using serum samples from patients with active vasculitis with clinical or pathologic features consistent with the diagnosis of active Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant, or Churg-Strauss syndrome.[8] All ELISAs have interassay and intra-assay coefficients of variation of less than 20%, determined ideally with specimens that have clinically significant values that also are within the linear part of the assay's standard curve.

- For in-house assays, the cutoff point for positivity is determined by using serum samples from age- and sex-matched patients with inflammatory diseases that usually are not associated with ANCA. The receiver operating characteristic plot analysis is used to determine a cutoff value that provides the best compromise between sensitivity and specificity. This value will reflect the laboratory's referral population.
- In-house and commercial ELISAs test negative and positive control samples, an in-house or nonkit commercial control sample, and additional control samples according to the requirements of local and/or national regulations or accrediting organizations. A well or wells without serum to which all other reagents including diluent are applied ("substrate blank") is also tested. For in-house and commercial assays, the optical density or derived numeric value of the control samples is recorded and plotted serially to determine whether the run should be accepted or rejected. The values of the control samples should remain within the predetermined levels of the interassay coefficient of variation. For each in-house assay, a dilution series is performed to determine any loss of sensitivity, and the within-run precision is checked.
- Samples are assayed in duplicate, and any serum sample that is positive in a single well only or for which ELISA results for the 2 wells differ by a predetermined amount is tested again by ELISA.
- Otherwise, the manufacturer's guidelines for commercial ELISAs are followed.

Optimal Recommendations

- Commercial assays are validated within each testing laboratory.
- The cutoff point for positivity for commercial ELISAs is confirmed within each testing laboratory as described for in-house assays.
- The test results are compared with international calibrated reference standards.

Comments

- A reference serum sample for C-ANCA with PR3 specificity is available from Statens Seruminstitut, Denmark (www.ssi.dk) but calibrated reference standards for PR3-ANCA and MPO-ANCA are not yet available.
- At present, there are no standardized units for PR3-ANCA and MPO-ANCA binding. All currently used units are arbitrary and vary from one assay to another. Reporting semiquantitative ranges such as low, moderate, and strong positive based on the laboratory's experience and interpretation, together with the numeric values, is helpful to the laboratory staff who generate the comments and to clinicians.

Routine Quality Assurance Program

Minimum Requirements

- The testing laboratory should conform to local regulatory requirements with regard to quality assurance guidelines.
- Laboratories must participate in local programs and, if these are not available, in international, external quality assurance programs. The performance of the laboratory in these programs must be reviewed regularly by laboratory management and the staff performing the assays.

Reporting of Results

Minimum Requirements

- IIF results may be issued before performance of the PR3-ANCA and MPO-ANCA ELISAs,[5] but the comments should indicate that antigen specificities are required for the interpretation of the ANCA test results.
- The comments indicate that the diagnosis of Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant, and Churg-Strauss syndrome should be confirmed histologically when possible.

The following are examples of comments for reporting ANCA results.

IIF Results Issued Before ELISA Performed

1. C-ANCA positive: This pattern is suggestive, but not diagnostic, of Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), and Churg-Strauss syndrome. Results of ELISAs for PR3-ANCA and MPO-ANCA will follow.
2. P-ANCA positive: This pattern occurs in microscopic polyangiitis (and its renal-limited variant) and in some cases of Wegener granulomatosis and Churg-Strauss syndrome. However, it also occurs in inflammatory bowel disease and other autoimmune diseases. Results of the ELISAs for MPO-ANCA and PR3-ANCA will follow.
3. C-ANCA (atypical) positive: This pattern is *not* typically associated with Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), or Churg-Strauss

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syndrome. It occurs in chronic infections, inflammatory bowel disease, and other autoimmune diseases. Results of the ELISAs for PR3-ANCA and MPO-ANCA will follow.

4. Atypical ANCA positive: This pattern is *not* typically associated with Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), or Churg-Strauss syndrome. It occurs in inflammatory bowel disease and other autoimmune diseases. Results of the ELISAs for PR3-ANCA and MPO-ANCA will follow.

5. ANCA negative: This result may occur in patients with treated or inactive Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), or Churg-Strauss syndrome. Patients with temporal arteritis, Henoch-Schönlein purpura, or cryoglobulinemic vasculitis and most patients with polyarteritis nodosa are ANCA-negative.

6. ANA positive: C-ANCA negative, but P-ANCA cannot be excluded because of the presence of antinuclear antibodies (ANA). Results of the ELISAs for PR3-ANCA and MPO-ANCA will follow.

IIF and ELISA Results Are Issued Together

1. C-ANCA positive—PR3-ANCA 3+ or 2+ (or MPO-ANCA 3+ or 2+): This result occurs in active Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), and Churg-Strauss syndrome. The diagnosis should be confirmed histologically whenever possible. Patients with systemic vasculitis in whom ANCA recur are more likely to relapse.

2. C-ANCA positive—PR3-ANCA negative (or 1+) and MPO-ANCA negative (or 1+): This result may occur in treated, inactive, or relapsing Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), and Churg-Strauss syndrome. It also occurs with chronic infections and occasionally in inflammatory bowel disease and other autoimmune diseases where its clinical significance is unclear.

3. C-ANCA (atypical) positive—PR3-ANCA negative and MPO-ANCA negative: This result is not specific to any diagnosis but occurs occasionally in chronic infections, inflammatory bowel disease, and other autoimmune diseases where its clinical significance is unclear.

4. P-ANCA positive—MPO-ANCA 3+ or 2+: This result occurs in active microscopic polyangiitis (and its renal-limited variant), Churg-Strauss syndrome, and sometimes Wegener granulomatosis. The diagnosis should be confirmed histologically when possible. Patients with systemic vasculitis in whom ANCA recur are more likely to relapse.

5. P-ANCA positive—MPO-ANCA negative (or 1+): This result may occur in treated, inactive, or relapsing microscopic polyangiitis (and its renal-limited variant), Wegener granulomatosis, and Churg-Strauss syndrome. Patients with systemic vasculitis in whom ANCA recur are more likely to relapse. This result also is common in inflammatory bowel disease and other autoimmune diseases where its clinical significance is unclear.

6. Atypical ANCA positive—PR3-ANCA negative or 1+ and MPO-ANCA negative or 1+: This result occurs in inflammatory bowel disease and other autoimmune diseases where its clinical significance is unclear. It does not occur in Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), or Churg-Strauss syndrome.

7. Atypical ANCA positive—MPO-ANCA 3+ or 2+: This result is uncommon enough to warrant an individualized comment. It occurs in drug-induced vasculitis.

8. C-ANCA negative—PR3-ANCA 1+; P-ANCA negative—MPO-ANCA 1+: This result may occur in treated, inactive, or relapsing Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), and Churg-Strauss syndrome. It also occurs occasionally in inflammatory bowel disease and other autoimmune diseases where its clinical significance is unclear.

9. ANA positive—MPO-ANCA 3+ or 2+: This result occurs in active microscopic polyangiitis (and its renal-limited variant), Churg-Strauss syndrome, and sometimes Wegener granulomatosis. The diagnosis should be confirmed histologically whenever possible. Patients with systemic vasculitis in whom ANCA recur are more likely to relapse.

10. ANA positive—MPO-ANCA 1+: This result is uncommon enough to warrant an individualized comment. It may occur in treated, inactive or relapsing microscopic polyangiitis (and its renal-limited variant), Wegener granulomatosis, and Churg-Strauss syndrome. Patients with systemic vasculitis in whom ANCA recur are more likely to relapse. This result is also common in inflammatory bowel disease and other autoimmune diseases where its clinical significance is unclear.

Optimal Recommendations

- Results are correlated with clinical details.
- Results are compared with those from previous assays for individual patients.
- Antibody levels are taken into account when interpreting the results.

Comments

- The International Consensus Statement advocates screening by IIF and confirmation of IIF positivity in PR3-ANCA and MPO-ANCA ELISAs. There are no published studies comparing this protocol with screening by ELISA and confirmation of the results by IIF.
- ANCA levels often help in the interpretation of results. The International Consensus Statement does not advocate titration of fluorescence, but rather the use of antigen-specific

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ELISAs that confirm PR3 or MPO specificity and also indicate antibody levels. PR3-ANCA and MPO-ANCA levels in Wegener granulomatosis, microscopic polyangiitis and its renal-limited variant, and Churg-Strauss syndrome are usually high (3+ or 2+) at diagnosis, decline, often disappear with treatment, and usually reappear before relapse.[9,10] When present, PR3-ANCA and MPO-ANCA levels in chronic infections, inflammatory bowel disease, and autoimmune diseases are usually low (1+) and do not correlate with disease activity.

- The International Consensus Statement distinguishes between C-ANCA and C-ANCA (atypical) (in which there is no central interlobular accentuation or cytoplasmic granularity), but many laboratories do not report C-ANCA (atypical).[5] C-ANCA is usually directed against PR3, but C-ANCA (atypical) often has specificity for bactericidal/permeability–increasing protein. This explains the comments about C-ANCA appearing to occur in chronic infections and inflammatory bowel disease.[11-13]
- Some laboratories may confuse a C-ANCA with an atypical ANCA (in which both cytoplasmic and perinuclear fluorescence are commonly present). The PR3-ANCA level will usually distinguish patients with Wegener granulomatosis from patients with a true atypical ANCA due to inflammatory bowel disease or other autoimmune disease.
- In the International Consensus Statement, P-ANCA describes all perinuclear fluorescence, whether nuclear extension (usually indicating MPO-ANCA) is present or not. This is because the demonstration of nuclear extension may depend on the neutrophil substrate, fluorescence intensity, and the observer's experience. The decision to use only P-ANCA acknowledges that most clinicians understand that this term does not necessarily indicate a vasculitis.
- The International Consensus Statement does not advocate testing on formalin-fixed neutrophils to confirm P-ANCA with MPO specificity.[5] This observation has been used in some laboratories as evidence for MPO-ANCA and, hence, the diagnosis of a systemic small vessel vasculitis. However some ANCA-positive serum samples with MPO specificity demonstrate no fluorescence on formalin-fixed neutrophils.[14,15]
- Serum samples with an ANA that may mask a P-ANCA should be tested for both PR3-ANCA and MPO-ANCA.[5]
- In drug-induced vasculitis, a P-ANCA or an atypical ANCA is often present with moderate or strong levels of ANCA with specificity for MPO and sometimes other antigens.[16,17]
- C-ANCA with MPO specificity accounts for fewer than 10% of all patients with Wegener granulomatosis, microscopic polyangiitis (and its renal-limited variant), or Churg-Strauss syndrome.[18] P-ANCA with PR3 specificity is rare.

ANCA Testing in Inflammatory Bowel Disease and Other Nonvasculitic ANCA-Associated Autoimmune Diseases

The International Consensus Statement has addressed ANCA testing when a small vessel vasculitis is suspected, but the demonstration of ANCA also may help in the diagnosis of inflammatory bowel disease, primary sclerosing cholangitis, autoimmune hepatitis type I, and Felty syndrome.[19-23] ANCA occur in 50% to 70% of patients with ulcerative colitis, 10% to 30% of patients with Crohn disease, 90% of patients with autoimmune hepatitis type I, and 90% of patients with Felty syndrome. ANCA also occur in up to 30% of patients with active rheumatoid arthritis.[24,25]

Ulcerative colitis and Crohn disease are diagnosed and differentiated most readily on clinical, radiologic, endoscopic, and histologic criteria, but 10% to 15% of patients have features that initially do not fit either diagnosis. ANCA are much more common in ulcerative colitis[26] and typically occur in Crohn disease when there is colonic involvement.[27] ANCA testing alone will not distinguish between ulcerative colitis and Crohn disease, but the combination of ANCA testing by IIF and anti–*Saccharomyces cerevisiae* antibody (ASCA) testing by ELISA often helps differentiate between these conditions.[28,29] Patients with ulcerative colitis are more likely to have P-ANCA–positive but ASCA-negative results, and those with Crohn disease are more likely to have P-ANCA–negative but ASCA-positive results. The presence of ANCA in ulcerative colitis and Crohn disease does not seem to correlate with disease activity, extent, or treatment. However, antibody levels may be higher with more active disease.[14] Individual studies in inflammatory bowel disease have suggested that certain ANCA antigenic specificities correlate with clinical features, but these observations await confirmation.[14] This also is true of ANCA in rheumatoid arthritis.[14]

In these nonvasculitic autoimmune diseases, the IIF pattern is usually described as P-ANCA, but there is a broad rim-like fluorescence of the neutrophil nuclear periphery without nuclear extension. This differs from the pattern seen when MPO is the target.[3] C-ANCA and atypical ANCA also occur. These antibodies usually have multiple specificities in which the antigens derive mainly from the nucleus, cytosol, and specific granules, rather than from the primary granules that are recognized by ANCA in systemic vasculitis. Targets include histone H1, nuclear membrane proteins, and high mobility group proteins (HMG1 and HMG2).[30-32] Other antigens include catalase, elastase, α-enolase, bactericidal/permeability–increasing protein, PR3, and MPO.

There is currently no single satisfactory technique for ANCA testing in inflammatory bowel disease,

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primary sclerosing cholangitis, autoimmune hepatitis type I, or Felty syndrome. However, adherence to the International Consensus Guidelines will demonstrate antibodies with the distinctive peripheral rim (or cytoplasmic or atypical) fluorescence on ethanol-fixed neutrophils and the absence of strong binding in the PR3-ANCA and MPO-ANCA ELISAs. This fluorescence often disappears on formalin-fixed neutrophils, but the results for individual serum samples may be inconsistent. Results also are inconsistent when serum samples are tested on deoxyribonuclease (DNase)-treated neutrophils. Thus, testing on formalin-fixed neutrophils and the use of DNase are not advocated for routine ANCA testing in these conditions, but specialist laboratories may choose to perform these techniques. It is not possible to recommend the use of ELISAs for ANCA with certain antigenic specificities until these findings are confirmed to correlate with clinical features in nonvasculitic autoimmune diseases.

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