Review
Autoantibodies in vasculitis
Allan Wiik

Department of Autoimmunology, Statens Serum Institut, Copenhagen, Denmark

Corresponding author: Allan Wiik (e-mail: aw@ssi.dk)

Abstract

Before the mid-1980s the only autoantibody widely used to assist in diagnosing vasculitic disease was IgG antibody to the α_3 domain of the noncollagenous part of type IV collagen (anti-glomerular basement membrane). Since that time, antineutrophil cytoplasmic antibodies (ANCAs) directed at the azurophilic granule proteins proteinase-3 and myeloperoxidase have been established as clinically useful autoantibodies to support a diagnosis of Wegener's granulomatosis, microscopic polyangiitis, Churg–Strauss syndrome and limited forms of these primary, small vessel necrotizing and often granulomatous vasculitides. The establishment of standardized methods for identifying those antibodies was needed before they could be used in clinical practice. The levels of both types of ANCAs tend to increase in parallel with the degree of clinical disease activity, and they decrease with successful immunosuppressive therapy. More than one assay may have to be used to discover imminent exacerbations in proteinase-3-ANCA associated syndromes. Although autoantibodies to endothelial cells may be important players in the pathogenesis of several vasculitic conditions, they have not gained clinical popularity because of lack of standardized detection methods.

Keywords: antiendothelial antibodies, antineutrophil cytoplasm antibodies, prognostics, vasculitis

Introduction
'Vasculitis' is the term used for inflammatory diseases that involve blood vessel walls and the surrounding interstitium. Vasculitis may affect large, medium and small sized arteries, arterioles, capillaries, venules and veins [1]. Only the vasculitides that involve arterioles, capillaries and venules give rise to production of autoantibodies, which can serve as practical surrogate markers of the condition in question. Hence, the primary focus of the present review is on autoantibodies that are characteristically found in small vessel vasculitides [2].

The lack of a unified terminology and classification for the various vasculitic conditions in the 1980s led to several attempts to set up a clinically applicable nomenclature. The American College of Rheumatology proposed classification criteria for some primary vasculitides and advocated the use of somewhat complicated diagnostic algorithms for establishing a precise diagnosis for each condition [3], but one clinically important vasculitic condition, namely microscopic polyangiitis [4], had been left out. A simplified nomenclature for primary vasculitic conditions, each one defined by their most common clinical, histological and immunological characteristics, was proposed by an *ad hoc* expert committee in 1994 [1]. This so-called Chapel Hill terminology has been widely accepted because of its simplicity and ease of use in clinical work up and is therefore used in this review.

The antineutrophil cytoplasmic antibodies (ANCAs) directed at proteinase-3 (PR3) and myeloperoxidase (MPO) were discussed at length at the Chapel Hill Conference. That they are very characteristic markers of systemic Wegener's granulomatosis (WG), Churg–Strauss syndrome and microscopic polyangiitis (as well as limited forms of these conditions, such as renal-limited necrotizing and crescen-

tic glomerulonephritis [NCGN]) was recognized, but it was not proposed that they be included in the criteria for these conditions.

The varying presentations of small vessel vasculitis (SVV) necessitate close collaboration between many different specialities in clinical medicine, as well as pathology and immunology experts, if an early and precise diagnosis is to be established that can serve as a rational basis for clinical decisions (e.g. prognosis estimation, follow-up strategy and therapy) [5]. What may appear to be a case of ANCA-positive WG limited to the upper airways may turn out to be a more systemic disease when the vasculitis team becomes involved. Finding ANCAs in the serum of a patient with seemingly skin-limited vasculitis can indicate the presence of systemic SVV, and therefore the vasculitis team should be involved to determine the disease extent and help to locate sites that should be biopsied to confirm the diagnosis. In contrast, generalized WG with severe central nervous system manifestations may be consistently negative for ANCAs [6]. The histopathological diagnosis rests on the presence of necrotizing vasculitis involving capillaries and venules but sometimes arterioles also, whereas larger arteries are less frequently involved [2]. In WG and Churg–Strauss syndrome perivascular granulomatous lesions with giant cells can be found in upper airway biopsies and bronchi, but often the picture is dominated by nonspecific inflammatory infiltrates [7]. Lesions in the kidneys are typically those of focal NCGN without immune deposits [8], sometimes termed pauci-immune NCGN.

Antineutrophil cytoplasmic antibodies

The first description of ANCAs came from Australia, where patients suffering from NCGN were found to harbour antibodies that reacted with neutrophilic granulocyte cytoplasm by indirect immunofluorescence (IIF) using ethanol-fixed human leucocytes as substrate [9]. These findings were corroborated by a Dutch–Danish study of patients with WG, most of whom had active systemic disease, including nephritis [10]. The classical granular cytoplasmic ANCA (C-ANCA) pattern [11] was found to be caused by ANCAs directed against PR3 [12], and high titers of C-ANCA were confirmed to be associated with active systemic WG [13]. A majority of patients with pauci-immune NCGN without a diagnosis of WG, however, produced ANCAs that selectively stained the perinuclear area of neutrophils and monocytes (P-ANCA) [11], and these antibodies were found to be predominantly directed against MPO [14]. Furthermore, in these patients, the levels of MPO-ANCA were dependent on disease activity, with high levels being found in active phases of the vasculitic disease [15].

These findings led to great interest in the practical use of PR3-ANCA and MPO-ANCA as tools for diagnosing and monitoring patients with SVV, but also gave rise to the elaboration of various methods to demonstrate and quantify these antibodies. It soon became apparent that different methodologies led to very different results, and therefore the First International Workshop on ANCAs was established in Copenhagen in 1988. It was agreed to identify one technique to be recommended for use when sera are screened for ANCA using IIF [16]. Follow-up studies revealed a clearly improved recognition of SVV-associated ANCAs and reproducible classification of the two IIF ANCA patterns, but not improved evaluation of titers [17]. However, PR3-ANCA and MPO-ANCA determination was inconsistent and a European multicenter project – the EC/BCR ANCA assay standardization study, supported by the European Commission – was initiated to achieve better standardization. At the end of this project it was concluded that enzyme-linked immunosorbent assay (ELISA) methods using purified native PR3 and MPO, directly coated onto microwells, had been standardized, and it was confirmed that these methods were useful in differentiating patients with recent onset SVV from those with other systemic inflammatory diseases. They also worked well in patients with a previously established diagnosis of WG, Churg–Strauss syndrome or microscopic polyangiitis [18]. It was a major step forward, and receiver operating curves were used to set cutoff values for the PR3-ANCA and MPO-ANCA ELISAs in order to attain satisfactory differentiation from inflammatory disease control patients [18].

Another important conclusion of this study was that ANCAs that are associated with SVV should only be reported as positive if both the IIF test and the direct ELISA for PR3-ANCA or MPO-ANCA are clearly positive. The importance of this combined ANCA testing approach was widely confirmed subsequently [19]. The background for this is the common presence of IIF-ANCA in many chronic inflammatory diseases (e.g. rheumatoid arthritis, Felty's syndrome, systemic lupus erythematosus, ulcerative colitis, chronic active hepatitis, primary sclerosing cholangitis, systemic HIV infection, active tuberculosis, cystic fibrosis, Sweet's syndrome and subacute bacterial endocarditis, among others) [20,21]. Most of these conditions do not result in diagnostically important production of PR3-ANCA or MPO-ANCA. The autoimmune response to neutrophils in these conditions can be regarded as an over-expanded innate immune response to neutrophils that are constantly being recruited to inflammatory sites, the antibodies probably playing a role in the active removal of neutrophil debris. Multiple neutrophil autoantigens both from the cytosolic, granule and nuclear compartments are targeted by such autoantibodies, which for clinical clarity's sake should be termed 'neutrophil-specific autoantibodies' (NSAs) and not ANCAs [22]. If it is locally preferred to retain the term ANCA for such antibodies, then laboratory reports should clearly state that a positive IIF-ANCA that is

combined with negative MPO-ANCA or PR3-ANCA ELISA results is atypical for a patient with SVV [23].

Immunoglobulin classes of antineutrophil cytoplasmic antibodies

Early studies indicated that WG-associated ANCAs determined using IIF mainly belonged to the IgG class [24]. IgM class ANCAs have been found in some patients with haemorrhagic renopulmonary capillaritis [25], but determination of IgM ANCAs has not become routine in most immunology laboratories. IgA class ANCAs have been reported in some patients with SVV, but these findings are controversial and IgA ANCA testing has never come into use.

Technical issues in testing for antineutrophil cytoplasmic antibodies

The basic concept underlying ANCA detection using IIF is to allow autoantibodies to react with conformationally preserved intracellular antigens in all compartments of the neutrophil and monocyte, cells that have many biological and functional properties in common and share similar antigens. To gain access to the interior of the cells and make them stay on the slide, some form of permeabilization and fixation is needed. Ethanol and acetone have very similar permeabilization and fixation properties and have both been used, but ethanol is the recommended reagent for this purpose [16]. The advantage of using whole buffy coat cells instead of isolated neutrophils is that lymphocytes and eosinophils can be useful as control cells. The former may be used to detect the presence of non-organ-specific autoantibodies that react with nuclei (antinuclear antibodies [ANAs]) or cytoplasm (anticytoplasmic antibodies), and the latter may be used as controls for ANCAs because neither lymphocytes nor eosinophils should react in the presence of SVV-associated ANCAs.

It is distressing that most commercial slide preparations for IIF ANCA detection are covered by purified neutrophils so that the internal control cells are missing, and accordingly other tests must be conducted to exclude ANAs and anticytoplasmic antibodies. P-ANCAs and ANAs are sometimes found in the same serum, but direct comparison of their titers by serum dilution is impossible if lymphocytes are absent. The use of HEp-2 cells for estimating ANA levels, commonly set at a cutoff of 1:160, cannot be compared with ANCA levels, which are judged to be positive at lower dilutions (e.g. a cutoff of 1:20) [26]. This problem has been very prominent in studies of ANCAs in patients with systemic lupus erythematosus, who regularly harbour antichromatin antibodies. These are seen as a homogeneous or peripheral staining of neutrophil, lymphocyte and eosinophil nuclei on whole buffy coat smears [16].

The P-ANCA pattern is the result of IgG antibodies reacting with cationic granule proteins (especially MPO, but also lysozyme, cathepsin G, lactoferrin, elastase and azurocidin) that have redistributed from their site of origin in the granules onto the nucleus by ionic attraction to the anionic nucleus upon ethanol fixation [14]. If cells are simultaneously treated by a cross-linking agent such as formalin before execution of the IIF test, then all antigens will stay *in situ* and MPO-ANCAs give rise to a classical C-ANCA reaction [14]. This technique works well for MPO-ANCAs but not for many other NSAs [26]. No consistent studies have been done to show the impact of formalin fixation of neutrophils on demonstration of various specificities of ANCAs and ANAs, but formalin treatment commonly destroys the reactivity of a sizeable number of NSAs with neutrophils [26,27]. NSAs that give rise to atypical C-ANCA and P-ANCA patterns in the standard IIF technique most likely represent a summation of reactivities with multiple neutrophil antigens [23].

Other methods used to determine antineutrophil cytoplasmic antibodies

There is clearly an agreement between investigators that IIF levels judged by titration are not paralleled by the levels found with use of ELISA methods. In addition, some sera from well-characterized patients with WG have been shown to have classically positive C-ANCA and persistently negative PR3-ANCA using direct ELISA. It has been hypothesized that the discrepancies may be explained by loss of conformational epitopes on the purified PR3 antigen. However, the fact that similarly purified PR3 is reactive with such sera if binding to the solid phase is mediated by a particular anti-PR3 mouse monoclonal antibody makes it more likely that at least one epitope on the PR3 molecule is hidden upon adsorption to the plastic surface [28]. This modification of the ELISA technique has been named 'capture' ELISA. The advantage of this technique for detecting PR3-ANCAs has been an increased nosographic sensitivity in patients with SVV, but positive reactions are also detected in cases where the disease relapses and the direct ELISA can become negative [28,29]. Even with the use of optimally expressed recombinant PR3 protein [30], the use of the capture principle appears to confer increased reactivity and allows detection of PR3-ANCA as well as PR3-ANCA complexed to its antigen [31]. Therefore, these observations may result from a shift in autoantibody epitope target, better conformation of the PR3 molecule upon monoclonal antibody presentation, and a widened reactivity by detection of immune complexes of PR3/PR3-ANCA. Recent studies suggest that PR3-ANCAs reacting with the proform of PR3 may reflect disease activity better than those directed at mature PR3 [32].

Another principle is to use conformationally preserved radiolabelled PR3 of a crude neutrophil extract as antigen in a precipitation assay [33], but this has not been used widely.

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Other antineutrophil cytoplasmic antibody autoantigens

Apart from the major autoantigens MPO and PR3, which are both located in the azurophilic granules of nonactivated neutrophils and monocytes, there are a few other antigens that can be targeted in SVV. Human leucocyte elastase has been found as a target in some patients [34,35], but at least some of these patients most probably have antibodies to a multitude of neutrophil granule antigens as part of a drug-induced syndrome that may present just like a primary SVV or a lupus-like syndrome [34,36]. The importance of discovering such a condition is that withdrawal of the offending drug mostly leads to remission of clinical symptoms and disappearance of the ANCAs.

Another rare but important antibody specificity is ANCAs directed at bactericidal/permeability-increasing protein, which are common in patients with cystic fibrosis and *Pseudomonas* infections [37] and in patients with other chronic airway infections [38], both conditions in which a secondary vasculitis may develop. ANCAs directed at bactericidal/permeability-increasing protein have also been found in patients with inflammatory bowel disease and primary sclerosing cholangitis, which may reflect an immune response to intestinal bacteria permeating through a leaking intestinal wall. IIF-ANCA may be negative in some of these patients although specific ELISA is positive. These patients must be identified in order to avoid risky treatment with immunosuppressive agents and be treated rationally with antibiotics.

Prognostic use of antineutrophil cytoplasm antibodies

Clinical studies of patients with ANCA-associated SVV have indicated that patients with PR3-ANCAs have a slightly different disease phenotype than those who have MPO-ANCAs [39]. PR3-ANCA-positive patients tend to have more upper airway, nose and ear disease, and perivascular granulomas in biopsy material, whereas MPO-ANCA-positive patients are older, have more peripheral nerve, lung and kidney involvement (rapidly progressive glomerulonephritis), and fewer granulomas. The mortality of patients with microscopic polyangiitis was clearly higher in patients positive for C-ANCA/PR3-ANCA than in those positive for P-ANCA/MPO-ANCA [40], which may relate to the particular predisposition of the former group to have disease relapses [39]. PR3-ANCAs indicate a worse prognosis for kidney function than do MPO-ANCAs [40]. It is important to appreciate that patients with microscopic polyangiitis are essentially different from patients with classical polyarteritis nodosa [1,41] as the latter do not produce ANCAs, have artery involvement only with no SSV, and they can often be treated less vigorously.

Some studies indicated that a disease flare in a PR3-ANCA associated vasculitis patient is preceded by a rise in PR3-ANCA levels [42,43] but this may only be seen in about half of the patients [44]. As stated above, the capture technique may be advantageous for using PR3-ANCAs as a predictor of and surrogate marker for a vasculitis flare [29,31,45]. Neither direct nor capture technique for MPO-ANCA quantification have been shown to be superior with regard to nosographic sensitivity and relationship to disease activity in SVV.

Other autoantibodies

Most patients who present with the clinical picture of a haemorrhagic renopulmonary syndrome (previously called Goodpasture's syndrome) have ANCAs in their serum (about 80%) [46,47], which are sometimes accompanied by anti-glomerular basement membrane (GBM) antibodies [46,48]. The rest of these patients have anti-GBM only and therefore have the classical Goodpasture's syndrome, and these patients are younger than ANCA-associated SVV patients [46]. Patients who coexpress ANCAs and anti-GBM antibodies are older and follow a disease course that is not different from that of ANCA-associated SVV [49]. Anti-GBM antibodies are directed at the noncollagenous α_3 domain of type IV collagen, which is selectively expressed in the GBM and lung basement membrane. They are quite distinct from coexpressed ANCAs because they can be differentially removed from serum by absorption. It is possible that MPO-ANCAs may aggravate the vasculitic process in patients with mild anti-GBM disease, as has been shown experimentally in rats [50], but there is no agreement as to whether anti-GBM antibodies or ANCAs come first.

For many years it has been known that antiendothelial cell antibodies (AECAs) are found in many patients with SVV [51,52]. These are independent from ANCAs with regard to antigen recognition [53,54], but they may be implicated in the pathogenesis of ANCA-associated SVV [54]. AECA and ANCA levels often fluctuate in parallel during disease relapses and remissions, and it has been suggested that AECAs may even be better predictors of relapses than ANCAs [52]. A major drawback is the lack of standardization of AECA assays, which limits their clinical use.

Autoantibodies involved in the pathogenesis of small vessel vasculitis

Many excellent studies and reviews have appeared in the literature on this topic [55–58]. The involvement of the various autoantibodies implicated in the pathogenesis of primary SVV is not dealt with in the present review. The recent advancement of an experimental model closely mimicking human MPO-ANCA associated vasculitis is a major step forward for gaining insight into key pathogenetic mechanisms and for testing new potential therapies [59].

Conclusion

The importance of ANCAs and anti-GBM antibodies as tools for diagnosing, prognosticating and monitoring

patients with primary SVV is unique in the field of systemic autoimmune disease. If an early diagnosis of SVV is to be established, a team of medical and laboratory specialists must collaborate with the aim of limiting vital organ damage through rational immunomodulating therapy. Complex interactions between autoantibodies, endothelial cells and inflammatory cells, including neutrophils, are likely to act in concert with immune cells in the pathophysiology of SVV and in the perpetuated production of the autoantibodies that are characteristically found in SVV patients. The efficacy of treatment is often mirrored by a decrease in or disappearance of autoantibodies.

Competing interests

None declared.

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Correspondence

Allan Wiik, MD, DSc, Department of Autoimmunology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. Tel: +45 3268 3546; fax: +45 3268 3876; e-mail: aw@ssi.dk