Laboratory Diagnostics in Vasculitis Patients

Allan Wiik

Department of Autoimmunology, Statens Serum Institut, Copenhagen, Denmark

Key words: anti-neutrophil cytoplasm antibodies, vasculitis, autoantigens, autoimmune serology, drug-induced syndrome

Abstract

Anti-neutrophil cytoplasm antibodies are important markers of certain small vessel necrotizing vasculitides, but the optimal use of laboratory results in daily clinical practice necessitates collaboration between clinicians and laboratory specialists. Physicians must familiarize themselves with ANCA tests in ANCA-related vasculitides as well as in differential diagnostic patient populations in order to define cutoff values. Indirect immunofluorescence with a consensus-agreed technique combined with standardized enzyme immunoassays is the modality for detecting the main SSV-associated ANCA specificities using cutoff values that can sufficiently distinguish SVV from non-SVV patients. The combined use of IIF and direct EIA to demonstrate proteinase 3-ANCA and myeloperoxidase-ANCA at significant levels leads to a very high diagnostic specificity towards SVV conditions such as Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, and limited forms of these such as renal-limited focal necrotizing glomerulonephritis. A strong reactivity of ANCA against several azurophil granule components indicates a drug-induced syndrome. ANCA-related SVV and drug-induced vasculitis or lupus syndromes have characteristic ANCA profiles that can help distinguish these conditions from other inflammatory diseases.

IMAJ 2001;3:275–277

Systemic forms of small vessel vasculitides are rare and the organs or tissues involved at the onset of disease vary considerably from patient to patient. Consequently, the patient's initial visit may be to a general physician or to any of a number of subspecialists. A clinical suspicion of SVV should prompt the ordering of ANCA tests to help establish early diagnosis based on agreed disease criteria and histopathology. It is important to remember that characteristic vasculitis symptoms may be scarce at onset but that the systemic nature of the disease is mainly indicated by the presence of constitutional symptoms such as loss of appetite and weight loss, fatigue, fever, arthralgias/myalgias, etc. Even in the early stage of systemic SVV strongly expressed ANCA are usually found. The presence of an increased C-reactive protein level also supports the diagnosis of systemic SVV.

Patients with Wegener's granulomatosis experience the initial disease symptoms mainly in the upper airways, ears or sinuses and are commonly referred to an otolaryngologist, whereas patients with microscopic polyangiitis frequently exhibit their disease onset in the lungs or kidneys and are seen by a pulmonologist or nephrologist. The diagnosis of SVV is ideally obtained by collaboration between several clinical specialists in a vasculitis team. Until now not enough emphasis has been placed on the requisite collaboration between clinical vasculitis specialists and serologists in preparation for the knowledge-based differential diagnosis of ANCA. This is the focus of the present review.

Laboratory testing for ANCA

Most laboratories around the world start out by screening for ANCA using IIF with commercial or in-house prepared ethanol-fixed human leukocytes as cellular substrate. The detailed methodology has been published elsewhere [1]. ANCA may be found in the form of either a granular neutrophil and monocyte fluorescence scattered throughout the whole cytoplasm, the so-called cytoplasmic ANCA, or a perinuclear/nuclear fluorescence in these cells, called perinuclear ANCA. In both cases lymphocytes in the preparation should be negative or differently stained. If a mixed or otherwise less distinct ANCA pattern is found the serum is likely to derive from a non-vasculitic patient [2].

In all cases where IIF is positive, the sera must be studied further using EIA to detect ANCA directed to proteinase 3 and myeloperoxidase [2]. Commercial assays with purified PR3 and MPO directly coated onto the wells of high binding microtiter plates are used in most routine serology settings. Several methods have been reported on the purification of PR3 [3] and MPO [4] from human sources, thus making in-house EIA testing feasible, inexpensive and standardizable [5]. Estimating the content of IIF ANCA is usually done by grading the strength of neutrophil staining into weak, intermediate and strong positive reactions, but occasionally titration is also used. EIA results are mostly related to in-house standards that have been designated a certain unitage/ml. International serum standards for PR3-ANCA and MPO-ANCA will be made

ANCA = anti-neutrophil cytoplasm antibodies
SSV = small vessel vasculitides
HF = indirect immunofluorescence
EIA = enzyme immunoassay

PR3 = proteinase 3
MPO = myeloperoxidase

available once candidate sera are selected in a multicenter study involving expert laboratories.

The European multicenter study on ANCA assay standardization demonstrated that the IIF technique agreed upon in 1988 gave center-to-center reproducible results regarding categorization of sera into negative C-ANCA and P-ANCA positive sera, whereas titers could not be compared [5,6]. EIA using direct EIA techniques were clearly superior to assays using autoantigen mixtures [7]. PR3 prepared by quite different purification procedures worked equally well in the direct EIA modifications prescribed by each supplier of PR3 [5]. It was ultimately shown that the direct EIA techniques for PR3-ANCA and MPO-ANCA could be standardized in a multicenter trial involving experienced as well as inexperienced laboratories [6]. Detailed direct EIA methodology has been described elsewhere [8].

Certain laboratories have used a mouse monoclonal anti-PR3 or anti-MPO antibody to capture PR3 and MPO respectively, on the microplate, from crude extracts of azurophilic granules [9], or anti-PR3 monoclonal capture of PR3 from native purified PR3 preparations [10]. Although an increased nosographic sensitivity may be reached with the capture EIA for PR3-ANCA detection in kidney patients [10], larger studies are needed to clarify whether a satisfactory diagnostic specificity can be attained in other patient materials. Also, comparison must be made between different monoclonal antibodies for the capture of PR3 [11].

Radioactively labelled neutrophil extracts were used early on for immunoprecipitation and identification of autoantigens involved in ANCA reactivity [12]. Such methods, however, are not suitable for routine serology. Solid-phase radioimmunoassay on neutrophil extracts was also used in early studies [13] but was never widely practiced. A more recent immunoprecipitation technique utilizes ^{3}H-difluorophosphate attached to PR3 as tracer in the immunoprecipitate [14]. This technique will also bring down labelled leucocyte elastase, if elastase-ANCA is present in the serum, due to the difluorophosphate-binding property of elastase. This technique is a “mixed bag” – it has the advantage that a whole-cell extract from neutrophils with added tracer can be used without isolation of antigen, but the disadvantage that a subsequent electrophoretic separation of the immunoprecipitate needs to be done to clearly identify which serine protease (PR3 or elastase) the IgG recognized [14]. Moreover, the sensitivity of this immunoprecipitation technique may be superior to that of direct EIA, but, again, larger studies are needed to evaluate its clinical utility.

Differential diagnostics of ANCA

In clinical diagnostics a certain disease entity can be recognized in conditions with similar or overlapping features only if the characteristics of differential diagnostic diseases are known. This pertains to both the setting and the strength of expression of its cardinal features. Similar rules apply in the differential diagnostics of ANCA. In order to be able to distinguish SVV-related ANCA from those commonly found in rheumatoid arthritis, ulcerative colitis, sclerosing cholangitis and chronic hepatitis, SVV sera must be compared with sera of these diseases. This must be done locally, since ethnic differences, together with the background influence of infections, irradiation, foods, toxic agents, allergies etc. vary in different geographic areas. Moreover, relevant disease controls differ from clinic to clinic due to different referral patterns and clinical subspecialties. Serum banks containing a representative number of vasculitis sera as well as a locally agreed spectrum of differential diagnostic sera from firmly diagnosed patients must be tested by the ANCA tests locally, irrespective of whether testing is done by in-house methodology or commercial assays. Following the manufacturer’s instructions and using the manufacturer’s cutoff values of positivity merely makes it possible to distinguish disease-related ANCA from those of healthy controls. In the clinical setting this competes with the ability to distinguish a sick WG patient from the healthy visitors in the clinic.

In the first study of C-ANCA in WG patients conducted [15], the disease controls comprised various granulomatous diseases, connective tissue diseases and infections, and the selected IIF cutoff values could clearly distinguish WG sera from disease control sera. In the European multicenter study [6], a number of inflammatory connective tissue diseases and secondary vasculitides were used as disease controls. Use of IIF alone did not satisfactorily differentiate SVV from non-SVV sera at the cutoff chosen. If PR3-ANCA and MPO-ANCA were called positive when cutoff had been set at a level selected from receiver operation curves to represent 90% specificity towards disease controls, a reasonable but incomplete separation of SVV from non-SVV patients could be attained. However, when a positive C-ANCA at dilution 1:20 was found together with a positive PR3-ANCA as defined above, this combination was 99% specific for SVV. Similarly, the combination of a positive P-ANCA and positive MPO-ANCA as defined above showed 99% specificity for SVV. There were no differences between newly diagnosed and earlier diagnosed patients [6].

A good example of clinically defined ANCA positivity is represented by the study of Westman et al. [10], who used various glomerulonephritides as controls towards focal necrotizing glomerulonephritis to disclose the diagnostic potential of the capture EIA principle for PR3-ANCA detection. While some disease controls were positive as expected, it was possible to distinguish between clinically and histopathologically defined vasculitic glomerulonephritis from other glomerulonephritides. With such narrowly selected controls, the question of course remains whether some PR3-ANCA-positive control patients were in reality patients who would later develop vasculitic glomerulonephritis.

It is important to remember that both vasculitic and lupus-like syndromes may develop in patients treated long term with certain drugs, and that these patients usually become strongly ANCA positive by both IIF and EIA [16–18]. Nowadays

C-ANCA = cytoplasmic ANCA
P-ANCA = perinuclear ANCA

propylthiouracil, penicillamine, sulfasalazine, tetracyclins and anti-tuberculous drugs are used as provoking agents more frequently than procainamide and hydralazine, which are scarcely used in most countries. Many other drugs, however, can provoke similar syndromes. Patients commonly develop strongly positive P-ANCA that simultaneously target a number of different azurophil granule constituents, most frequently MPO, PR3 and elastase [16,18]. Such multi-target specificity is almost never seen in patients with primary ANCA-associated SVV. P-ANCA or atypical ANCA with weak reactivity towards a number of different neutrophil antigens are very common in long-standing inflammatory diseases characterized histologically by neutrophil and monocyte invasion at sites of involvement [19–21]. IIF ANCA-positive sera in most laboratories derive from such inflammatory disease patients and not from SVV patients. This is the primary reason to recommend that positive IIF ANCA results alone not be reported to clinicians, but rather to await EIA results – which will decisively indicate whether or not SVV is present [2].

It is possible that recombinant proteins in the future may become useful for the detection of ANCA [22–24].

Conclusion

In summary, the optimal information on the clinical utility of ANCA requires in-depth knowledge on the way positivity is defined, and this definition must be based on data obtained in differential diagnostic diseases relative to those in SVV.

References

1. Wiik A, Rasmussen N, Wieslander J. Methods to detect autoantibodies to neutrophilic granulocytes. In: van Venrooij WJ, Maini RN, eds. Manual of Biological Markers of Disease. The Netherlands: Kluwer Academic Publishers, 1993:1–14.
2. Savige J, Gillis D, Benson E, Davies D, Esnault V, Falk RJ, Hagen EC, Javne D, Jennette JC, Paspaliaris B, Pollock W, Pusey C, Savage CO, Silvestrini R, van der Woude F, Wieslander J, Wiik A. International consensus statement on testing and reporting of antineutrophil cytoplasmic antibodies (ANCA). International group for consensus statement on testing and reporting of antineutrophil cytoplasmic antibodies. *Am J Clin Pathol* 1999;111:507–13.
3. Stummann L, Wiik A. A high yield procedure for purification of proteinase 3: the autoantigen in Wegener's granulomatosis. *J Immunol Methods* 1997;206:35–42.
4. Olsson I, Olofsson T, Odeberg H. Myeloperoxidase-mediated iodination in granulocytes. *Scand J Haematol* 1972;9:483–91.
5. Hagen EC, Andrassy K, Csernok E, Daha MR, Gaskin G, Groos WL, Hansen B, Heigl Z, Hermans J, Javne D, Kallenberg CGM, Lesavre Ph, Lockwood CM, Lüdemann J, Mascart-Lemone F, Mira[eix E, Pusey CD, Rasmussen N, Sinico RA, Tzioufas A, Wieslander J, Wiik A, van der Woude FJ. Development of solid phase assays for the detection of anti-neutrophil cytoplasmic antibodies (ANCA). A report on the second phase of an international cooperative study on the standardization of ANCA assays. *J Immunol Methods* 1996;196:1–15.
6. Hagen EC, Daha M, Hermans J, Andrassy K, Csernok E, Gaskin G, Lesavre P, Lüdemann J, Rasmussen N, Sinico RA, Wiik A, van der Woude FJ. The diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies (ANCA) in idiopathic systemic vasculitis. Results of an international collaborative study. *Kidney Int* 1998;53:743–53.
7. Hagen EC, Andrassy K, Chernok E, Daha MR, Gaskin G, Gross W, Lüdemann J, Pusey CD, Rasmussen N, Savage COS, Sinico A, Wiik A, van der Woude F. The value of indirect immuno-fluorescence and solid phase techniques for ANCA detection. A report on the first phase of an international cooperative study on the standardization of ANCA assays. *J Immunol Methods* 1993;159:1–16.
8. Wiik A. Antineutrophil cytoplasmic antibodies. Manual of Clinical Laboratory Immunology. Washington DC: ASM Press, 1997:954–9.
9. Cohen Tervaert JW, Goldschmeding R, Elema JD, van der GM, Huitema MG, van der Hem GK, The T.H., dem Borne AE. Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. *Kidney Int* 1990;37:799–806.
10. Westman KWA, Selga D, Bygren P, Sagelmark M, Baslund B, Wiik A, Wieslander J. Clinical evaluation of a capture ELISA for detection of proteinase-3 antineutrophil cytoplasmic antibody. *Kidney Int* 1998;53:1230–6.
11. Sommarin Y, Rasmussen N, Wieslander J. Characterization of monoclonal antibodies to proteinase-3 and application in the study of epitopes for classical anti-neutrophil cytoplasm antibodies. *Exp Nephrol* 1995;3:249–56.
12. Goldschmeding R, Cohen Tervaert JW, van der Schoot CE, van der Veen C, Kallenberg CGM, von dem Borne AEGK. ANCA, antimyeloperoxidase, and anti-elastase: three members of a novel class of autoantibodies against myeloid lysosomal enzymes. *APMIS* 1989; 97(Suppl.6):48–9.
13. Savage COS, Winearls CG, Jones S, Marshall PD, Lockwood CM. Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. *Lancet* 1987;i:1389–93.
14. Lucena-Fernandes F, Dalpe G, Dagenais P, Richard C, Calvert R, Boire G, Menard HA. Detection of anti-neutrophil cytoplasmic antibodies by immunoprecipitation. *Clin Invest Med* 1995;18:153–62.
15. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985;i:425–9.
16. Dolman KM, Gans ROB, Vervaadt ThJ, Zevenbergen G, Maingay D, Nikkels RE, Onker AJ, dem Borne AE, Goldschmeding R. Vasculitis and anti-neutrophil cytoplasmic autoantibodies associated with propylthiouracil therapy. *Lancet* 1993;342:651–2.
17. Nässberger L, Sjöholm AG, Jonsson H, Sturfelt G, Akesson A. Autoantibodies against neutrophil cytoplasmic compounds in systemic lupus erythematosus and in hydralazine-induced lupus. *Clin Exp Immunol* 1990;81:380–3.
18. Choi HK, Merkel PA, Walker AM, Niles JL. Drug-associated antineutrophil cytoplasmic antibody-positive vasculitis: prevalence among patients with high titers of antimyeloperoxidase antibodies. *Arthritis Rheum* 2000;43:405–13.
19. Brimnes J, Halberg P, Wiik A, Heegaard NHH. Specificities of anti-neutrophil autoantibodies in patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 1997;110:250–6.
20. Brimnes J, Nielsen OH, Wiik A, Heegaard NHH. Molecular targets of autoantibodies to neutrophils in patients with ulcerative colitis. *Dig Dis Sci* 1999;44:415–23.
21. Wiik A, Brimnes J, Heegaard NHH. Distinct differences in autoantigen specificity of anti-neutrophil cytoplasm antibodies in systemic vasculitides and other inflammatory diseases. *IMAJ* 1999;1:4–7.
22. Specks U, Fass DN, Fautsch MP, Hummel AM, Viss MA. Recombinant human proteianse 3, the Wegener's autoantigen, expressed in HMC-1 cells is enzymatically active and recognized by c-ANCA. *FEBS Lett* 1996;390:265–70.
23. Sun J, Fass DN, Viss MA, Hummel AM, Tang H, Homburger HA, Specks U. A proportion of proteinase 3 (PR3)-specific anti-neutrophil cytoplasmic antibodies (ANCA) only react with PR3 after cleavage of its N-terminal activation dipeptide. *Clin Exp Immunol* 1998;114:320–6.
24. Short AK, Lockwood CM, Bollen A, Moguilevski N. Neutrophil and recombinant myeloperoxidase as antigens in ANCA positive systemic vasculitis. *Clin Exp Immunol* 1995;102:106–11.

Correspondence: Dr. A. Wiik, Dept. of Autoimmunology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. Phone: (45) 3268-3362, Fax: (45) 3268-3876, email: aw@ssi.dk