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L28. Relevance of detection techniques for ANCA testing

Introduction

Diagnosis of ANCA-associated vasculitis is based on clinical assessment supported by investigation results, with tissue biopsy as the gold standard and ANCA testing. To understand
the significance of ANCA, clinicians need to know about the methods used for their detection, associated problems and pitfalls. The problems and pitfalls associated with ANCA testing may be summarized as follows:

- 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;
- ANCA tests were developed in populations with high disease prevalence, but were often applied clinically in settings with low clinical suspicion of AAV;
- the development of novel detection technologies and automated platforms affect the international recommendations current validity and made it necessary to update the standardization process and to revise existing ANCA detection strategies;
- the usefulness of serial measurements for monitoring of patients is still debated.

This article reviews the role of ANCA tests in the diagnosis and management of AAV.

**ANCA and diagnosis**

**ANCA testing by indirect immunofluorescence technique**

Up to now, indirect immunofluorescence technique (IFT) has remained the standard method for the routine determination of ANCA in vasculitis, using ethanol-fixed human neutrophils as substrate. Subsequently, two main immunofluorescence patterns are distinguished, a cytoplasmic (C-ANCA) or a perinuclear (P-ANCA). Because antinuclear antibodies (ANA) can react with neutrophils and give false-positive ANCA results, and to avoid the artifactual P/A pattern, formalin-fixed neutrophils can be used.

Image analysis is an automated alternative to conventional IFT. This technique quantifies fluorescence in a single dilution of a patient sample in comparison with the intensity of standardized calibrators. This technique is less prone to observer error and has high diagnostic accuracy. Recently, computer-based image analysis of IIF patterns by pattern recognition algorithms has been successfully applied for automated analysis of ANCA by neutrophil-based assays. However, the evaluation of the image analysis for the potential to detect ANCA should be done in multicentre comparative studies.

Today, the diagnostic performance of IFT might be questioned given that the high sensitive PR3-/MPO-ANCA ELISAs are readily available, and a positive immunofluorescence ANCA result is less frequently associated with a diagnosis of systemic necrotising vasculitis than a positive PR3-/MPO-ANCA ELISA. Furthermore, the lack of expertise in ANCA pattern interpretation in even experienced laboratories is an ongoing issue. Recent studies found that the variation in reporting of ANCA IFT patterns was partial due to technical factors, but misinterpretation was more common. IFT is labour intensive and the automation is not yet worldwide available. A new strategy to detect ANCA must be considered that encompasses accuracy, reproducibility and cost-effectiveness.

**ANCA testing by antigen-specific assays**

The “International consensus statement on testing and reporting ANCA” requires all laboratories to screen for ANCA by IFT, and to confirm the specificity of fluorescent sera by ELISA. Almost all diagnostic testing laboratories screen for ANCA by IFT and confirm positive results in direct ELISAs for PR3- and/or MPO-ANCA. Today, the second-generation tests so called capture ELISAs and the third-generation ANCA assays (anchor ELISA, or high sensitivity) are superior in overall diagnostic performance compared to direct ELISA and even to IFT. Recently, the diagnostic accuracy of PR3-ANCA detection by this assay to identify patients with AAV has been further evaluated. Sera from 980 consecutive patients from one academic hospital were tested for the presence of ANCA by IFT and hsPR3-ANCA ELISA. This assay displayed a higher sensitivity and specificity compared to IFT (sensitivity: 80.2% vs. 78%, specificity: 98.1% vs. 91%). Based on published results concerning the diagnostic performance of the novel generation of ANCA assays, which may replace the need for a combined analysis with IFT and ELISA in the future, we propose that these assays could be the principal serological test for the diagnosis of AAV.

During the last years, novel immunometric ANCA assays such as fluorescent enzyme immunoassays, chemiluminescence technology and latex turbimetry have been developed. Furthermore, recently introduced bead-based multiplex technology offers a unique opportunity to detect the presence of multiple autoantibodies at the same time and in the same sample. Rapid screening ELISA, in which results are available in less than 60 minutes, and “near patient” testing assays that use whole blood are also available. These new technologies represent the beginning of a new era in ANCA automated immunoassays and future studies are needed to establish whether the assays could be used for routine ANCA screening.

**Role of ANCA in monitoring**

In general, there is agreement on the rough association of ANCA positivity and disease activity, while the usefulness of serial measurements for the monitoring of patients is still debated. Recently, a meta-analysis study examined two specific aspects of serial ANCA testing on future disease relapse: (a) the predictability of a rise in ANCA titres, and (b) the persistence of ANCA. They found that both a rise in and a persistence of ANCA during remission is only modestly predictive of future disease relapse and that there is limited use to serial ANCA measurements during disease remission to guide treatment decisions for individual AAV patients.

**Summary**

ANCA are a good serologic marker for AAV, especially for GPA and MPA. Today, the best diagnostic performance is obtained...
when indirect immunofluorescence test is combined with antigen-specific assay. The new developed more sensitive and specific methods for ANCA detection in AAV, which may replace the need for a combined analysis with IFT and ELISA in the future, should be evaluated in multicentre studies. ANCA testing can be improved by restricting the use of the tests to clinical situations with a rather high pretest probability for AAV, and carefully searching for conditions such as drug exposures and infections that are known to be associated with the occurrence of ANCA, whether the vasculitis is present or not. Therefore, a rational strategy for ANCA testing is needed and the international consensus statement on testing of ANCA should urgently be revised.

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Further reading

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L29. Relevance of anti-LAMP-2 in vasculitis: Why the controversy

Introduction
The description of autoantibodies to neutrophil cytoplasmic components antigens (ANCA) [1] and the identification of myeloperoxidase (MPO) and proteinase-3 (PR3) as their principal targets has [2,3] revolutionised understanding of small-vessel vasculitis. Assays for ANCA are central components of the diagnosis and led naturally to the term ANCA-associated vasculitis (AAV) to describe granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) [4]. Despite uniform treatment recommendations based on multinational prospective randomised controlled trials, relapses are common and mortality remains high [5], emphasising the need for therapies targeted to the underlying immunopathogenesis and disease activity.

Clinical and experimental studies provide overwhelming evidence for the involvement of autoantibodies to MPO and PR3 in the pathogenesis of AAV (reviewed in [6]). Despite this, there is compelling evidence that autoimmunity to MPO and PR3 is not the complete answer: ANCA and conventional assays for autoantibodies to MPO or PR3 are negative in at least 10% of patients with apparently identical disease. More importantly, neither the presence nor the titre of antibodies to MPO and PR3 correlate with disease activity during long term follow up [6] which suggests additional factors are needed for injury. The clinical data are supported by experimental studies. Thus the in vitro effects of antibodies to MPO and PR3 depend critically on the simultaneous use of TNF-α or LPS; and anti-MPO antibodies in rodent models similarly require additional triggers such as cytokines, LPS or binding of small amounts of anti-GBM antibodies to induce severe injury (reviewed in [7–9]).

These uncertainties together with the fact that neither MPO nor PR3 are expressed by endothelium prompted the original systematic search for autoantibodies to glomerular and neutrophil membrane proteins that led to the original discovery of autoantibodies to human lysosome associated membrane glycoprotein 2 (hLAMP-2) in AAV [10]. Our subsequent studies demonstrated a very high prevalence of antibodies to hLAMP-2 in those patients presenting with AAV and provided evidence for their pathogenicity [11,12] but the findings have been challenged [13], leaving the role of anti-hLAMP-2 antibodies controversial [14,15]. Here we summarise current data on antibodies to hLAMP-2 in AAV, highlighting areas of consensus and disagreement. Finally we consider the evidence for the pathogenicity derived from rodent studies.

Autoantibodies to hLAMP-2 and their measurement
LAMP-2 is a heavily glycosylated type 2 membrane protein with critical roles in lysosomal integrity and cellular homeostasis more generally (reviewed in [16]). It has three domains: an 11 amino acid cytoplasmic domain responsible for lysosomal targeting; a 24 amino acid transmembrane domain; and a heavily glycosylated 324 amino acid luminal (or extracellular) domain that is the invariable target for the patients’ autoantibodies. Carbohydrate moiety accounts for 50 to 75% of the molecular mass of the extracellular domain with the extent of glycosylation depending both on cell type and activation state.