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Antineutrophil Cytoplasmic Antibodies (ANCA): Diagnostic Utility and Potential Role in the Pathogenesis of Vasculitis

Branko Malenica, Marija Rudolf, Ana Kozmar

Division of Immunology, Clinical Institute of Laboratory Diagnosis, Zagreb University Hospital Center, Zagreb, Croatia

Corresponding author:

Branko Malenica, MD, PhD

Division of Immunology

Clinical Institute of Laboratory Diagnosis

Zagreb University Hospital Center

Kišpatićeva 12

10000 Zagreb, Croatia

b_malenica@yahoo.com

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SUMMARY Antineutrophil cytoplasmic antibodies (ANCA) are a heterogeneous group of circulating antibodies directed toward the cytoplasmic constituents of neutrophils and monocytes. ANCA have been described in various diseases including idiopathic systemic vasculitides, connective tissue diseases, inflammatory bowel diseases, autoimmune liver diseases, infectious diseases, and some drugs. ANCA recognize different target antigens such as proteinase 3 (PR3-ANCA), myeloperoxidase (MPO-ANCA), cathepsin G, lactoferrin, bactericidal/permeability-increasing protein (BPI), and some others. However, only PR3-ANCA and MPO-ANCA are closely associated with systemic vasculitides, in particular Wegener s granulomatosis, microscopic polyangiitis and its renal limited manifestation, and Churg-Strauss syndrome. Both *in vitro* and *in vivo* experimental data strongly support a pathogenic role for ANCA in vasculitis and glomerulonephritis.

KEY WORDS ANCA; PR3-ANCA; MPO-ANCA; vasculitis; pathogenesis

INTRODUCTION

Antineutrophil cytoplasmic antibodies (ANCA) are a heterogeneous group of circulating antibodies directed toward the cytoplasmic constituents of neutrophil granules and monocytes. ANCA were first described in 1982 by Davies *et al*. in a few patients with segmental necrotizing glomerulonephritis (1,2). The importance of this observation had not been recognized until 1985, when a distinct granular cytoplasmic fluorescence pattern, first called ACPA (anticytoplasmic antibodies) and later termed C-ANCA (cytoplasmic), was shown to be associated with Wegener s granulomatosis (WG) (3). Later, a second ANCA fluorescence pattern (later called perinuclear, P-ANCA) was described in patients with microscopic polyangiitis (MPA) (4) and in patients with the idiopathic form of pauci-immune necrotizing crescentic glomerulonephritis (iNCGN) (5). These data have now been confirmed by many groups and support the view that ANCA-associated vasculitis and glomerulonephritis are indeed a distinct disease category (6-11). Subsequently, a third, less clearcut ANCA subtype (later termed 'atypical' or very perinuclear, a/P-ANCA) was described in a wide range of connective tissue diseases (CTD), inflammatory bowel (IBD) and autoimmune liver diseases, and infectious diseases (8-13).

Today the various ANCA serve as useful seromarkers. They can be used as clinical tools to aid in the diagnosis of WG and to distinguish new entities within the large spectra of vasculitis/glome-

rulonephritis and chronic inflammatory bowel and liver diseases. Recent studies support the hypothesis that ANCA and their target antigens may be implicated in the pathogenesis of these diseases, at least in vasculitis (7-11,14-20).

In this review we will evaluate background information about the evolution of ANCA methodology, the diagnostic application of ANCA testing, and the current understanding of the pathogenic role of different subtypes of ANCA. We also discuss ANCA in the context of cutaneous vasculitis.

ANCA TEST METHODOLOGY AND THEIR TARGET ANTIGENS

Currently, three basic assay principles are applied for the detection of ANCA. The original method of ANCA detection is indirect immunofluorescence (IIF) (21). While it remains the most widely used method, it does not identify the specific antigen responsible for the ANCA immunofluorescence. Enzyme-linked immunosorbent assay (ELISA) is used for target-specific ANCA determination (22). There are two popular types of such solid-phase assays. The target antigen can be coated directly onto the plastic reaction well (standard ELISA), or it can be linked to the reaction well *via* target antigen-specific mouse monoclonal or rabbit polyclonal antibodies (capture ELISA or sandwich ELISA) (23). Other detection methods such as radioimmunoassay (RIA), immunoblotting (IB) or immunoprecipitation techniques are not widely used for routine ANCA testing (24).

ANCA are routinely detected by IIF on ethanol-fixed neutrophils (21,25,26). Recent consensus statements on testing and reporting of ANCA recognize four different fluorescence patterns: a coarse granular cytoplasmic fluorescence with accentuation between the nuclear lobes – classic cytoplasmic or C-ANCA (Fig. 1A, e, f); a typically perinuclear fluorescence with some nuclear extension (Fig. 1B, e) and granular cytoplasmic fluorescence on formalin-fixed neutrophils (Fig. 1B, f) – perinuclear or P-ANCA; pronounced nuclear rim fluorescence with minimal nuclear extension, center of nucleus unstained (Fig. 1C, e) and non-reactivity with formalin-fixed neutrophils (Figure 1C; f) – very perinuclear or 'atypical' a/P-ANCA; and atypical ANCA which include all other IIF reactivity, most commonly a combination of cytoplasmic and perinuclear fluorescence (not shown).

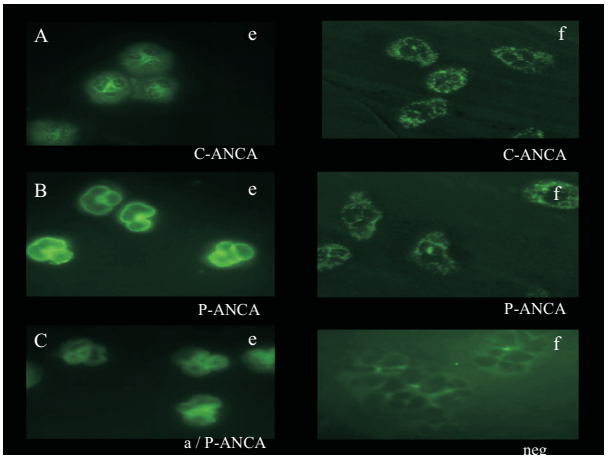

Figure 1. Characteristic fluorescence patterns of anti-neutrophil cytoplasmic antibodies (ANCA) on ethanol (e) and paraformaldehyde (f) fixed human neutrophil cytospin preparations. (A) a coarse finely granular fluorescence present throughout the cytoplasm and with accentuation between nuclear lobes (e and f)-cytoplasmic-C-ANCA; (B) a typically perinuclear fluorescence with some nuclear extension (e) and a granular cytoplasmic fluorescence on formalin fixed neutrophils (f)-perinuclear-P-ANCA ; (C) a prononuced nuclear rim fluorescence, center of nucleus unstained, without the nuclear extension (e) and negative fluorescence on formalin fixed neutrophils (f) -"atypical" perinuclear -a/P-ANCA or very perinuclear P-ANCA.

The characteristic C-ANCA pattern has been recognized as a characteristic staining pattern produced by the sera of most patients with WG, but also of some 50% of patients with MPA and a minority of patients with other necrotizing vasculitis or idiopathic NCGN (27). Differentiating the classic granular cytoplasmic fluorescence pattern with interlobular accentuation (C-ANCA) from diffuse flat cytoplasmic fluorescence pattern without interlobular accentuation (C-ANCA-atypical) pattern may at times be difficult.

P-ANCA were originally described in patients with idiopathic and/or vasculitis associated NCGN (5). Further studies showed that the P-ANCA pattern could also be produced by the sera from patients with vasculitis without renal involvement and patients with IBD, autoimmune liver disease, infectious diseases such as HIV infection, and very rarely connective tissue disease (CTD) such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (11,12). The perinuclear pattern

cannot always be distinguished from the nuclear staining pattern. Thus, discrimination between antinuclear antibodies (ANA) and P-ANCA is not possible in most sera that are ANA positive when isolated ethanol-fixed neutrophils are used as a substrate. In fact, antibodies reacting with nuclei of granulocytes only, and not with those of other substrates, have been detected in patients with RA long before ANCA had been described, and were named granulocyte-specific antinuclear antibodies (GS-ANA). Recent studies, however, have shown that the P-ANCA fluorescence pattern represents an artifact of ethanol fixation which allows for rearrangement of positively charged granule constituents around and on the negatively charged nuclear membrane (28). The use of crosslinking fixatives, such as paraformaldehyde, during the preparation of neutrophil substrates prevents the perinuclear rearrangement of charged antigens and thereby allows for distinction of true P-ANCA from ANA with or without granulocyte specificity. On formalin-fixed neutrophils, true P-ANCA will display diffuse granular cytoplasmic staining, whereas an ANA-containing serum sample will again display nuclear staining. The simultaneous testing for ANA using standard substrates, such as HEp-2 cells, is not sufficient for this distinction because P-ANCA and ANA can occur simultaneously.

'Atypical' P-ANCA with different frequency were found in patients with IBD, autoimmune liver diseases and infectious diseases such as HIV infection (11,12). Distinction between these two P-ANCA similar fluorescence patterns is possible on formalin-fixed neutrophils where the 'atypical' P-ANCA pattern displays non-reactivity, whereas true P-ANCA display diffuse granular cytoplasmic staining (Fig. 1).

IIF should be performed on serum samples from all 'new' patients, since about 10% of ANCA-positive serum samples in patients with WG or MPA can be demonstrated only by IIF.

To circumvent the problems associated with IIF, i.e. the lack of target antigen specificity and operator dependence, target antigen-specific solid-phase immunoassays have been used. ELISA and IB techniques enable identification of many target antigens that are associated with autoantibodies causing different immunofluorescence patterns (Table 1). The target antigen recognized by most C-ANCA positive sera has been identified as proteinase 3 (PR3), a neutral serine protease present in the azurophilic granules of neutrophils. Proteinase 3 has been cloned and shown to be a 29 kD glycoprotein of 228 aminoacids (29-33). Human antibodies to PR3 appear to recognize conformational determinants on the molecule (34). Very rarely, sera with PR3-ANCA reactivity can also cause P-ANCA fluorescence pattern (35). Myeloperoxidase (MPO) represents the P-ANCA target antigen with the greatest clinical utility because of the frequent association of MPO-ANCA with MPA and iNCGN (36). All serum samples should be assayed in PR3-ANCA and MPO-ANCA ELISAs, since about 5% of serum samples are positive only by ELISA. However, many sera that produce P-ANCA or a/P-ANCA staining pattern on ethanol-fixed neutrophils do not contain autoantibodies to MPO or PR3 as tested by antigen-specific assays. It has now been recognized that a number of these sera contain antibodies to other granular constituents of neutrophils. In particular, autoantibodies have been demonstrated to human leukocyte elastase (HLE) (37,38), cathepsin G (CG) (39-42), lactoferrin (LF) (43-46), lysozyme (47), azurocidin (48), -enolase (49-51), actin (52-54), tropomyosin (53, 54), high motility groups of nonhistone chromosomal proteins 1 and 2 (HMG1 and HMG2) (55-57), bactericidal/ permea-

Table 1. Target antigens for antineutrophil cytoplasmic antibodies (ANCA)

ANCA target antigen	Immunofluorescence pattern
PR3	C-ANCA, very rarely P-ANCA
MPO	P-ANCA, very rarely C-ANCA
Eosinophylic peroxidase	a/C-ANCA
HLE	P-ANCA
-Enolase	P-ANCA
Azurocidin	P-ANCA
Cathepsin G	P-ANCA
Lactoferrin	P-ANCA
Lysozyme	P-ANCA, a/C-ANCA
Actin	a/C-ANCA, a/P-ANCA
Tropomyosin	a/C-ANCA, a/P-ANCA
HMG1/2	P-ANCA
BPI	a/C-ANCA, a/P-ANCA
Lamin B1	a/P-ANCA
Histone H1	a/P-ANCA

bility-increasing protein (BPI) (58-61), lamin B1 (13, 47) and histone H1 (13,62).

DIAGNOSTIC UTILITY OF ANCA TESTING

Since the first description of ANCA, the different ANCA fluorescence patterns have been described in various diseases. In addition to systemic vasculitides, this list now includes various CTDs, IBD, autoimmune liver diseases, infections, malignancies, myelodysplastic processes and many others, even diabetes mellitus. The interpretation of many reports is compromised by limited information about the methods utilized for ANCA detection. Reports that include data on different fluorescence patterns and specific target antigens allow for more meaningful analysis of clinical associations and utility.

ANCA in primary systemic vasculitides

The most clear-cut association of a disease with ANCA directed against a specific target antigen remains the association between WG and PR3-ANCA (Table 2). Between 80% to 95% of all ANCA found in WG are C-ANCA (8,12,63-65). The use of more sensitive PR3-ANCA specific methods (capture ELISA) of detection has confirmed that the C-ANCA in WG is almost always associated with anti-PR3 (23,66). It has been estimated that 5%-20% of ANCA in WG may be P-ANCA, which are mostly directed against MPO (67,68) and only rarely against other known target antigens such as human leukocyte elastase (69). The sensitivity of C-ANCA/PR3-ANCA for WG is related to the extent, severity and activity of disease (64). In a meta-analysis of C-ANCA in WG, the pooled sensitivity was 91% for the subset of patients with active disease compared to 63% for those with inactive disease (70). Some longitudinal studies have shown that titers of C-ANCA/PR3-ANCA correlate with clinical disease activity (71-74), although this has been disputed by other authors (75,76). Additionally, disease relapse can be anticipated and prevented by intensifying immunosuppression in response to rising titers (76,77). Persistent or intermittent C-ANCA positivity is an independent risk factor for relapse (78-81).

Table 2. Disease associations of C-ANCA (PR3-ANCA) and P-ANCA (MPO-ANCA) in systemic vasculitis

Patients	Sensitivity of			
with	C-ANCA	PR3-ANCA	P-ANCA	MPO-ANCA
WG	80-90	85	5-20	24
MPA	35-45	26	40-50	58
iNCGN	30-40	50	46-65	58
CSS	14-33	33	33-42	50
PAN	3-10	0	5-30	38

Most patients with MPA are ANCA positive, either with specificity for MPO or for PR3 (5,11,12,64, 67). The interpretation of data about the association of ANCA and MPA is complicated by the lack of consensus about the definition of MPA. Since there continues to be controversy about which features clearly separate MPA from PAN, it remains unclear whether ANCA also occur in some patients with classic PAN (82). However, most data support the perception that MPA, which is frequently associated with GN and/or pulmonary capillaritis, is often associated with MPO-ANCA, whereas classic PAN is not (64,83,84). The relation between titers of ANCA and disease activity has not been studied systematically in MPA (70,78,80,85).

Other target antigens for ANCA, such as BPI and azurocidin, have recently been detected in patients with systemic vasculitis (48,58,60,86). These antibodies may also simultaneously occur with PR3- or MPO-ANCA.

Most patients with iNCGN are positive for anti-MPO, the remaining generally being positive for anti-PR3 (5,11,12,68,87). Many of these patients have constitutional symptoms together with signs of systemic involvement so that the distinction between WG, MPA and iNCGN is far from absolute.

ANCA have been detected with variable frequency in patients with CSS (88-90). Both MPO and PR3 have been described as target antigens.

Although Ig-A-ANCA have occasionally been noted in Henoch-Schönlein purpura (91-93), most studies have not shown this to be a significant association with regard to either frequency or clinical feature of illness (94).

Patients with Kawasaki disease may have ANCA and antiendothelial cell antibodies (AECA) but these antibodies occur in the minority of pa-

tients and do not occur more often than in other pediatric febrile illneses (95,96).

Other forms of vasculitis, particularly giant cell arteritis, Takayasu arteritis and Behcet s disease, are not ANCA-associated (97,98).

ANCA in connective tissue diseases (CTD)

ANCA have been described in patients with a variety of CTD, including RA (99-104), SLE (105-107), PM/DM (108), juvenile chronic arthritis (109), reactive arthritis (110,111), relapsing chondritis (112,113) and anti-phospholipid syndrome (108). ANCA in scleroderma is uncommon and is of doubtful clinical significance (114,115). The fluorescence patterns in CTD are mostly P-ANCA. To a lesser extent, atypical fluorescence patterns may be found, and true C-ANCA patterns are rare (108,116). A multitude of target antigens, including LF, HLE, LZ, CG, MPO, HMG1 and HMG2, have been described in these diseases (Table 3). However, a significant proportion of target antigens for ANCA encountered in this setting remain to be identified. PR3-ANCA occur extremely rarely in these patients (116).

Table 3. Target antigens of ANCA in connective tissue diseases (CTD)

Patients	Target antigens in ELISA (%)				
with	PR3	MPO	LF	CG	HMG1/2
SLE	0	1	11	37	37/18
RA	0	1	35	33	40/25
SS	0	0	0	22	44/11
SSc	0	18	5	5	36/18
PM/DM	0	0	27	9	18/18

SLE=systemic lupus erythematosus; RA=rheumatoid arthritis; SS=Sjögren's syndrome; SSc=systemic sclerosis; PM/DM=polymyositis/dermatomyositis; PR3=proteinase 3; MPO=myeloperoxidase; LF=lactoferrin; CG=cathepsin G; HMG1/2=high mobility group (HMG) nonhistone chromosomal proteins 1 and 2

While patients with CTD have an increased frequency of vasculitis, data to suggest that ANCA positivity enhances the risk of vasculitis are contradictory (108,116). Non-vasculitis aspects of rheumatic disease activity, severity and chronicity also fail to consistently correlate with ANCA status (100,102,103,108,116). Consequently, there is little clinical utility for ANCA testing in patients with CTD in whom one of the ANCA-associated systemic vasculitides is not suspected.

ANCA in inflammatory bowel disease (IBD) and autoimmune liver diseases

Shortly after the discovery of ANCA in WG and MPA, ANCA were discovered in IBD and autoimmune liver diseases (13,117-125). On IIF these ANCA produce perinuclear and less often atypical cytoplasmic fluorescence patterns. MPO is not a prominent target antigen for these P-ANCA (Table 4). A multitude of other target antigens, particularly LF, CG, actin, BPI, catalase and HMG1/2, have been identified as target antigens in IBD (13,39-41,45,126-128). Data from most series suggest that the prevalence of P-ANCA is higher in ulcerative colitis (40%-80%) than in Crohn s disease (10%-40%). Some studies have suggested a higher association of ANCA in patients with Crohn s disease and colonic involvement (129,130), treatment-resistant left-sided ulcerative colitis (131) or chronic pouchitis (132,133). The overlap of ANCA findings between the various clinical groups of IBD patients is significant. Therefore, ANCA testing does not facilitate the differential diagnosis of patients with IBD. Neither is the correlation of titers with disease activity sufficiently reliable to be useful for monitoring IBD activity (13,123,124,129).

ANCA are also frequently found in patients with primary sclerosing cholangitis (PSC) and autoimmune hepatitis type 1 (50%-90%). ANCA are generally not associated with primary biliary cirrhosis

Table 4. Target antigens of ANCA in patients with inflammatory bowel and autoimmune liver diseases

Patients	Target antigen in ELISA (%)						
with	MPO	LF	CG	Actin	BPI	Catalase	HMG1/2
UC	2-29	5-50	0-1	NT	43	38	32
CD	0-6	1-21	0-2	NT	26	26	26
PBC	NT	NT	NT	NT	25	NT	NT
PSC	0-33	0/50	NT	NT	35	NT	NT
AIH type 1	NT	8	0	47-52	29	NT	NT

UC=ulcerative colitis; CD=Crohn's disease; PBC=primary biliary cirrhosis; PSC=primary sclerosing cholangitis; AIH=autoimmune hepatitis; BPI=bactericidal/permeability increasing protein; HMG1/2=high mobility group (HMG) nonhistone chromosomal proteins 1 and 2; NT=not tested

(13,121,122,134-136). ANCA reactivity in autoimmune hepatitis type 1 most often appears to be directed to actin (13,54,136).

ANCA in infectious diseases

ANCA, mainly with 'atypical' cytoplasmic or P-ANCA immunofluorescence patterns, have been described in human immunodeficiency virus (HIV) infection (137,138), cystic fibrosis with bacterial airway infections (139,140), chromomycosis (141), acute malaria (42,142), invasive amebiasis (143), and in patients with some other infections (144, 145). These ANCA are not caused by antibodies against PR3 and MPO. A recent report has described the occurrence of BPI-IgG-ANCA and BPI-IgA-ANCA in the majority of patients with cystic fibrosis (146). Anti-BPI titers were directly related to the severity of airway destruction. Cathepsin G has been identified as the most significant target antigen for ANCA, causing an atypical cytoplasmic fluorescence pattern in patients with acute malaria (42,142). Invasive amebiasis is the only infection that has been reported to be associated with PR3-ANCA (143). The presence of autoantibodies causing cytoplasmic fluorescence on ethanol-fixed neutrophils from patients with infection shows the importance of excluding infectious processes before initiating immunosuppressive therapy. These observations may suggest that ANCA, which occur transiently in the setting of infection, and the persistent ANCA in patients with vasculitis may be the results of molecular mimicry in susceptible hosts (147). Subsequent diversification of T- and B-cell responses ('epitope spreading') may lead to response against different epitopes on the same target molecule (intramolecular spreading) or may extend to other molecules (intermolecular spreading) (148,149).

ANCA associated with drugs

Increasing numbers of case reports and reports of small series have identified associated diseases that are presumed to be induced by certain drugs with ANCA reactivity. The following drugs have been connected to autoimmune clinical sequels and ANCA: hydralazine-induced lupus with anti-MPO and elastase antibodies, hydralazine-associated vasculitis with anti-MPO and anti-LF antibodies and minocycline-induced arthritis, fever, and livedo reticularis with anti-MPO antibodies (150-156). Vasculitis occurring after propylthiouracil therapy has been associated with ANCA specificities to several different target antigens, including PR3, MPO and elastase (152,153,157-160).

Diagnosis of cutaneous manifestations of Wegener s granulomatosis (WG) and microscopic polyangiitis (MPA)

WG and MPA are the prototype systemic diseases associated with ANCA. Cutaneous lesions may occur in up to one-half of patients with WG (Table 5) (161-163). In general, cutaneous lesions are more likely in patients who have multiple organ system involvement. Cutaneous lesions may also be the first manifestation of WG and may not be vasculitic. Patients may develop cutaneous lesions before the onset of upper airway or pulmonary or renal symptoms, making the diagnosis of WG very difficult for dermatologist. Some patients may develop more than one type of cutaneous lesions, which may change over time or with treatment. In order to best utilize laboratory testing for ANCA, it is essential to recognize the cutaneous patterns of WG and MPA, and, more important, to recognize those disorders that can simulate cutaneous lesions seen in WG (Table 6). Specific lesions are defined as being directly related to WG and not as the results of infection, drug sensitivity or other definable causes other than WG. The most common specific manifestation of cutaneous WG are palpable purpura and ulceration with the histopathologic correlation of leukocytoclastic vasculitis (LCV), most often without granuloma formation, and with a histo-

Table 5. Characteristic involvement of Wegener's granulomatosis by organ/system

Organ/system	%
Upper airway	90-95
Pulmonary	54-85
Pulmonary hemorrhage	5-15
Glomerulonephritis	51-80
Gastrointestinal	>5
Ocular	35-52
Nervous system	20-50
Cardiac	8-16
Cutaneous lesions	15-50

Table 6. Diagnostic entities with cutaneous manifestations to be considered on differential diagnosis of cutaneous Wegener's granulomatosis

Leukocytoclastic or granulomatous vasculitis
Pyoderma gangrenosum
Neutrophilic dermatoses
Infection (bacterial, fungal, mycobacterial)
Infestation (e.g., amebiasis)
Malignancy (lymphoma, lymphomatpoid granulomatosis, leukemia cutis)
Cutaneous Crohn's disease
Drug reactions (including hydralazine and minocycline)
Panniculitis (alpha-1 antitrypsin deficiency, erythema nodosum)
Connective tissue syndromes (inclusive of SLE)
Acneiform lesions
Rheumatoid nodules/papules
Granuloma annulare
Calciphylaxis

pathologic correlate of acute and/or granulomatous inflammation, respectively. Many other diagnostic entities with cutaneous manifestations are to be considered on the differential diagnosis of WG (Table 6). Diagnostic algorithm for cutaneous WG includes careful clinical examination and clinical pathologic correlation to confirm the diagnosis (Table 7). Testing of ANCA may play a significant role in the evaluation of patients suspected to have systemic vasculitis or another illness with presentation similar to WG. Only a positive C-ANCA (IIF) with a positive PR3-ANCA (ELISA) result, and a positive P-ANCA (IIF) with a positive MPO-ANCA (ELISA) result are sensitive and specific for WG and MPA. Repeat ANCA testing is warranted to follow the course of the disease in patients who have proven WG or MPA as ANCA levels correlate loosely with disease activity and relapses in the absence of ANCA are rare. A patient with LCV and other cutaneous findings suggestive of WG with a positive C-ANCA/PR3-ANCA or P-ANCA/MPO-ANCA test result should be followed carefully for subsequent development of systemic disease manifestations even if there is no sign of internal organ involvement at the time of initial presentation.

Table 7. Diagnostic algorithm in differential diagnosis of cutaneous Wegener's granulomatosis

Characteristic clinical findings (purpura, ulcerations, nodules, etc.)
Biopsy for routine microscopy and special stains for microorganisms
Biopsy for tissue culture*
Serologic tests including ANCA (IIF and ELISA)
Longitudinal follow up of ANCA

*Recommended in the majority of cases if histopathology or clinical findings may also be compatible with infectious cause.
ANCA-antineutrophil cytoplasmic antibodies; IIF-indirect immunofluorescence; ELISA-target antigen-specific solid-phase assay

DEVELOPMENT OF ANCA AND THEIR ROLE IN THE PATHOGENESIS OF VASCULITIS

Interaction of ANCA with their target antigens

PR3-ANCA are able to inhibit enzymatic activity of native and recombinant PR3 by binding near the catalytic domain (164). Anti-PR3 antibodies prevent the inactivation of PR3 by its natural inhibitor alfa-1 antitrypsin (1-AT) (165). The inhibitory activity differs between sera and is not directly related to the amount of specific antibody. In a longitudinal study it has been shown that disease activity in patients with WG correlates with the amount of inhibitory activity of the serum rather than with the titer of PR3-ANCA (166). Likewise, MPO-ANCA may interfere with binding of MPO to its natural inhibitor ceruloplasmin (167). This suggets that escape of the enzyme from its inactivation may contribute to the inflammatory process.

The phenotypic expression of 1-AT is highly polymorphic: severely, medium and non-deficient proteinase inhibitor phenotypes can be distinguished (168,169). The prevalence of ANCA of diverse specificity was remarkable among a population with the severely deficient phenotype (170). These data suggest that deficient 1-AT activity is involved in the induction of ANCA.

Interaction of ANCA with endothelial cells

As mentioned above, the antigens recognized by MPO-ANCA and PR3-ANCA are constituents of granules from myeloid cells and are thought to be specific for cells of the monomyeloid lineage. Re-

cent studies have demonstrated, however, that PR3 can be expressed in other cell types as well, in particular in renal carcinoma cells (171) and human endothelial cells (172). These cells, primed by cytokines (TNF- , IL-1, IFN-), express PR3 in their cytoplasm and cell surface making PR3 available for interaction with ANCA. Others have not been able to show endothelial PR3 expression (173). MPO and PR3 are cationic proteins and as such may bind to anionic structures such as the glomerular basement membrane (GBM) and the surface of endothelial cells. It has, indeed, been shown that ANCA can bind to cultured human endothelial cells incubated with MPO (174). Under these conditions, anti-MPO can induce complement-dependent endothelial cell lysis. *In vitro* incubation of endothelial cells with PR3 induces production of IL-8, endothelial cell apoptosis, and detachment and lysis of the endothelium (175-177). Although these *in vitro* studies have demonstrated that ANCA can interact, directly or indirectly, with endothelial cells, direct immunofluorescence studies from lesional tissue have failed to show significant deposition of IgG along the endothelium or the glomerular capillary wall. Thus, the *in vivo* relevance of those *in vitro* studies has yet to be established.

ANCA-mediated neutrophil and monocyte activation

In vitro activation of neutrophils by PR3- and MPO-ANCA requires prior priming of cells by low doses of proinflammatory cytokines, such as TNF- , IL-1 or LPS. Priming is associated with translocation of ANCA target antigens on the surface of neutrophils, which makes them accessible for interaction with antibodies (178-180). ANCA (anti-PR3 or anti-MPO), bound to appropriately displayed target antigens, have been shown to enhance neutrophil oxidative burst and degranulation (178,179,181,182). Released neutrolytic enzymes may produce tissue injury by means that include induction of endothelial cell apoptosis, detachment and cytolysis (176,177,183,184). In addition, they secrete inflammatory mediators such as TNF- , IL-1, IL-8 and leukotriene B4 (185,186). Upon activation by ANCA, PMN express increased levels of adhesion molecules, including 2-integrins, that facilitate binding to and transmigration through the endothelial monolayer (187,188).

The precise mechanism underlying ANCA-induced neutrophil activation is unclear. It appears that neutrophil activation involves both specific binding of the antibodies *via* F(ab)2-fragments to surface expressed PR3 or MPO and interaction of their Fc-fragments with Fc R on neutrophils, particularly with Fc RIIa-receptor (189,190). Very recently, it has been shown that ligation of Fc RIIa and Fc RIIIb is necessary for ANCA-induced neutrophil activation, but that signaling cascades by ANCA were different from the signal pathways used by Fc R engagement only (191).

The effect of ANCA on monocytes has been studied to a lesser extent. ANCA have been shown to activate monocytes to the production of reactive oxygen species (192), IL-8, a potent attractant for PMN (193), and monocyte chemoattractant protein-1(MCP-1), even without prior priming (194). Increased production of MCP-1 at the site of inflammation could play an important role in the formation of granulomas by amplification of local monocyte recruitment. Monocytes from the peripheral blood of patients with WG showed the presence of MPO and PR3 on their cell surface during active disease, and monocytes do express the Fc RIIa receptor and CD18 (195).

T-cell reactivity to ANCA-associated antigens

The presence of cellular infiltrates in inflammatory lesions, frenquently also in the form of granulomas, in patients with WG and some other vasculitis, such as polyarteritis nodosa, Kawasaki and Takayasu s disease, suggests an important role of cell-mediated immunity in the pathogenesis of these diseases (196-200). Immunopathologic studies have shown that the inflammatory infiltrate is composed mainly of activated T-lymphocytes, the majority of which are CD4+ and macrophages (196,197,199,201-203). In agreement with these data, the levels of soluble IL-2 receptor are elevated in WG patients and increase prior to major relapses, providing indirect evidence for a role of activated T-cells in systemic vasculitis (204,205). T-lymphocytes isolated from WG patients proliferate in response to a crude neutrophil extract containing PR3 and MPO (206,207). WG patients re-

sponded more frequently and strongly to PR3 than controls (208,209). However, not all WG patients responded to PR3, and T-cell proliferation was also found in healthy controls (209). In this study, strong IL-10 production elicited by PR3 *in vitro* may have an important function *in vivo*. Recently, it has been shown that B-lymphocyte activation is related to active disease, whereas T-lymphocyte activation persists during remission of the disease in patients with WG, which points to an intrinsically disordered immune system in this disease.

The in vivo role of ANCA in experimental models

Although all of the aforementioned mechanisms may be operative *in vivo* in idiopathic vasculitis, conclusive evidence for the pathogenicity of ANCA awaits a convincing animal model of ANCA-induced vasculitis. Several animal models have now emerged that may be relevant in this respect (17). Unfortunately, human anti-PR3 antibodies only react with neutrophils of baboons and not with those of lower species. Also, immunization of rats with human PR3 does not result in antibodies reacting with rat neutrophils. Interestingly, a recent study has shown that rats injected with syngeneic rat apoptotic neutrophils develop ANCA, but the specificity of these ANCA could not be established and they did not seem to be PR3 or MPO (210). Recently, PR3 has been detected and cloned in mice (211). Unfortunately, monoclonal and polyclonal antibodies to human PR3 do not react with cloned mouse PR3. Antihuman PR3 was induced in mice by idiotypic manipulation (212,213). The mice were immunized with affinity-purified PR3-IgG from two WG patients (212). After two weeks, mice developed anti-idiotypic (mouse anti-human anti-PR3 antibodies, ab2) and, after four months, anti-anti-idiotypic antibodies reacting with human PR3 (ab3). In addition, the sera derived from these mice also reacted with human MPO and endothelial surface proteins. Histopathologically, sterile microabscesses developed in the lungs after eight months, and mice died at 8-15 months after immunization with anti-PR3-IgG. The ab3 antibodies were tested for their neutrophil activating ability showing that these antibodies were capable to induce adhesion of human neutrophils to fibronectin and to initiate a respiratory burst (213). These studies demonstrate that dysregulation of the idiotypic network may lead to the development of pathogenic autoantibodies. Whether this theory is relevant for the induction of ANCA-associated vasculitis cannot, however, be proven until pathogenic idiotypes on bacterial or viral antigens are identified.

Several animal models for anti-MPO-associated vasculitis/glomerulonephritis have been described. Brown Norway rats (the Th2-responder type), exposed to mercuric cloride (HgCl2), developed a generalized autoimmune disease, mediated by T-cell dependent polyclonal B-cell activation (214). The HgCl2-induced autoimmune syndrome is characterized by lymphoproliferation, high IL-4 production and hypergammaglobulinemia (215). In addition, a multitude of IgG autoantibodies appeared in these rats, including antibodies to DNA, collagen, thyroglobulin and components of the glomerular basement membrane (216,217). In addition to the above autoantibodies, HgCl2-treated rats developed antibodies to MPO (218). These rats also developed widespread tissue injury including necrotizing vasculitis in the submucosal vessels of the duodenum and cecum (219). Subsequent studies have demonstrated an important role for autoreactive CD4+ T cells which, upon transfer into healthy animals, can induce the disease (220). On the other hand, it was shown that CD4+OX22 high T-cells were protective since depletion of these cells aggravates the severity of tissue injury, although the autoantibody response was not affected (221). Treatment with cyclosporine during the early phase of the disease diminished tissue injury and delayed the rise in anti-MPO and anti-GBM autoantibodies (222). In contrast, treatment with cyclosporine during the late phase of the disease exacerbated tissue injury although the development of anti-MPO and anti-GBM autoantibodies was completely suppressed. Taken together, these observations suggest that HgCl2-induced vasculitis is primarily T-cell dependent.

The MRL/lpr strain of mice spontaneously develop lymphoproliferation, proliferative glomerulonephritis and systemic necrotizing vasculitis of small and medium-sized arteries and arterioles, that particularly affect the kidney and gallbladder (223,224). The spontaneous development of tissue injury in these mice is immunologically characterized by accumulation of T-cells with polyclonal

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B-cell stimulation resulting in the appearance of rheumatoid factors and autoantibodies of diverse specificity including autoantibodies against ds-DNA, ss-DNA, histones, smooth muscle and ANCA (225). However, monoclonal IgG antibodies derived from these mice were found to be polyreactive, recognizing MPO, LF and DNA (226).

SCG/KJ strain of mice, derived from the F1 hybrid of BXSB and MRL/lpr mice, spontaneously develop rapidly progressive glomerulonephritis and necrotizing vasculitis (227). The sera from these mice produce a perinuclear ANCA staining pattern on human and murine neutrophils and react with murine neutrophil extract and human MPO by ELISA (228,229). Transfer of MPO specific hybridomas derived from SCG/KJ mice into the peritoneum of nude mice induced proteinuria, although histologically no evidence for glomerulonephritis or vasculitis was found (228).

Induction of a selective autoimmune response to MPO is a more direct method of studying the pathophysiologic role of anti-MPO. Immunization of Brown Norway rats with human MPO in complete Freund s adjuvant results in the development of anti-human MPO antibodies cross-reacting with rat MPO and delayed-type hypersensitivity to human MPO (230,231). The autoimmune response alone does not result in clinical lesions. However, after unilateral perfusion of the left kidney with products of activated neutrophils consisting of proteolytic enzymes (HLE and PR3), MPO and its substrate H2O2, these MPO-immunized rats develop necrotizing crescentic glomerulonephritis with interstitial tubulonephritis and vasculitis. Systemic injection of lysosomal enzymes, MPO and H2O2 in the jugular vein of MPO-immunized rats also resulted in small vessel vasculitis and giant cell formation in the lungs and guts but not in the kidneys (231). The localization of the inflammatory process in the lungs and guts suggests that local factors, e.g., mucosa-associated microbial elements, are involved.

The pathogenic potential of anti-MPO antibodies *in vivo* has also been demonstrated in glomerular basement membrane (anti-GBM) nephritis in Brown Norway rats previously immunized with human MPO (232). Immunized rats not only developed antibodies to human MPO but to rat MPO as well. Following injection of a subnephritogenic dose of rabbit anti-rat GBM antibodies, severe glomerulonephritis developed characterized by hematuria, proteinuria, fibrinoid necrosis of glomerular capillaries, extensive fibrin accumulation of monocytes/macrophages and crescent formation. In control immunized rats injected with the anti-GBM antibodies, only mild glomerulonephritis was found without evidence for crescent formation, suggesting that the autologous anti-rat MPO antibodies are responsible for the exacerbation of the anti-GBM disease.

These studies suport the hypothesis that the presence of ANCA itself is not sufficient to cause tissue injury. Additional factors inducing a proinflammatory environment are needed, which initially result in priming of neutrophils and monocytes and activation of the endothelium. After that, ANCA activate primed neutrophils/monocytes and form *in situ* immune complexes with ANCA antigens released from activated neutrophils, thereby exacerbating the inflammatory response and finally leading to vasculitis and tissue destruction.

INTEGRATIVE VIEW OF THE POTENTIAL IMMUNE MECHANISMS IN THE PATHOGENESIS OF ANCA-ASSOCIATED VASCULITIS

Schematic presentation of an integrative view of ANCA-mediated vascular tissue damage is shown in Figure 2. The model is based on the four prerequisites for endothelial cell damage by ANCA: 1) the presence of ANCA; 2) expression of target antigens for ANCA on primed neutrophils and monocytes; 3) the necessity of an interaction between primed neutrophils and endothelium by means of 2-integrins; and 4) activation of endothelial cells (7,8,16-20).

Infection or other tissue injury induces an inflammatory response associated with cytokine release (TNF- , IL-1) resulting in priming of neutrophils and/or monocytes. Priming of these cells prompts expression of ANCA target antigens (PR3, MPO) on their surface. In this manner the autoantigen becomes accessible to the circulating ANCA. Primed neutrophils and monocytes also express Fc -receptors on their surface. Consequently, ANCA can bind to their surface *via* their constant region (Fc). Simultaneous binding of circulating antigen released from activated inflammatory cells may cause crosslinking of Fc -receptors initating the

Fc -receptor engagement-dependent activation events.

The mechanisms by which ANCA production is triggered and perpetuated remain unclear. It has been postulated that superantigens and defect(s) in apoptosis or in the removal of apoptotic cells may lead to the development of ANCA through bypassing normal mechanisms of tolerance (233,234). It is well known that superantigens from bacteria and viruses activate large populations of T-cells expressing -chain variable gene segments of the T-cell receptor (TCR-V). These T-cells produce large amounts of proinflammatory cytokines and help the autoreactive B-cells produce ANCA. It has been reported that the majority of patients with WG are chronic nasal carriers of *Staphylococcus aureus* and that carriers experience a relapse nearly eight times more frequently than noncarriers (79). Some studies in these patients have demonstrated skewing of the TCR-V repertoire in peripheral T-cells, suggestive of superantigen triggering of T-cells (235). Also, the clustering of autoantibodies has been observed on the surface of apoptotic cells in systemic vasculitis. Morever, it has been found that ANCA interact with clustered granule constituents on the surface of apoptotic neutrophils (236,237).

The binding of ANCA to primed neutrophils and monocytes induces release of cytokines (IL-8, MCP-1) and possibly other factors that are strong chemoattractants for more inflammatory cells. ANCA also induce a respiratory burst with release of reactive oxygen species (ROS) and degranulation with release of lysosomal enzymes (HLE, PR3, MPO), which leads to endothelial cell injury

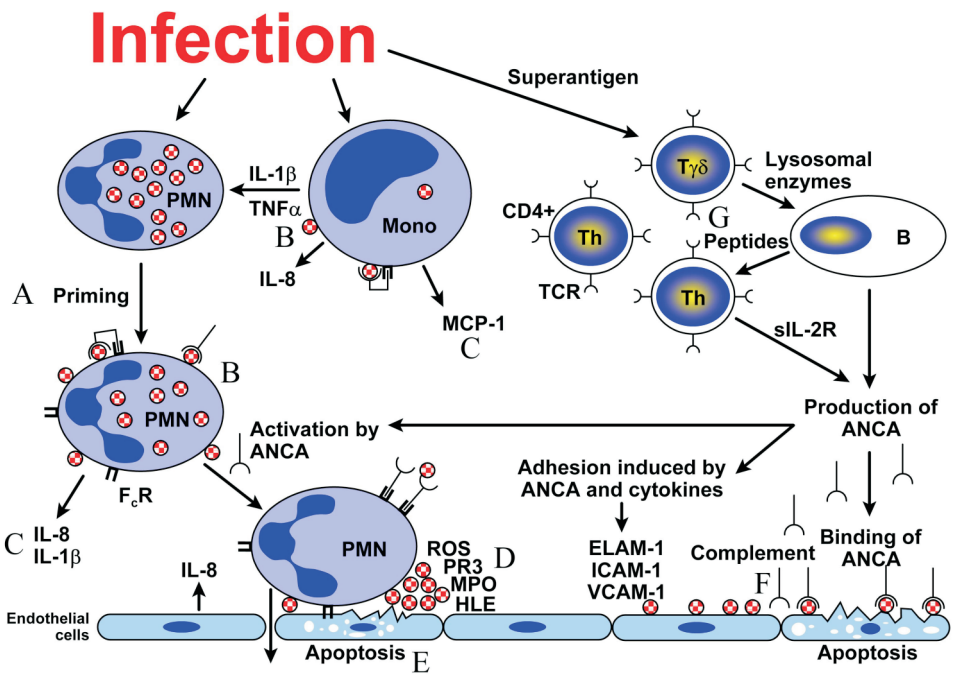

Figure 2. Schematic presentation of an intergrative view of the immune mechanisms involved in the pathogenesis of ANCA-associated vasculitis. Cytokines released due to infection or other tissue injury cause priming of neutrophils and/or monocytes (A) and upregulation of adhesion molecules (ELAM-1, ICAM-1, VCAM-1) on the endothelium. Circulating primed neutrophils and/or monocytes express ANCA antigens (PR3, MPO), adhesion molecules (LFA-1, VLA-4) and Fc R on the cell surface (B). Binding of ANCA to primed neutrophils and monocytes induces release of cytokines such as IL-8, IL-1 , MCP-1 and possibly others factors that are strong chemoattractants for more inflammatory cells possibly leading to granuloma formation (C). Adherence of primed neutrophils and/or monocytes to the endothelium followed by activation of these cells by ANCA. Activated neutrophils and monocytes release reactive oxygen species (ROS), which leads to endothelial cell injury and eventually to necrotising inflammation (D). PR3 and MPO from ANCA-activated neutrophils and/or monocytes results in endothelial cell activation, endothelial cell injury, or even endothelial cell apoptosis (E). PR3 and MPO serve as planted antigens resulting in in situ immune complexes, which in turn attract other neutrophils (F). The mechanism by which ANCA production is triggered and perpetuated remain unclear. However, T-cells are though to play a significant role in mediating the production of ANCA by plasma cells, which are derived from antigen-specific B-cells (G). Adapted from ref. 163.

(apoptosis) and eventually to necrotizing inflammation. Except for cytokines, ANCA stimulation also activates endothelial cells resulting in increased adherence of primed and activated neutrophils to their surface. Consequently, the release of cytotoxic neutrophil constituents can directly cause endothelial cell damage by direct cytotoxicity, apoptosis and complement activation by immune complex bound on the endothelial cell surface.

This conceptual scheme is consistent with clinical observations and animal model data, which clearly indicate that ANCA alone do not necessarily lead to disease manifestations. Cofactors that lead to activation of inflammatory and endothelial cells seem to be required to activate various pathogenic mechanisms leading to small vessel vasculitis.

CONCLUSION

The significance of ANCA as diagnostic markers for systemic necrotizing vasculitis or idiopathic necrotizing crescentic glomerulonephritis is well established. Detection of PR3-ANCA strongly suggests a diagnosis of Wegener s granulomatosis, and changes in serum levels of these autoantibodies seem to mirror disease activity. MPO-ANCA are associated with various forms of systemic necrotizing vasculitis including idiopathic necrotizing crescentic glomerulonephritis. The diagnostic significance of ANCA against other target antigens is not clear. Their usefulness in measuring disease activity is at present not clear, although some studies suggest that rises in anti-MPO levels precede relapses.

Many questions remain concerning the pathophysiologic role of ANCA, although both *in vitro* and *in vivo* experimental data strongly support a pathogenic role for ANCA in vasculitis and glomerulonephritis. However, ANCA alone are not sufficient and other, probably exogenous factors seem necessary for disease activation and (re)activation. Circumstantial evidence from human (*Staphylococcus aureus* carriage) and experimental (e.g., the mercuric chloride model) studies suggest that exogenous factors, possibly of a microbial nature, are involved in disease expression.

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Abbrevations

AECA antiendothelial cell antibody
ACPA anticytoplasmic antibody
a1-AT a1-antitrypsin
AIH autoimmune hepatitis
ANA antinuclear antibody
ANCA antineutrophil cytoplasmic antibody
a/P-ANCA 'atypical' perinuclear ANCA
C-ANCA cytoplasmic ANCA
P-ANCA perinuclear ANCA
BPI bactericidal/permeability-increasing protein
CD Crohn's disease
CG cathepsin G
CTD connective tissue diseases
CSS Churg-Strauss syndrome
ELAM endothelial adhesion molecule
ELISA enzyme-linked immunosorbent assay
FcgR Fc-gamma receptors
GBM glomerular basement membrane
GS-ANA granulocyte-specific ANA
HLE human leukocyte elastase
HMG1 and 2 high motility group of nonhistone chromosomal proteins 1 and 2
H2O2 hydrogen peroxide
IB immunoblot
IBD inflammatory bowel disease
ICAM-1 intercellular adhesion molecule-1
IIF indirect immunofluorescence
IFN- interferon-
IL-1b interleukin-1b
IL-8 interleukin-8
iNCGN idiopathic necrotizing crescentic glomerulonephritis
LF lactoferrin
LCV leukocytoclastic vasculitis
LFA-1 lymphocyte function antigen-1
LPS lipopolysaccharide
LZ lysozyme
MCP-1 monocyte chemoattractant protein-1
MPA microscopic polyangiitis
MPO myeloperoxidase
MPO-ANCA myeloperoxidase - antineutrophic cytoplasma antibody
PAN polyarteritis nodosa
PBC primary biliary cirrhosis
PM/DM polymyositis/dermatomyositis
PR3 proteinase-3
PR3-ANCA proteinase-3 antineutrophic cytoplasma antibody

PSC primary sclerosing cholangitis
RA rheumatoid arthritis
RIA radioimmunoassay
ROS reactive oxygen species
SLE systemic lupus erythematosus
SS Sjögren's syndrome
SSc systemic sclerosis
TCR-V -cell receptor
TNF- tumor necrosis factor a
UC ulcerative colitis
VCAM-1 vascular adhesion molecule-1
VLA-4 very late antigen-4
WG Wegener's granulomatosis

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