Twenty-eight years with antineutrophil cytoplasmic antibodies (ANCA): how to test for ANCA – evidence-based immunology?

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Abstract Wegener’s granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, and primary pauci-immune crescentic glomerulonephritis are associated with circulating antineutrophil cytoplasmic autoantibodies (ANCA) (collectively called ANCA-associated vasculitides, AAV). Two types of ANCA, one with a cytoplasmic fluorescence pattern (C-ANCA) and specificity for proteinase 3 (PR3-ANCA) and the other with a perinuclear pattern (P-ANCA) and specificity for myeloperoxidase (MPO-ANCA), account for this association and are highly specific markers for these vasculitides. AAV most often require therapy with cytotoxic and antiinflammatory agents, and hence a well-established diagnosis is mandatory to avoid unnecessary and risky treatment. The widespread use of ANCA screening in the past decade has resulted in the occurrence of greater numbers of false-positive results and has led to greater difficulty in test interpretation. Methods for ANCA detection have been standardized internationally in large multicentre studies and an international consensus statement on testing and reporting of ANCA has been published (1999 and 2003). Despite these advances, problems with the extended use of ANCA testing in daily clinical practice remain. They may be summarized as follows: (1) the basic standards for ANCA testing are not uniformly met; (2) there is still controversy over the value of formalin fixation of neutrophils in differentiating P-ANCA from antinuclear antibodies (what is the place of this substrate in ANCA testing?); (3) the new generation of PR3-ANCA and MPO-ANCA ELISAs are more sensitive and specific than immunofluorescence testing (should ELISAs replace the immunofluorescence test?); and (4) should alternative methods for ANCA detection such as image analysis and/or multiplex immunoassays be used for screening? In this paper, we review these issues, identify areas of uncertainty, and provide practical guidelines where possible.

Keywords ANCA • Screening • Proteinase 3 • Myeloperoxidase • ANCA-associated vasculitides

Introduction

The role of antineutrophil cytoplasmic antibodies (ANCA) in the clinical diagnosis of systemic inflammatory disease has been increasing since their description 28 years ago, and they have become an important tool for supporting a diagnosis of systemic necrotizing small-vessel vasculitis: Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome, collectively called ANCA-associated vasculitides (AAV) [1]. There are two types of ANCA, one with a cytoplasmic fluorescence pattern (C-ANCA) and specificity for proteinase 3 (PR3-ANCA) and the other with a perinuclear pattern (P-ANCA) and specificity for myeloperoxidase...
dase (MPO-ANCA). The first publications on the clinical utility of ANCA as a diagnostic marker for WG were evaluated in a meta-analysis by Rao et al. [2]. The sensitivities of C-ANCA testing for WG ranged from 34% to 92%, and the specificities range from 88% to 100%. The pooled sensitivity was 66%, and the pooled specificity was 98%. Several more recent studies have been conducted over the past few years, most of them retrospective, and the reported sensitivities and specificities of ANCA tests range widely, depending not only on the test characteristics of the assays used but also on the population under study. Between 60% and 95% of all ANCA found in WG are C-ANCA and PR3-ANCA.

The European Vasculitis Study (EUVAS) study group showed that the value of ANCA testing rises when a combined procedure is followed. The results of immunofluorescence testing (IFT) for ANCA detection can be greatly increased by the addition of a well-standardized antigen-specific ELISA. When the results of IFT are combined with those of the ELISA (C-ANCA/PR3-ANCA and P-ANCA/MPO-ANCA), the diagnostic specificity increases to 99%. The sensitivities of the combination of C-ANCA/PR3-ANCA and P-ANCA/MPO-ANCA for WG, MPA and primary pauci-immune crescentic glomerulonephritis are 73%, 67% and 82%, respectively [3]. The sensitivity of C-ANCA/PR3-ANCA for WG is related to the severity and activity of the disease at the time of sampling. The association between MPA and P-ANCA/MPO-ANCA is reported to be in the range 40–80% [3]. Few patients with WG are positive for P-ANCA/MPO-ANCA and few patients with MPA are positive for C-ANCA/PR3-ANCA. ANCA have been detected with variable frequency (10–70%) in Churg-Strauss syndrome. Both PR3 and MPO have been described as target antigens.

In general, ANCA levels are usually high at presentation and a rather solid association between ANCA titres and disease activity has been described by many groups. Because there is a strong publication bias against negative results, the relationship between ANCA levels and disease activity appears even less clear. Furthermore, one must keep in mind that among these studies, most of them retrospective, the definition of relapse, the intensity of clinical screening for disease activity, the interval for follow-up visits, and the methods and interval used for sequential ANCA detection varied widely. Generally, a relapse is likely with high antibody levels and unlikely when the levels are low. Furthermore, treatment decisions should be based on the clinical presentation and the histological findings, and not the results of ANCA testing alone.

Kain et al. found yet another ANCA with reactivity toward the antigen lysosome-associated membrane protein 2 (LAMP-2) in patients with focal necrotizing glomerulonephritis. Antibodies toward LAMP-2 were detected in nearly all patients with pauci-immune necrotizing glomerulonephritis (sensitivity 93%), but only in 1 of 20 patients with WG in the localized disease stage. Furthermore, the presence of these antibodies correlated very well with disease activity [4]. The diagnostic relevance of LAMP-2 ANCA for other rheumatic diseases has yet to be evaluated. In addition to their diagnostic value, LAMP-2 ANCA have also contributed to our understanding of the pathogenesis of autoimmune vasculitis. Kain et al. found that rats immunized with human LAMP-2 produced antibodies to the protein and developed pauci-immune necrotizing glomerulonephritis [4].

The current method for the determination of ANCA is indirect IFT of human neutrophils and antigen-specific ELISAs. However, there is still room for improvement. A new strategy to detect ANCA must be considered that is accurate, reproducible and cost-effective.

**Standardization of ANCA assays**

In general, IFT and/or antigen-specific direct ELISAs (PR3- and MPO-ANCA) are used for routine determination of ANCA in vasculitis. Standardization of PR3- and MPO-ANCA ELISAs and evaluation of their clinical utility by EUVAS [3] has substantially improved the detection of ANCA, and together with the recommendations of the International Consensus Statement on Testing and Reporting of ANCA [5] can minimize the occurrence of false-positive ANCA results. However, a major problem with the current application of ANCA assays is that they are not standardized to international standards.

**Formalin fixation of neutrophils does adequately differentiate between antinuclear antibody and ANCA staining**

The International Consensus Statement on Testing and Reporting of ANCA requires all serum samples to be examined by IFT on ethanol-fixed neutrophils [5]. However, technically it is difficult to differentiate P-ANCA/A-ANCA patterns from the staining shown by antinuclear antibody (ANA) with this type of fixation. Several laboratories have incorporated a protocol in which formalin-fixed neutrophils are used. Unfortunately, there is still controversy over the value of formalin fixation of neutrophils in differentiating autoantibodies. Recently, we have compared the IFT patterns of ethanol- and formalin-fixed neutrophil preparations, in
order to distinguish vasculitis-related P-ANCA/A-ANCA from ANA in the routine diagnosis of ANCA. We also sought to determine if the main target antigens of ANCA are destroyed by formalin fixation. The diagnostic performance of three commercial IFT ANCA kits for several ANCA subtypes has been evaluated. Serum from 100 patients with a variety of rheumatic and inflammatory diseases and 20 healthy controls was analysed. The 100 patients included 50 with systemic vasculitis (AAV and cryoglobulinaemic vasculitis), and 50 with rheumatoid arthritis with and without rheumatoid vasculitis, systemic lupus erythematosus, systemic sclerosis, inflammatory diseases that mimic vasculitis. Patients were selected according to their clinical diagnosis. Patients with ANCA-associated disease fulfilled the criteria of the Chapel Hill Consensus Definitions and at least two of four criteria of the American College of Rheumatology classification. The serum samples were examined by IFT using commercial ANCA kits (Binding Site, Heidelberg, Germany; Bio-Rad Laboratories, Hercules, CA; and Euroimmun, Lübeck, Germany) and one in-house assay, and for PR3-/MPO-ANCA specificity using ELISA and immunoblotting, as previously described [6]. ANA were detected on HEp-2 cells. The ANCA target antigens proteinase 3, myeloperoxidase, elastase, lactoferrin, bactericidal increasing protein (BPI) and cathepsin G were detected by a double-staining technique using specific monoclonal antibodies. The stained slides were investigated by confocal laser scanning microscopy.

Of 17 samples positive for C-ANCA, 15 were positive for PR3-ANCA, 1 for MPO-ANCA and 7 for ANA. All samples positive for C-ANCA showed the cytoplasmic pattern of formalin-fixed neutrophils by the in-house assay and the BioRad assay, and two samples were negative by the Binding Site and Euroimmun assays. All 23 serum samples positive for P-ANCA showed a perinuclear and/or nuclear staining of ethanol-fixed neutrophils, and 16 were positive for MPO-ANCA or PR3-ANCA and 13 were positive for ANA. Of the 23 samples, all converted to the cytoplasmic pattern on formalin fixation with the BioRad assay, 22 with the in-house assay and only 17 with both the Binding Site and Euroimmun assays. Of 55 serum samples positive for ANA, 41 showed positive nuclear/perinuclear staining with ethanol fixation, but 25 were positive for both ANA and ANCA. Of the 55 ANA-positive samples, 30 were exclusively positive for ANA and 16 showed a perinuclear/nuclear or speckled nuclear staining of ethanol-fixed neutrophils and 15 of these samples became negative on formalin fixation. The commercial kits showed more than 95% agreement but the fluorescence intensity varied between them.

Antibodies directed specifically against myeloperoxidase, BPI, cathepsin G, elastase and lactoferrin produced a perinuclear or nuclear staining of ethanol-fixed neutrophils. All antibodies converted to a cytoplasmic pattern on formalin fixation. Thus, formalin fixation prevented the redistribution of cytoplasmic molecules while retaining their reactivity. Only anti-PR3 antibodies induced a cytoplasmic pattern on neutrophils with both type of fixation (Figs. 1 and 2).

Thus, we demonstrated that the formalin fixation of neutrophils does adequately differentiate between ANA and P-ANCA/A-ANCA staining and can be used in the routine diagnostic laboratory. Surprisingly, all commercial IFT kits evaluated provided acceptably accurate results. Recently, Pollock et al. have shown that testing P-ANCA-positive serum on formalin-fixed cells is highly sensitive for confirming MPO-ANCA in active vasculitis, but less sensitive and specific in treated vasculitis and nonvasculitic disease [7]. This study showed some limitations of this technique, and we conclude that a multicentre comparative study is necessary.

A new assay type: the “anchor” ELISA for detection of ANCA

The published guidelines on ANCA testing demand that in the case of a positive IFT for ANCA, an ELISA test is obligatory as a minimum requirement [5]. However, many hospital/referral laboratories only use commercially available ELISA kits to detect ANCA. The performance of commercial direct ANCA ELISAs varies significantly and, in some cases, differs from that of IFT ANCA.
assays. There are significant differences in sensitivity, specificity and predictive value among available commercial ELISA kits [8]. Furthermore, conventional direct ELISAs using PR3 immobilized to the surface of the ELISA plate, show great variation in performance and often lack sensitivity (Fig. 3). For detection of PR3/MPO-ANCA, the three-dimensional structure of the antigen may be crucial for the recognition and binding of antibodies, and thus for the sensitivity of the assay. Capture ELISAs are superior in overall diagnostic performance to direct ELISAs [3]. However, the sensitivity of capture ELISAs may also be reduced by the capturing antibodies, which also may hide relevant epitopes.

In an attempt to preserve the native structure of the antigen and hence to increase the sensitivity of the solid-phase assay, a novel ELISA method, the so-called “anchor” ELISA, for the detection of PR3-ANCA has been developed. The anchor PR3-ANCA ELISA immobilizes PR3 via a bridging molecule to the plastic plate, thus preserving all epitopes for the binding of ANCA, and has been shown to be superior to direct ELISAs and capture ELISAs in a study testing for PR3-ANCA in patients with WG [9].

Recently, the diagnostic accuracy of PR3-ANCA detection by this assay in identifying patients with AAV has been further evaluated. Serum samples from 980 consecutive patients in one academic hospital were tested for the presence of ANCA by IFT and hsPR3-ANCA ELISA. The hsPR3-ANCA ELISA displayed a higher sensitivity and specificity than IFT (sensitivity 80.2% vs. 78%, specificity 98.1% vs. 91%). The positive predictive value of IFT was 0.73, and the positive predictive values for C-ANCA by IFT and hsPR3-ANCA by ELISA were 0.89 and 0.92, respectively. The positive likelihood ratio was highest for hsPR3-ANCA by ELISA (35), compared to C-ANCA by IFT (33) and IFT (8.6) [10].

These results show that this novel hsPR3-ANCA ELISA is a superior method for the detection of PR3-ANCA, and may be used as a screening test in patients with suspected systemic vasculitis in routine clinical practice, especially if WG is suspected. However, the initial screening of patients for ANCA with IFT is still considered necessary. As the novel generation of PR3-ANCA ELISAs does not screen for MPO-ANCA and the bead-based multiplex assays for the detection of both MPO- and PR3-ELISA at the same time need to be further evaluated, the initial screening of patients for ANCA with IFT with subsequent performance of an ELISA is still necessary.

**Alternative methods for ANCA detection**

In recent years, several methods have been published as alternatives to conventional methods for detecting ANCA by IFT and ELISA.

Image analysis is an automated alternative to conventional IFT. This technique quantifies the fluorescence intensity in a single dilution of a patient sample in comparison with the intensities of standard calibrators. In a cohort of consecutive WG patients positive for PR3-ANCA, the image analysis method detected ANCA levels in 75% of the patients at the time of renal relapse. These results suggest a lower diagnostic sensitivity as compared with IFT.
(100%) and the capture ELISA (100%) [11]. The value of ANCA measured by quantitative image analysis for predicting relapse is somewhat better than that of IFT, but comparable with that of direct ELISA [11]. However, multicentre comparative studies are lacking.

Recently introduced multiplex technology offers a unique opportunity to detect the presence of multiple autoantibodies at the same time and in the same sample. This new technology built on a synthesis of multiplex beads and flow cytometry technology represents the beginning of a new era in automated immunoassays. Trevisin et al. compared the performance of a flow cytometric immunoassay for PR3-ANCA and MPO-ANCA with IFT and ELISAs in active and treated vasculitis and inflammatory bowel disease [12]. The immunoassay specificity was 88% compared with 96% for IFT and 94% for both ELISAs. The PR3-ANCA and MPO-ANCA immunoassay was almost as sensitive as IFT, and more sensitive than, but just as specific as, most ELISAs, in detecting ANCA in active and treated vasculitis [12]. A major advantage of this assay is its ability to be further modified to simultaneously screen for a panel of autoantibodies relevant to vasculitis. However, rigorous prospective studies are urgently needed to further evaluate this new fully automated method for initial screening of patients suspected of having vasculitis.

Conclusion

In summary, the best means of determining vasculitis-associated ANCA is IFT in combination with a PR3-ANCA and MPO-ANCA ELISA. The newly developed methods for ANCA detection in AAV, which may replace the need for a combined analysis with IFT and ELISA in the future, should be urgently evaluated in multicentre studies.

The solution to problems in the diagnosis of ANCA is focusing on fundamentals, i.e. the correct implementation of IFT and ELISA, the cautious use of commercial assays, and the restriction of the use of the tests to clinical situations with a rather high pretest probability of AAV. Furthermore, the International Consensus Statement on Testing and Reporting of ANCA urgently needs to be revised, and training workshops aimed at improving ANCA tests should be conducted.

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