Full Review

Pathogenesis of anti-neutrophil cytoplasmic antibody-associated vasculitis: challenges and solutions 2014

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ABSTRACT

Anti-neutrophil cytoplasmic autoantibodies (ANCA) with specificity for proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA) are a defining feature of ANCA-associated vasculitides (AAV). They play a pivotal role in disease pathophysiology and have strongly improved early diagnosis and treatment of these infrequent, but potentially fatal diseases. Neutrophils and their products are major players in initiating the autoimmune response and tissue destruction in vasculitic as well as granulomatous inflammation. This review highlights recent findings on old and novel players (ANCA, neutrophils, neutrophil extracellular traps, fibroblasts, immune cells and complement) and puts them into context with the current understanding of disease mechanisms in AAV.

Keywords: ANCA, NETs, pathogenesis, vasculitis

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) encompass granulomatosis with polyangiitis (GPA, formerly known as Wegener’s granulomatosis), microscopic polyangiitis (MPA) and eosinophilic GPA (EGPA, formerly known Churg–Strauss syndrome) [1]. They are characterized by necrotizing small-vessel vasculitis and glomerulonephritis with absence or paucity of immunoglobulin deposits, together with neutrophil (polymorphonuclear leukocytes, PMNs) or eosinophil-enriched granulomatous inflammation, in GPA and EGPA, respectively, and the presence of circulating ANCA.

ANCA was first detected by indirect immunofluorescence technique (IFT) in undifferentiated vasculitis. Then, in two small GPA cohorts a distinct fluorescence pattern, later called C-ANCA, was shown to be associated with GPA [2, 3]. A second major pattern, now called P-ANCA, has been associated with MPA [4] and to EGPA. Using IFT and more sophisticated techniques, the denominating autoantibodies reacting with the distinct molecular structures within the PMNs, proteinase 3 (PR3) and myeloperoxidase (MPO) were described and have allowed a more molecular approach to define AAV subgroups.

Recent studies in GPA and MPA have given evidence for a genetic contribution to disease susceptibility, supporting further the role of PMNs and ANCA in this disease [5]. Scarce findings support environmental factors precipitating the diseases [6]. However, infectious triggers and certain drugs can lead to PR3-ANCA and MPO-ANCA, to vasculitis and/or granuloma formation, phenotypically similar to GPA and MPA.

The pathogenesis of both the vasculitis and the granulomatous inflammation is still not completely understood. Here we will focus on the role of PMNs, their products and their autoantibodies, which all obviously play a major role in directing the concert of inflammatory cells and their cytokines observed in the blood and tissues of GPA and MPA. PR3 and MPO are important molecules of the neutrophil extracellular traps (NETs) formed by activated PMNs. The contribution of NETs to the development of autoimmunity with ANCA and their deposition within the inflammatory lesions seen in situ will be described. In this short review, we can highlight only a few aspects in detail and will focus on the pathophysiology of GPA and MPA.

SHORT STORY OF ANCA DISCOVERY

ANCA were first reported by two Australian groups in necrotizing and crescentic glomerulonephritis, or unclassified vasculitis [7, 8]. Then, van der Woude et al. [2] presented
diagnostic evidence for the presence of autoantibodies seen with a distinct IFT pattern in the sera of patients with Wegener’s granulomatosis. The diagnostic value was soon confirmed and extended by analysing the GPA cohort (Figure 1) and the importance of the new seromarker was highlighted: ‘In WG the diagnosis depends on a combination of characteristic clinical features and histological appearance. In fulminant cases (active extensive disease) both are difficult to establish, and we congratulate the Dutch–Danish group for developing a new marker for an old disease—one that will soon become a third criterion in the diagnosis of WG.’ [3]

The first data given by IFT only were extended and substantiated by newly developed enzyme-linked immunosorbent assays [9, 10]. Subsequently two different fluorescence pattern (‘classic’ or central accentuated, and perinuclear) have been differentiated at the First ANCA Workshop in 1989, leading to C-ANCA and P-ANCA, respectively. At the Second ANCA Workshop in 1990, PR3 was introduced as a target for C-ANCA [11]. Soon, by ‘decoding of Wegener’s autoantigen’, the genetic structure of PR3 was defined [12]. In addition, the second ANCA fluorescence pattern, P-ANCA, was induced by autoantibodies against MPO [4]. P-ANCA occurred in most of the MPA patients and was also reported in renal-limited pauci-immune necrotizing and crescentic glomerulonephritis.

The improvement of methods for ANCA detection and their clinical utility was recently reviewed elsewhere [13]. With this new diagnostic tool, the AAV turned out to be much more common than formerly believed: in northern Germany, doubled prevalence rates of AAV have been documented between 1994 and 2006 [14]. Furthermore, other new diagnostic and management strategies have fundamentally improved their outcome.

**AAV SUBGROUPS: NEW MOLECULAR DEFINITION**

GPA is associated primarily with PR3-ANCA, whereas MPA and renal-limited vasculitis are associated principally with MPO-ANCA [15]. However, in the various cohorts worldwide there is a broader range for PR3-ANCA in MPA than earlier expected, and about 5–30% MPO-ANCA in GPA. Moreover, the prevalence of the different clinical variants of AAV and their association with ANCA subtypes is geographically different [15].

**FIGURE 1:** Anti-cytoplasmic antibodies (later renamed C-ANCA) in the German GPA cohort. In the second patient cohort tested (using IFT) C-ANCA was observed in 18 of 34 patients with GPA, but in none of the controls. C-ANCA was negative in ‘limited’ disease (now localized GPA) and always positive in active generalized GPA. In addition, a positive association between disease activity and the presence of ANCA has been shown. The high rate of relapses in GPA under the ‘standard (NIH)’ treatment regimen and the time from first signs/symptoms to diagnosis is still today a challenge. Reprinted with permission from *The Lancet*, volume 327, 1986, Gross et al. [3].
PR3-ANCA-associated and MPO-ANCA-associated diseases have with respect to vasculitic manifestations common clinical phenotypes and similarities in their tissue lesions induced by vasculitis. However, ANCA antigen specificity appears to be more closely associated with disease phenotype and prognosis than with the former clinical diagnosis of the 'old entities', GPA or MPA. PR3-ANCA is associated with granulomatous inflammation, respiratory tract involvement, more extensive extra-renal organ manifestation and a higher relapse rate [16, 17]. In contrast, MPO-ANCA is more frequent in kidney-limited disease, displays more severe renal scaring and a worse renal prognosis [18].

In addition, genome-wide association studies (GWAS) have shown that the autoantigen specificities PR3 and MPO correlate better with different HLA risk genes (PR3-ANCA with HLA-DP, MPO-ANCA with HLA-DQ) than with the clinicopathologic phenotypes of GPA and MPA [5]. Polymorphisms within the HLA region further highlight the evidence of a genetically determined background of these autoimmune diseases and the role of (auto)antigen recognition. Moreover, the association with the genes encoding PR3 (PTN3) and its inhibitor α-1-antitrypsin (SERPINA1) with PR3-ANCA disease and/or GPA additionally supports a central pathogenetic role of PMN products and their neutralizing counterparts.

In view of these differences and the awareness of long-term outcome observations, it has been suggested to use both ANCA specificity and the clinicopathologic phenotype to reclassify AAV (e.g. PR3-ANCA GPA, MPO-ANCA GPA, etc.) [1]. However, it should be kept in mind that not all patients with the diagnosis of GPA, MPA or EGPA have detectable ANCA. In GPA, a variant without detectable vasculitis is seen in ∼5% of patients [19, 20].

**FIGURE 2:** PMNs and their NETs as central players within the pathogenesis of ANCA-associated vasculitis. Both environmental (infections) and genetic factors contribute to autoimmunity and the disease phenotype. Activated or dying PMNs present via NETs autoantigens (PR3, MPO) to DCs and induce a proinflammatory milieu by integrating other inflammatory cells, activating the complement system (via properdin) as well as the coagulation cascade (via TF). NETs have been documented in the characteristic organ lesions seen in AAV, e.g. in glomerular crescents. Primed PMN activated by ANCA contribute to necrotizing granulomatous inflammation and vasculitis. Neutrophilic microabscesses dominate the early phase of the 'granuloma', a mixed infiltrate of palisading histiocytes, multi-nucleated giant cells, PMNs, DCs, clusters of T and B cells and ectopic lymphoid-like tissue neoformation surrounding 'geographic' central necrosis. Bone destruction is mediated by fibroblasts. B, B cell; BAFF, B-cell-activating factor; C5a, complement factor 5a; DC, dendritic cell; M, monocyte/macrophage; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PMN, neutrophil; PR3, proteinase 3; ROS, reactive oxygen species; T, T cell; TF, tissue factor.
There is substantial evidence of in vitro experiments and animal studies supporting a role of PMNs and, at least in part, their autoantibodies (ANCA) in the pathogenesis of AAV (Figure 2). PMNs are the dominant infiltrate within vasculitic as well as granulomatous lesions. Depleting PMNs and/or complement (activated by PMNs properdin) in experimentally induced vasculitis stop the disease [21].

In AAV, the ANCA target antigens are located intracytoplasmic and are also constitutively expressed on the cell membrane of PMNs. Epigenetic modification in ANCA target antigen-encoding genes results in an increased expression of PR3 and MPO in PMNs of ANCA patients [22]. PMN activation and/or the release of NETs effectively display the autoantigens PR3 and MPO to dendritic cells (DCs) as well as lead to inflammation in situ [23, 24]. By expression of neutrophil tissue factor and involving platelets and blood coagulants, NETs are implicated in thrombogenesis, which could explain the increased risk of venous thromboembolic events in AAV [25, 26].

Priming with cytokines (e.g. TNF-α) results in the translocation of the ANCA target antigens on the surface of PMNs where they are accessible to interaction with ANCA. This observation has led to the ANCA Cytokine Sequence Theory [27, 28]. The binding of ANCA activates PMNs through Fab and Fc receptor engagement which then release reactive oxygen species (ROS), proinflammatory cytokines, complement activators (e.g. properdin) and enhances neutrophil–endothelial interaction. The complement activation generates C5a and the recruitment of further PMNs at the site of inflammation [29]. ANCA-activated PMNs cause endothelial damage and acute inflammation with fibrinoid necrosis and leucocytoclasis (‘necrotizing vasculitis’). Furthermore, activated PMNs release B-cell-stimulating factors promoting B-cell survival [30] as well as other cytokines participating in the recruitment, activation and programming of DCs, monocytes and lymphocytes [31]. B cells and the complement C5a receptor are now major targets in new treatment strategies [32].

In vitro, ANCA are able to activate primed PMNs and modulate PMN apoptosis or NETosis. Animal models have convincingly shown the pathogenicity of MPO-ANCA in inducing pauci-immune necrotizing and crescentic glomerulonephritis and small-vessel vasculitis [21]. However, no convincing animal model for PR3-ANCA AAV has been developed. As murine PR3 shows only 68% homology with human PR3 and is not constitutively expressed on the cell surface, there is a need for humanized murine models. Recently, the combination of low-dose lipopolysaccharide and human PR3-ANCA in a humanized mouse model induced pulmonary haemorrhage and mild proliferative glomerulonephritis, but lacking granulomatous inflammation [33].

The GWAS results mentioned before have confirmed the distinct genetic difference between GPA and MPA and between PR3-ANCA-positive versus MPO-ANCA-positive patients. These and other new insights into the molecular basis of systemic vasculitis will challenge further on the idea of a strictly common pathway of vasculitis probably induced by both autoantibodies.

Upon stimulation PMNs actively release webs of nuclear-derived chromatin fibres called NETs into the extracellular space. These NETs are decorated with histones and antimicrobial cytoplasmic proteins, including PR3 and MPO, and are able to capture and kill microbes extracellularly. The current evidence suggests that NETs are directly involved in endothelial damage and thrombosis as well as ANCA induction. Persistent NET exposure at sites of tissue lesions could further augment inflammation. Because aggregated NETs may also promote resolution of neutrophil inflammation by degrading cytokines and chemokines and disrupting neutrophil activation and recruitment there is some debate about their pathophysiological role [34].

Recently, Kessenbrock et al. [23] could demonstrate the release of NETs by ANCA-stimulated PMNs as well as the deposition of NETs in kidney biopsies of GPA patients. Increased levels of circulating MPO-DNA-complexes were detected in patients with AAV, correlating with disease activity. These NET-derived products can activate DCs and autoreactive B cells in a toll-like receptor 9-dependent manner [23] and form a platform to initiate the autoimmune response. Uploading of myeloid DCs with NET components induces ANCA and autoimmunity in susceptible mice [24], suggesting that NET structures containing cytoplasmic proteins are highly immunogenic. Inhibition of the NET production with DNase prevented uploading of DCs and induction of autoimmunity.

In comparison to healthy controls, PMNs from AAV patients are able to produce more ROS and more extensive NET formation [35]. Sera from patients with MPA exhibited lower activity of DNase I and a lower rate of NET degradation suggesting a prolonged exposure of the ANCA autoantigens to the immune system [36]. Recently, it was shown that NET formation can be suppressed by engagement of signal inhibitory receptor on leukocytes-1 [37]. Inhibition of NET formation and extracellular DNA degradation have also been suggested with PAD4-specific inhibitors [38]. Therefore, PMNs and NET formation appear as upcoming new therapeutic targets in AAV.

Several drugs, including hydralazine, propylthiouracil (PTU) and levamisole-adulterated cocaine, are able to induce ANCA in some and vasculitic manifestations mimicking AAV in few patients [39]. They are now categorized as ‘vasculitis with probable etiology’ [1]. Here, ANCA are usually present with high-titre and MPO specificity, frequently accompanied by ANCA directed against other autoantigens (elastase, lactoferrin, bacterial/cellular/ permeability increasing protein or PR3). Moreover, common side effects of these drugs are leucopenia and/or agranulocytosis.

Using PTU, an anti-thyroid drug which is associated with the development of MPO-ANCA vasculitis, Nakazawa et al. demonstrated an abnormal conformation and impaired degradation of NETs in vitro. Immunization of Wistar-Kyoto rats with PTU-induced NETs induced the production of MPO-
ANCA and a typical vasculitis with pulmonary and renal involvement [40].

The vicious circle of NET formation may be further enhanced by bacterial infections, e.g. with Staphylococcus aureus, which is known to strongly induce NETs and which has been linked with the pathogenesis and the occurrence of relapses in AAV. In line with this observation, ANCA are also present in a substantial proportion of patients with infective endocarditis [41].

Therefore, the central role of NETs in AAV is demonstrated in vivo and in vitro by recently published studies and all the data provide support for the new and attractive hypothesis that the NET-associated autoantigens initiate ANCA production. Furthermore, the published data support the concept that PMNs and their aberrant NET formation promote vasculitis by inducing autoimmunity and accelerating inflammation in situ.

**ANCA—PATHOGENIC VERSUS NON-PATHOGENIC AUTOANTIBODIES**

Although clinical observations, in vitro data and animal studies support the idea of a pathogenic role of ANCA, the occurrence of ANCA-negative patients with AAV, the presence of naturally occurring ANCA in healthy individuals, the lack of a strong correlation between ANCA titres and disease activity and the absence of an all accepted animal model for PR3-ANCA disease argue against pathogenicity. Recent studies have answered some of these puzzling questions and pointed out that not all ANCA are pathogenic.

Proposed mechanisms for the autoantibody response to produce ANCA include molecular mimicry between bacterial peptides and self-antigens, and the initiation of the immune response by complementary peptides to autoantigen epitopes or a mimic of the antisense peptide [29]. The detection of autoantibodies to an epitope of human lysosome-associated membrane protein-2 (hLAMP-2), which shows 100% homology to a bacterial adhesion molecule, FilH, supports the molecular mimicry theory. Furthermore, antibodies to complementary peptides of an autoantigen or a mimic can elicit an anti-idiotypic antibody response with cross reaction to the autoantigen [29]. Unfortunately, both concepts still have to be proven.

The pathogenic potency of ANCA seems to strongly depend on the epitope specificity. Natural autoantibodies against MPO and PR3 can be detected in healthy individuals with low titre and low avidity [42]. In AAV, both PR3-ANCA and MPO-ANCA recognize a restricted number of epitopes and a restricted epitope spreading within their corresponding autoantigen has been described [43]. Especially the creation for PR3-ANCA chimeric variants was helpful in defining relevant conformational epitopes [44, 45]. For PR3-ANCA, it was recently shown that ANCA compete with the binding of α1-antitrypsin to PR3 and that differences in epitope specificity of ANCA could influence the inactivation of PR3 [44]. Although several studies could demonstrate that ANCA epitopes change during the course of the disease, these changes were not consistent so far. Anyway, these findings have led to a comeback of the first and dominating concept which postulated an interference of ANCA with PR3 activity and/or with the interaction of PR3 and α1-antitrypsin 25 years ago [27].

Recently, Roth et al. found that only certain MPO-ANCA epitopes were highly specific for active diseases. Epitopes of natural antibodies and epitopes exclusive to active disease where located within close proximity, suggesting that epitope spreading from asymptomatic natural autoantibodies to disease-causing antibodies could occur. Reactivity against one linear epitope, aa447-459, was exclusively found in active disease, declined with remission and was also observed in the total immunoglobulin fraction of ANCA-negative patients. This was explained by masking of the epitope in serum by ceruloplasmin, an endogenous inhibitor of MPO. The pathogenic capacity was further supported in a transgenic animal model [46].

The ANCA autoimmune response is further augmented by an impaired T-cell and B-cell suppression and the enhanced release of B-cell-stimulating factors by activated PMNs.

From these studies, we can conclude that not all PR3- or MPO-ANCAs are pathogenic. Intra-molecular epitope spreading, masking of epitopes by serum factors and concealing them from routine testing and the failure of current ANCA assays to discriminate between pathogenic and non-pathogenic antibodies could explain at least in part the unresolved questions.

**LOCALIZED GPA DUE TO GRANULOMA FORMATION**

Extravascular granuloma formation is the major factor differentiating MPA from GPA and has been extensively reviewed by Muller et al. [47]. GPA usually starts with granulomatous inflammation affecting the upper and/or lower respiratory tract (‘localized GPA’) and progresses in the majority of patients to systemic vasculitis (‘generalized GPA’). There is also evidence for a persistent localized GPA variant with localized granulomatous manifestations restricted to the upper respiratory tract, lung or eye, and without progression to systemic disease, representing ~5% of GPA patients in a monocentric German cohort [19] as well as in a French cohort [20]. In this subgroup of patients, ANCA is less frequently detectable, however, frequent relapses, treatment refractory disease course and damage induced by granulomatous inflammation in the ENT region are observed [48].

The pathogenesis of granuloma formation is not really understood and seems to result from an aberrant immune response obviously directed by predominating PMNs. Acute lesions in the early phase are dominated by PMNs and show intense neutrophilic infiltration, the formation of neutrophilic microabscesses and only scattered multi-nucleated giant cells. Later classic features of the granulomatous inflammation are, aside from the PMN infiltration, the development of palisades by histiocytes/macrophages and scattered multi-nucleated giant cells surrounding irregularly outlined (‘geographic’) necrotic zones. The mixed inflammatory infiltrate around these zones typically includes clusters of T and B cells, partially with follicular organization, as well as plasma cells.
Our understanding of the pathogenesis of AAV has tremendously broadened over the last decades. Genetic factors and infectious triggers as well as PMNs, cellular immunity with impaired T and B cell response, vascular endothelial cells, complement and inflammatory mediators play a role in the pathogenesis. PMNs are a key player in the development of necrotizing vasculitis as well as in the initial granulomatous inflammation. Recent data provide support for the attractive hypothesis that NET-associated autoantigens initiate ANCA production. PR3-ANCA and MPO-ANCA are not only a defining feature of AAV and have improved early diagnosis, but are directly pathogenic, although not all ANCA seem to be equal. The success of targeted B-cell therapy further proves the importance of B cells and ANCA. However, further studies are needed to answer still open questions and find new treatment options targeting, e.g., complement or NETs.

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CONFLICT OF INTEREST STATEMENT

None declared.

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