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REVIEW

ANCA and associated diseases: immunodiagnostic and pathogenetic aspects

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SUMMARY

The past decade has seen an explosion of data on the new group of autoantibodies known collectively as ANCA (anti-neutrophil cytoplasmic antibodies). ANCA are specific for granule proteins of granulocytes and monocytes and induce distinct fluorescence patterns, e.g. the cytoplasmic (classic) cANCA and the perinuclear pANCA. cANCA is induced by antibodies directed against Proteinase 3 (PR3; PR3-ANCA) in about 90% of all ANCA-positive sera, and pANCA is induced by antibodies against myeloperoxidase (MPO; MPO-ANCA) in about 40%. A further staining pattern, which does not have a clear cut association with a distinct granule protein, is sometimes seen in chronic inflammatory bowel diseases. PR3-ANCA are serological markers for Wegener's granulomatosis (WG) and MPO-ANCA are associated with certain subtypes of primary vasculitides. Evidence exists that both the autoantigen and ANCA participate in the pathogenesis of at least the group of 'ANCA-associated vasculitides'.

Keywords anti-neutrophil cytoplasmic antibodies granule proteins vasculitis glomerulonephritis chronic inflammatory bowel disease autoimmunity

INTRODUCTION

Ten years ago autoantibodies directed against the cytoplasmic constituents (ANCA) of polymorphonuclear leucocytes (PMN) and monocytes were found in a small number of patients with non-classified vasculitis [1,2]. The importance of this observation was not recognized until 1985, when a distinct fluorescence pattern (ACPA, later called cANCA) was shown to be associated with Wegener's granulomatosis (WG) [3]. Soon after this initial report, extended studies dramatically increased the immunodiagnostic knowledge of ACPA and WG [4,5]. In 1988 a second ANCA-fluorescence pattern—later termed pANCA (see below)—was described to be associated with (renal) systemic vasculitis [6]. One year later, a third, less clear-cut ANCA subtype was seen in ulcerative colitis [7].

Today the various ANCAs 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 spectrums of vasculitis/glomerulonephritis (ANCA-associated, 'pauci-immune' necrotizing vasculitis/glomerulonephritis) and chronic inflammatory bowel diseases, and to monitor the disease activity of these disorders. Recent studies support the hypothesis that ANCAs and their target antigens may be implicated in the pathogenesis of these diseases, at least in vasculitis [8–11].

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This review article focuses on recent developments in the field of ANCA and includes data presented at the 4th International Workshop on ANCA and the 2nd International Colloquium on Wegener's Granulomatosis and Related Vasculitis Disorders held in Lübeck, Germany.

DETECTION OF ANCA

Several methods are used to detect ANCA: indirect immunofluorescence (IIF), radioimmunoassay (RIA), ELISA, Western blotting, dot blotting and immunoprecipitation. The first method to detect ANCA was IIF and it remains the 'gold standard' of ANCA screening. The reliability of the immunofluorescence assays depends on the type of substrate employed, the source of cells, fixation, storage, incubation and washing steps [12]. Using IIF on alcohol-fixed neutrophils, two different staining patterns can be differentiated: the classic 'cytoplasmic' pattern (cANCA), seen in WG, and the perinuclear pattern (pANCA), which is associated with necrotizing and crescentic glomerulonephritis (renal vasculitis) and microscopic polyarteritis [6]. In addition, there appears to be a further staining pattern which represents a peculiar mixture of both cANCA and pANCA ('snow-drift') pattern [13], called atypical or xANCA by some [14]. Clinically, this pattern is associated with a subgroup of patients with chronic inflammatory bowel disease [7,13].

SOLID PHASE ASSAYS

Problems of interpretation resulted initially from too little experience in reading the fluorescence pattern [15]. To improve the specificity and to circumvent problems with the interpretation of the IIF pattern, solid-phase immunoassays with highly purified target antigens, such as ELISAs, have been applied [6,16,17]. The first solid-phase assays for the detection of ANCA used crude preparations (acid extract and total nitrogen cavited PMN) and preparations of azurophil granules [18]. These extracts are not suitable for differentiating between various autoantibodies. The solid-phase assays were greatly improved when it was detected that the cANCA antigen is mainly Proteinase 3 (PR3) [19] and after the discovery that a major pANCA antigen is myeloperoxidase (MPO) [6]. MPO and other well characterized proteins, such as human leucocyte elastase (HLE) and lactoferrin (LF), have been used in solid-phase assays for pANCA differentiation in many laboratories. IIF correlates well with solid-phase assay with respect to the number of positive sera, but its titres show a low correlation to solid-phase readings. Between 80% and 90% of IIF-positive samples are positive in ELISA, and about 90% of ELISA-positive samples are positive in IIF [20]. Standardization of the currently available ELISA assays is underway in a multicentre study sponsored by the European Community. The initial data recently presented underline the need for further improvement of most ELISA assays [21]. Today, depending on the degree of purification of the antigens used, ELISA may be used as a complementary method to IIF in order to determine the specificity of ANCA, e.g. PR3-ANCA, MPO-ANCA, etc.

Molecular cloning of autoantigens has been developed as an alternative for the purification of intracellular proteins. Expression of PR3 as recombinant fusion proteins in *Escherichia coli* and synthesis by wheat germ ribosomes *in vitro* have been done. Interestingly, cANCA sera showed little or no binding to rPR3 [22].

cANCA, WEGENER'S AUTOANTIGEN AND WG

WG was first distinguished as a clinicopathologic entity by Friedrich Wegener in 1936 [23]. He established that this 'rhinogenic granulomatosis' preferentially exhibits granulomatous lesions in the respiratory tract in addition to vasculitis. Clinical and histopathological studies have revealed that WG follows a two-phase course [4,24]. In most patients, the upper and lower respiratory tract is affected first (initial, predominantly granulomatous phase). If left untreated, this limited form can turn into a generalized vasculitic disease leading to necrotizing crescentic glomerulonephritis, pulmonary capillaritis, and associated constitutional symptoms. In its full-blown phase the disease is life-threatening and requires highly aggressive immunosuppressive treatment [25]. Today, the diagnosis of WG is based on typical clinical findings and supporting histologic data (American College of Rheumatology classification) [26]. In some patients, especially those with limited disease, the diagnosis can be difficult to substantiate. Limited forms of WG appear to be more common than previously thought, probably because of increased awareness of the disease and because of the widespread use of ANCA testing [27]. No specific diagnostic laboratory test was available until cANCA were described. The specificity of cANCA for biopsy-proven WG was shown to be around 90%. The sensitivity depends on the extent and activity of disease: it is about 50% for patients in the initial phase and close to 100% for patients with active generalized disease (systemic phase). In complete remission, cANCA is not detectable in most patients, while in partial remission titres are usually very low [3,28–30]. It has been shown that rising titres may predict relapse [31,32] and help to differentiate relapses from superimposed (opportunistic) infections [28,33]. However, since in a minority of patients cANCA titres do not follow disease activity [34], titres alone should not be used as the major criterion for changing treatment protocols as discussed [31].

OTHER DISEASE ASSOCIATION

However, cANCA can be seen in a minority of patients with closely related disorders (Table 1) such as microscopic polyarteritis (mPA), Churg–Strauß syndrome (CSS) and classic polyarteritis nodosa (cPAN) [29,35,36]. Of these sera, only 50% react with PR3 [30]. Clinical studies must demonstrate whether these cANCA-positive, non-WG vasculitides differ from pANCA-positive vasculitides in respect of their clinical course and response to treatment. The same is true of pANCA (MPO-ANCA)-associated CSS and cPAN [37]. Alternatively, some of these vasculitides may comprise overlap syndromes, which can not be classified on clinical grounds. However, 'false positive' cANCA have been reported in infective disorders such as HIV infection [38–40], endocarditis [41], pneumonia, and infections in cystic fibrosis [42]. Furthermore, they were seen in monoclonal gammopathy [43], and in a few cases of malignancy

Table 1. Anti-neutrophil cytoplasmic antibodies (ANCA) subtypes, specificities and associated diseases

Acronyms	Target antigen(s)	Associated diseases
cANCA	Proteinase 3 (CAP 57)	Wegener's granulomatosis, and in a minority of: microscopic polyarteritis, Churg–Strauß syndrome, classic polyarteritis nodosa
pANCA	Myeloperoxidase, elastase, cathepsin G, lysozyme, lactoferrin	Renal vasculitis (microscopic polyarteritis), RPGN rheumatic and collagen vascular disorders
Atypical (x)ANCA or pANCA	Lactoferrin, lysozyme, beta-glucuronidase, cathepsin G	Ulcerative colitis, autoimmune hepatitis, primary sclerosing cholangitis

cANCA, Cytoplasmic anti-neutrophil cytoplasmic antibodies; pANCA, perinuclear anti-neutrophil cytoplasmic antibodies.

without signs of secondary vasculitis [30]. On the other hand, vasculitis can complicate cystic fibrosis [44], infections associated with HIV [45,46] and haematological neoplasia and solid malignant tumours [47]. Additionally, we and others have found cANCA in patients presenting with symptoms compatible with WG, such as sinusitis, nasal septum perforation, gingivitis, subglottic stenosis, episcleritis, glomerulonephritis, etc. [48–50]. Because some of these patients have developed a full blown WG after a couple of years, it is possible that they suffered from mono- or oligosymptomatic variants of WG. Additionally, ANCA has been reported to occur after immunoglobulin replacement therapy in a patient with uveitis [51].

NATURE OF THE cANCA AUTOANTIGEN

After the first report on the association of ANCA with WG, several groups started to investigate the autoantigen(s) involved. In 1989 Goldschmeding *et al.* [52] characterized the target antigen of cANCA as a diisopropylfluorophosphate (DFP)-binding serine protease, which is distinct from neutrophil elastase and cathepsin G (CG) and is located in the primary granules of human neutrophils. Subsequently, Lüdemann, Utecht & Gross [12] identified this serine protease as PR3, an elastinolytic enzyme of 29 kD [53]. By N-terminal amino acid sequencing and cDNA studies, PR3 has been shown to be identical to functionally different molecules, called variously azurophil granule protein (AGP7) [54], p29 [55], and myeloblastin. The multifunctional protein PR3 was renamed 'Wegener's autoantigen' [56] (Table 1).

PR3 has been detected not only in cells derived from bone marrow stem cells. Recently, it was described in a human renal cancer line (SK-RC11) [57]. PR3 consists of 229 amino acid residues. The isoelectric point is at 9·5 [58], which is in contrast to the isoelectric point of 7·9 as calculated from the primary amino acid sequence. This phenomenon might reflect the preferential distribution of positively charged amino acids on the surface of the protein. Distribution of surface charges might be important for the appearance of amphiphilicity, which is a functionally significant characteristic suspected in a number of antimicrobial polypeptides [58]. Indeed, a weak antimicrobial activity has been described for AGP7 against *E. coli* and *Candida albicans* [59] (Table 2). Thus, AGP7 may be part of the oxygen- and MPO-independent antimicrobial system of leucocytes, which includes granular cationic proteins. The question arises, however, whether autoantibodies to PR3 might inhibit the antimicrobicidal role of the enzyme, thus leading to a defect in the leucocyte's defence mechanism. This is certainly of interest in a disease which in some features resembles chronic infectious diseases, e.g. lues (granulomatous inflammation with secondary vasculitis), and which responds at least during its initial phase to the antimicrobial substance cotrimoxazol [60,61].

Myeloblastin, a PR3 homologue isolated from the human leukaemia cell line HL-60, participates in the regulation of myeloid differentiation. Down-regulation of myeloblastin expression by an antisense oligonucleotide stops the proliferation of HL-60 cells and induces differentiation [62] (Table 2). Recently it was demonstrated that myeloblastin mRNA is also expressed in cells from patients with acute myeloid leukaemia [63]. It is possible that inhibition of PR3 by cANCA might also cause changes in granulocyte or monocyte differentiation. The excessive leukocytosis seen in fulminant WG might be the consequence of this autoantibody-induced differentiation process [56]. However, such speculations must be regarded with scepticism because a single defect in the lysosomal protein production (e.g. in MPO deficiency) is compensated for by other defence mechanisms and leukocytosis in generalized WG could

Table 2. Characterization of Proteinase 3 'Wegener's autoantigen'

Synonyms	p29 Myeloblastin AGP7 Wegener's autoantigen
Localization	Azurophil granules of PMN and MPO-positive granules of monocytes HL60 promyelocytic cell line and cells from patients with acute myeloic leukaemia Renal human kidney carcinoma cells (SK-RC11)
Biochemical characterization	Neutral serine protease (trypsin/chymotrypsin superfamily) Molecular weight *ca* 29 kD Isoelectric point >9·5 229 amino acids, 54% sequence homology with elastase
Functional characteristics	Enzymatic activity (proteolytic activity): degradation of elastin, haemoglobin, fibronectin, laminin, vitronectin, and collagen type IV Inhibitor profile: alpha-1AT, alpha-2-macroglobulin, elafin Antimicrobial effect: against *E coli* and *C. albicans* Regulation of myeloid differentiation Serves as an autoantigen Pathophysiological implications

References are given in the text.
AGP7, Azurophil granule protein; PMN, polymorphonuclear leucocytes; MPO, myeloperoxidase.

as well be induced by the proinflammatory cytokines seen in this disease state.

Another important feature of PR3 is its enzymatic activity and substrate specificity. It degrades elastin, haemoglobin [12], fibronectin, laminin, vitronectin and collagen type IV [64] (Table 2). Furthermore, Kao *et al.* [53] showed that PR3 produces emphysema in hamsters. Moreover, serine proteases from activated neutrophils, monocytes and macrophages appear to be at least partially responsible for tissue injury seen in vasculitis and glomerulonephritis [65]. Elastase isolated from experimentally induced granulomas in mice is reported to digest elastic fibres in vessel walls [66]. In WG, evidence is accumulating that lysosomal proteins can be observed in the circulation (e.g. lysozyme) and in tissue sections (C. Mrowka, personal communication, [67–69]), where they might be responsible for tissue injury. Neutrophil serine proteases, e.g. HLE and CG, can mediate glomerular injury *in vivo* and lead to proteinuria [70]. In Kawasaki's syndrome, the immunoreactive PMN elastase/alpha-1AT complex was detected in the circulation [71] and eosinophil cationic protein was found to be deposited in vascular lesions of temporal arteritis [72].

In humans, the elastinolytic activity of PR3 is inhibited by various potent natural inhibitors, such as alpha-1-antitrypsin and alpha-2-macroglobulin [12]. Another very potent natural inhibitor of PR3 is elafin, as recently described by Wiedow *et al.* [73]. We found that most but not all anti-PR3-IgG isolated from the serum of several WG patients inhibited the elastinolytic activity of PR3, which indicates that some but not all of the antibodies bind directly to the active site of the enzyme, as recently confirmed by van der Wiel *et al.* [74]. Furthermore, we found that cANCA are not able to prevent binding of alpha-1AT to PR3. This is in contrast to recent findings of van der Wiel *et al.* [74], who reported that, in contrast to pANCA IgG, cANCA IgG inhibits the formation of PR3-alpha-1AT complexes and thus prevents the inactivation of PR3. From the clinical point of view this observation is of interest because alpha-1AT deficiency can be associated with vasculitis [75].

GRANULOMA FORMATION AND (AUTOREACTIVE) T CELLS

As mentioned above, WG typically begins not as a vasculitic but as a granulomatous disease ('initial phase'). Granuloma was the name originally given to a chronic inflammatory mass resembling a tumour. The use of the term is now restricted to histological lesions largely composed of lymphocytes and cells of the myelomonocyte lineage. Granulomas are found in infectious diseases (e.g. tuberculosis, lues), immunodeficiency states (e.g. hypogammaglobulinaemia), in chronic granulomatous disease of children, and in diseases of unknown etiology (e.g. Crohn's and Boeck's diseases). In chronic granulomatous disease, neutrophils do not develop a respiratory burst after phagocytosis, and the granuloma may represent a cellular attempt to eliminate infectious agents. WG resembles chronic granulomatous disease in a few clinical aspects and in its response to treatment (e.g. with cotrimoxazole and corticosteroids) [24]. It may or may not be induced by infectious agents. If PR3 is involved in defence mechanisms (e.g. against microbial infections), inhibition of PR3 by cANCA could result in an impairment of the defence system. Thus granuloma formation in WG, as in chronic granulomatous disease, could be a compensatory mechanism for eliminating infections, although this would not explain those cases of WG with granuloma formation which are ANCA-negative in the initial phase (*ca* 50%).

The clinical response of WG to corticosteroids does not argue against this model, since in chronic granulomatous disease granulomas can be dramatically reversed by corticosteroids. However, the infectious agents are not eliminated by this treatment. Thus the granuloma can be reduced but generally the disease will not be stopped [25].

Granuloma formation usually indicates a state of T cell hypersensitivity, and immunohistological studies in WG have revealed a predominance of $CD4^+$ cells in biopsies from the kidneys [76]. In cellular crescents of rapidly progressive glomerulonephritis (RPGN) kidney biopsies and in the peripheral blood of WG patients, activated ($CD25^+$) T cells are also elevated. Increased levels of serum cytokines (e.g. tumour necrosis factor-alpha (TNF-α), IL-6) during the acute disease phase are indirect indicators of T cell activation [77,78]. Elevated concentrations of soluble IL-2 receptor (sIL-2R) have been detected in WG sera, and the levels were shown to correlate with disease activity. It has been demonstrated that even in complete remission, sIL-2R levels tend to be elevated in WG [79]. However, T cell responses to neutrophil extract did not differ between vasculitis patients and controls. There were only low levels of antigen-specific proliferation, and these could not be amplified by *in vitro* selection [80]. In contrast to these findings, T cells from WG patients can proliferate after exposure to highly purified PR3 (W. Mayet, personal communication). The IgG subclass distribution of cANCA shows a high prevalence of IgG4-class antibody [76] together with increased total IgG4. This suggests repeated antigen stimulation in a T cell response and contrasts with the IgG subclass distribution of, for example, antinuclear antibodies, which are mainly restricted to IgG1 and IgG3 [11] (Table 3).

cANCA AND INFECTION

Indirect evidence suggests that microbial infections may be involved in the pathogenesis of ANCA-associated diseases; Wegener himself postulated a relationship between infection and WG [23]. In most patients with ANCA-associated systemic vasculitides, the prodromal phase resembles a flue-like syndrome [35] and infections appear to occur shortly before and during relapses of WG. In addition, beneficial effects of

Table 3. Indications of the involvement of T cells in Wegener's granulomatosis (WG) and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis

Granuloma formation in WG
Renal interstitial T cell infiltration
$CD4^+$ T cells in the respiratory tract and kidneys
Elevated levels of $CD25^+$ T cells
Elevated concentrations of sIL-2R
Prevalence of IgG4 subclass antibodies within cANCA
Proliferation of T cells after stimulation with PR3

cANCA, Cytoplasmic ANCA; PR3, Proteinase 3.

antibiotic therapy with sulphamethoxazole-trimetoprim have been observed in (initial phase) WG [60,61]. cANCA and pANCA were described in infectious disorders without evidence of vasculitis (septicaemia, bacterial pneumonia) [42], but these findings have not been confirmed by others [81]. Stegemann *et al.* [82] observed that chronic nasal carriage of *Staphylococcus aureus* made WG patients more prone to relapse. Recently, Esaguy *et al.* [83] demonstrated cross-reactivity between human LF and a 65-kD protein of tubercle and leprosy bacilli; both tuberculosis and leprosy can resemble WG histomorphologically in some aspects and can be associated with secondary vasculitis [84]. In summary, however, there is no direct evidence that any identified infectious agent can induce ANCA and/or ANCA-associated diseases.

pANCA, MPO-ANCA AND VASCULITIS

Besides the classic cytoplasmic staining pattern, a second, perinuclear pattern can be seen in IIF on alcohol-fixed neutrophils. This so-called pANCA pattern is an artefact of alcohol fixation caused by the translocation of basic (positively charged) proteins to the negatively charged nucleus [6,80]. If fixation is done with formaldehyde, which prevents the translocation of proteins, pANCA-like cANCA exhibit a cytoplasmic staining pattern. Because pANCA-fluorescence can resemble that of antinuclear antibodies (ANA), these have to be excluded by additional testing on HEp2 cells. Furthermore, sera in which pANCA is suspected should be analysed on formaldehyde-fixed PMN. This is supported by a recent observation of Cats *et al.* [85]: sera from patients with active giant-cell arteritis did not stain alcohol-fixed neutrophils, but showed a cytoplasmic fluorescence pattern on PMN fixed with paraformaldehyde/aceton. Using sequential fractions of neutrophil extracts isolated by high performance liquid chromatography (HPLC), antibodies directed against a 200-kD fraction were found in Takayasu's arteritis, although IIF did not show a typical ANCA fluorescence pattern [86]. Thus, the list of ANCA-associated (primary) vasculitides is still growing.

The most important target antigen of pANCA is myeloperoxidase. MPO-ANCA are found in about 60% of cases with microscopic polyarteritis and necrotizing glomerulonephritis. They facilitate the diagnostic procedure if these diseases are suspected [87] and may be helpful in monitoring disease activity. In contrast to PR3-ANCA, the correlation of MPO-ANCA with disease activity has not been documented by extensive studies, but has been described in several cases. Together with H_2O_2 and halide, MPO constitutes a potent microbicidal system within neutrophil granulocytes. However, additional target antigens such as HLE, CG, and LF have been described to be associated with this fluorescence pattern [17,30]. Additionally, lysozyme has been shown to be a target of pANCA. But still in about 50% of all pANCA-positive sera the target antigens remain to be identified [88]. Recently, Martensson & Nässberger [89] detected circulating autoantibodies directed against beta-glucuronidase (an enzyme located in primary granules of PMN). Thus, the list of target antigens for pANCA will continue to grow.

CLINICAL ASSOCIATIONS OF pANCA

In contrast to cANCA, pANCA are not specific for a single disease entity but tend to be associated with disease groups which share several common clinical and histological features [90]. pANCA are found in: (i) systemic vasculitides such as microscopic polyarteritis, Churg–Strauß syndrome, and in a minority of cases of classic polyarteritis nodosa, and rarely in secondary vasculitis; (ii) necrotizing crescentic glomerulonephritis and rarely in other forms of glomerulonephritis; (iii) chronic inflammatory rheumatic disorders, e.g. rheumatoid arthritis, and in Still's and Felty's syndromes; (iv) collagen vascular diseases such as systemic lupus erythematosus (SLE), and in Sjögren's syndrome without obvious signs of vasculitis; and (v) chronic inflammatory bowel disease and associated disorders. On the other hand, we found pANCA in less than 3% of 400 cases with biopsy-proven WG [30]. cANCA and pANCA are rarely combined in one serum [90]. Additionally, pANCA (MPO-ANCA) can be induced by drugs (hydralazine, clozapine, L-tryphtophan) [91–93] which can induce diseases associated with vasculitis (e.g. glomerulonephritis [91], pulmonary vasculitis [45,94,95]). Occupational exposure to environmental factors such as silica dust may provoke MPO-ANCA-associated RPGN: in these patients, the frequency of silica exposure was found to be 10 times that in a control group [96]. In 30% of patients suffering from Goodpasture's syndrome, ANCA were detected in addition to antibodies directed against the glomerular basement membrane [97]. However, improved methods of antibody detection drastically reduced the frequency of sera exhibiting both groups of antibodies [98]. These serological observations correspond with earlier case reports of the coexistence of anti-glomerular basement membrane disease and WG, and with the presence of vasculitis in renal biopsies from Goodpasture patients [99]. Furthermore, MPO-ANCA were reported in infective disorders [100].

pANCA AND RENAL DISEASE

Glomerular and other vascular lesions in ANCA-associated systemic and/or limited renal vasculitis are identical [101]. In the kidney, they consist of a necrotizing and/or crescentic glomerulonephritis. Independently of their localization (e.g. kidney, lung, muscle, etc.), the vascular lesions are characterized by their focal distribution, infiltration by granulocytes (at least during the acute phase), necrotizing inflammation and the absence of immunohistological evidence of immunocomplex deposition ('pauci-immune' vasculitis). One of the most life-threatening clinical manifestations of systemic involvement is the pulmonary haemorrhage syndrome caused by a necrotizing alveolar capillaritis and the RPGN leading to renal failure within a few days or weeks (pulmonary renal syndrome, PRS). Although all these symptoms can occur in WG—either together or as single events—these vasculitides do not exhibit Wegener's granuloma, although they can rarely be associated with cANCA (PR3-ANCA). This constellation has been called 'Wegener's vasculitis' [87], but this term is not generally accepted. In conclusion, complete and incomplete PRS can be induced by several immune-mediated mechanisms, e.g. by glomerular basement membrane autoantibodies (Goodpasture's syndrome), by immune complexes (e.g. SLE) and by ANCA. Thus, ANCA represent a 'third' group of autoantibodies associated with this

life-threatening condition, and are therefore of major diagnostic importance.

pANCA not directed against MPO are of special interest for rheumatologists and gastroenterologists. Coremans *et al.* [102] demonstrated pANCA directed against LF in 45% of patients suffering from rheumatoid vasculitis. In control patients with rheumatoid arthritis not complicated by vasculitis, LF-ANCA were seen in only 2%. Recent data indicate that pANCA-positive rheumatoid arthritis has a clinically more aggressive course with a higher frequency of systemic complications [103]. Juby *et al.* [104] found pANCA of undetermined specificity in 30% of patients with Felty's syndrome.

PR3/MPO-ANCA AND PATHOGENESIS OF 'PAUCI-IMMUNE' VASCULITIS

The etiology and pathophysiology of idiopathic (primary) systemic necrotizing vasculitides without immune deposits *in situ* ('pauci-immune' vasculitides as well as the renal-restricted variant, idiopathic crescentic glomerulonephritis) are scarcely understood. This contrasts with vasculitis mediated by immune complexes, such as in collagen vascular diseases (e.g. SLE), allergic or infectious diseases (e.g. hepatitis-associated polyarteritis, drug-induced vasculitis). Evidence has been accumulated over the past few years pointing to the possible pathophysiological role of both humoral and cellular autoimmunity against PR3, MPO and other granule proteins in ANCA-associated diseases (Table 1). The presence of these autoantibodies reflects the loss of self tolerance to granule proteins during the development of the vasculitic process, but the cause for the breakdown of the (self) tolerance to PR3 or MPO remains unknown.

It has been suggested that a complex interaction of genetic factors, microbial infections and idiotypic network regulation could be involved in the development of autoimmunity against myeloid granule proteins. In WG, genetic factors seem to be of minor importance: no association was found between any specific HLA antigen and WG [105], and the occurrence in siblings is a rare exception [106]. Neutrophils are involved in the initial vascular (glomerular) injury: this is clearly indicated by histological observations in early glomerular lesions [107], the marked reduction in renal damage following neutrophil depletion in experimental models of glomerulonephritis and by the clinical response to cyclophosphamide [25]. The infiltration of PMN and monocytes was detected in inflammatory lesions of ANCA-associated vasculitis and glomerulonephritis [108]. The vasculitic lesions found in WG and pANCA-associated polyarteritis are morphologically very similar and can not be distinguished by light or immunofluorescence microscopy [101,107]. Moreover, in the absence of granuloma, the clinical picture of both WG and mPAN is indistinguishable if predominantly small vessels are affected. This observation suggests a common immunopathological mechanism for both diseases which may be induced by the common autoantibodies (ANCA). The different pathological manifestations of MPO- and PR3-ANCA-associated disorders might be caused by the different biochemical and functional properties between these two myeloid enzymes. The remainder of this section will be devoted to a review of the most recent concepts of ANCA-mediated vascular injury.

Recently, two groups presented a model in which ANCA and their targets antigens could be involved as a major pathogenetic event in vascular tissue damage [9,10]. The most important phases in this model for ANCA-mediated vascular injury (ANCA-cytokine-sequence theory, Fig. 1) are discussed below. The model is based on ANCA, cytokines and adhesion molecules, PMN (granule proteins) and vascular endothelial cells. ANCA target antigens expressed on the cell surface in response to proinflammatory cytokines may interact with ANCA and lead to excessive activation of PMN (degranulation, generation of reactive oxygen species), and cell lysis and thus may induce necrotizing vasculitis.

In resting human PMN and monocytes, PR3 is mainly located intracellularly in MPO-positive granules [109]. Consequently, the target antigen of cANCA is not accessible to the circulating autoantibody *in vivo*. The following stages may occur during the pathological process:

1 Infection (or as yet unknown disease inherent factor) induces the production of proinflammatory cytokines (e.g. TNF-α, IL-1, IL-8), which are elevated in systemic vasculitis [78]. *In vitro* exposure of PMN to TNF-α/IL-8 leads to a time-dependent translocation of PR3 from their intragranular loci to the cell surface [69]. In this manner the autoantigen becomes accessible to the ANCA *in vivo*. In *ex vivo* studies, we have demonstrated that PR3 can be detected on the cell membrane of PMN from WG and sepsis patients [110]. Thus, the presence of PR3 on PMN plasma membranes is not specific for ANCA-associated disorders but it enables circulating ANCA to bind to their target antigens.
2 Any pathogenetic role of ANCA-activated PMN in systemic vasculitis demands a close connection of PMN to the endothelial cells, the target structure of vasculitis. Cytokine-induced expression of adhesion molecules (eg. LFA-1 and ICAM-1) allows a close contact of PMN to the endothelial monolayer, with resultant shielding from alpha-1AT. Immunohistological studies can demonstrate these molecules in tissue sections from these disease groups [111]. In addition, elevated levels of soluble ICAM-1 were demonstrated in active WG [112]. Recently, it was shown *in vitro* that Fab_2 fragments from ANCA increase neutrophil adhesion to endothelial cells, augmented by prestimulation with TNF-α [113]. Interestingly, increased neutrophil adhesion to endothelium in patients with active vasculitis (WG and cPAN) has been demonstrated [114].
3 Binding of ANCA to granule proteins located on the cell surface of PMN enhances the PMN–endothelial cell interaction and further activates PMN to degranulate and to release toxic oxygen species into the immediate surroundings, e.g. into the isolated microenvironment between PMN and endothelial cells, which may contribute to enhanced tissue damage. Falk *et al.* [115] first demonstrated *in vitro* that ANCA activate neutrophils, indicated by induction of a respiratory burst and degranulation. In addition, Lai *et al.* [116] described how ANCA may selectively affect the signal transduction pathway (inositol phosphate generation and translocation of protein kinase C) in human PMN upon stimulation with a chemotactic peptide. Furthermore, it was detected that in the presence of low levels of cytokines, ANCA stimulate PMN to damage endothelial cells *in vitro*, as measured by ^{51}Cr and ^{111}In release [9,32,117]. This might be

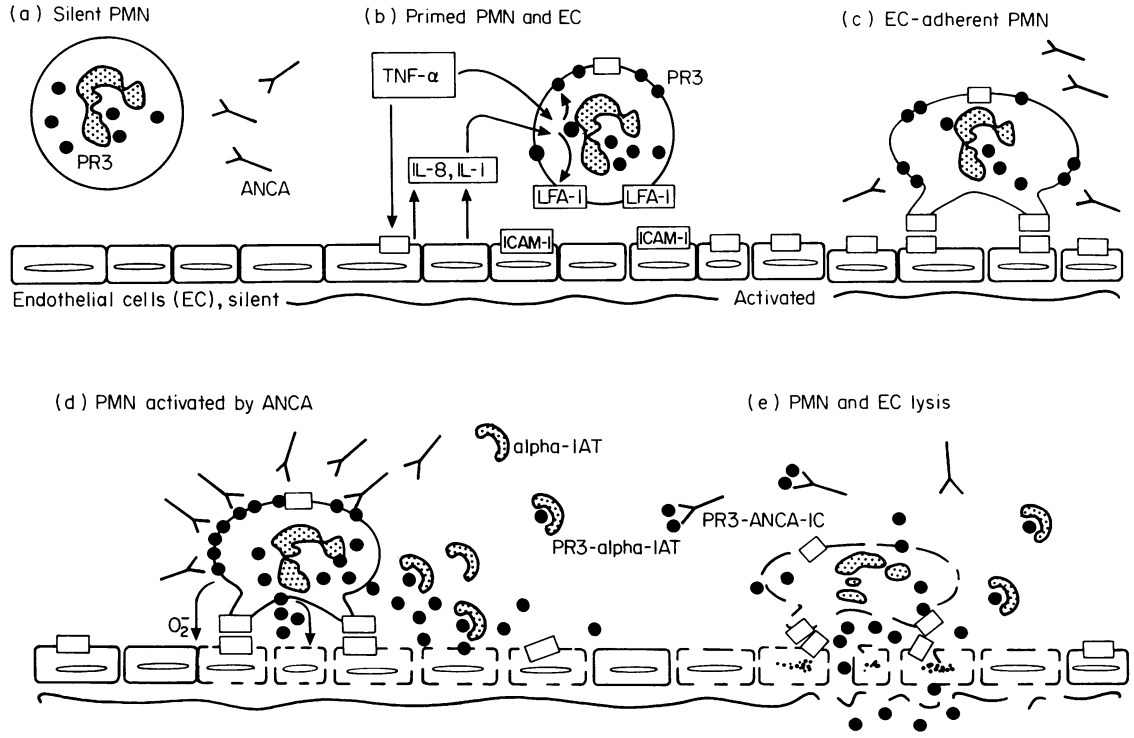

Fig. 1. Anti-neutrophil cytoplasmic antibody (ANCA)-cytokine sequence theory. (a) Resting neutrophil-Proteinase-3 (PR3) mostly sequestered in azurophil granules. (b) Priming of polymorphonuclear leucocytes (PMN) by cytokines. Intracytoplasmic PR3 is translocated to the cell surface and becomes accessible to ANCA. Expression of adhesion molecules. (c) Adhesion of PMN to endothelial cells. (d) Interaction between ANCA and PR3 leads to activation of neutrophils with degranulation, generation of oxygen radicals and endothelial cell injury. (e) This finally results in intravascular lysis of PMN and necrotizing vasculitis.

caused by granule enzymes (e.g. PR3, HLE, MPO), which may bind via charge interaction to the endothelial cell membrane. Here, they can lead to endothelial injury and subsequent necrotizing inflammatory lesions of the vessel wall. Deposition of neutrophil-derived cationic enzymes (e.g. MPO, PR3) on vascular endothelial cells was recently demonstrated *in vitro* [118,119]. Furthermore, ANCA-antigen bound to endothelial cells can be recognized by ANCA and can enhance complement-dependent cytotoxicity [118]. This may result in further vascular damage. In a rat model, Kiser *et al.* [120] observed that intradermal injection of polyclonal rabbit anti-MPO antibodies resulted in increased vascular permeability. It was not determined whether this effect was direct or mediated by anti-MPO-induced leucocyte activation. The role of Fc receptors in this model has to be investigated.

4 Finally, cell lysis of ANCA-activated PMN happens inside the vessel. This is in contrast to localized inflammation, which is characterized by transendothelial migration and chemotaxis of PMN to the inflammatory site. Donald, Edwards & McEvoy [121] found intravascular lysis of PMN in the vessels of WG lesions; in their report, organelles thought to originate from neutrophil cytoplasm were found to be coated by electron-dense material, indicating the presence of immunoglobulin on their surface. In this context, it is interesting that PMN from patients with active WG contain intracytoplasmic IgG as demonstrated by direct immunofluorescence [69].

In an alternative model proposed by Mayet *et al.* [122] vascular endothelial cells may be a direct target of ANCA. They demonstrated by laser scanning microscopy that PR3 is present in small amounts within the cytoplasm of cultured human umbilical vein endothelial cells. A time-dependent translocation of PR3 to the cell membrane was induced by stimulation with TNF-α. This observation was recently confirmed by polymerase chain reaction (PCR) technique. In contrast, by immunohistological method PR3 does not appear to be expressed within endothelial cells in the vessels from WG and healthy tissue [68]. Furthermore, no immune deposits can be observed in the ANCA-associated ('pauci-immune') vasculitis, which makes this highly interesting observation more doubtful with respect to its pathogenetic role.

ANCA AND ANTI-ENDOTHELIAL ANTIBODIES

Three years ago, antibodies directed against endothelial cells were demonstrated by RIA and cell ELISA in WG and MPO-ANCA-associated microscopic polyarteritis [123]. The target antigen(s) remain(s) unknown, but preadsorption studies indicate that anti-endothelial cell antibodies (AECA) and ANCA are two distinct antibody populations [124,125]. Contradictory results were published concerning the cytotoxic effects of AECA in systemic vasculitis [123,125b]. The role of this new group of

antibodies in the pathogenesis of systemic vasculitides remains to be investigated.

ANIMAL MODELS

So far, no animal models are available that closely resemble ANCA-mediated vasculitis. Recently, Quasim *et al.* [126] reported that Brown Norway (BN) rats treated with low doses of mercuric chloride develop necrotizing vasculitis in the gut together with MPO-ANCA. Interestingly, anti-CD4 treatment did not abrogate the disease. BN and Lewis rats immunized with human MPO develop anti-human-MPO antibodies that cross-react with rat-MPO. Histology of the ears showed neutrophil (BN rats) and mononuclear (Lewis rats) infiltrates [127]. However, animal models of WG are complicated by the fact that PR3 is only found in primates (unpublished observation).

ANCA IN CHRONIC INFLAMMATORY BOWEL DISEASE

In addition to the well defined staining patterns of cANCA and pANCA, a third, variable pattern was recently described (atypical or xANCA) [7,13,14,128]. However, most laboratories include this fluorescence pattern in the group of pANCA [7,129,130], because (i) it is not easy to distinguish from the perinuclear pattern in some cases, and (ii) the main target antigen is still unknown.

Clinically, this atypical ANCA is associated with chronic inflammatory bowel disease and related disorders; in ulcerative colitis, they have been reported in between 50% [128,129] and 70% of cases [131]. Rump *et al.* [128] noticed that ANCA titres correlate with disease activity, but this has not been confirmed by others [129,132]. In primary sclerosing cholangitis, a disease frequently associated with ulcerative colitis, ANCA were observed in up to 60% of cases [133]. In contrast, Crohn's disease was reported to be less frequently associated with ANCA, ranging from 2% [128] to 20% [130]. Recently, the spectrum of x/pANCA-associated gastroenterologic diseases was extended to autoimmune hepatitis, where ANCA were found in 50–80% of cases [133,134].

Using specific antigens, this type of ANCA has been shown to be directed against human neutrophil elastase, CG, LF, MPO or even PR3. LF was identified as a target antigen of ANCA in ulcerative colitis in 30% [134] to 50% [135] of sera. However, in more than 80% of x/pANCAs the antigen remains obscure [30].

Recently, an animal model (cotton top tamarin) of spontaneous colitis was investigated for the presence of ANCA to evaluate its potential as a model for the study of the immune response in human ulcerative colitis. Results show that cotton top neutrophils have antigens recognized by IgG from ANCA-positive human ulcerative colitis serum. However, the animals did not develop ANCA themselves [136].

Although it now appears unlikely that ANCA are directly involved in the pathogenesis of chronic inflammatory bowel disease, they may serve as a useful marker analogous to the antimitochondrial antibody in primary biliary cirrhosis.

CONCLUSION

In summary, IIF still remains the 'gold standard' of ANCA detection. However, after the screening procedure done by IIF, the fine specificity (e.g. PR3-ANCA, MPO-ANCA) should be assessed by additional analyses, e.g. ELISA. The detection of ANCA has improved the diagnostic procedure and follow up in patients with WG and has lead to a new diagnostic group within the so-called primary systemic vasculitides ('ANCA-associated vasculitides'). The study of the antibodies has extended understanding of the pathogenesis of 'pauci-immune' vasculitis, as described in the 'ANCA-cytokine sequence theory' [10]. Further studies should focus on the pathogenetic role of antibodies and their interference with the physiological functions of their respective target antigens. In particular, their possible involvement and/or their corresponding autoantigens in granuloma formation, as well as the role of T cells in these recently recognized autoimmune disorders, need to be elucidated. In pANCA, especially, further target antigens must be identified and characterized. Of particular importance is the understanding of the inductive process leading to autoimmunity to granule proteins.

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