Anti-neutrophil cytoplasmic antibodies

Kallenberg, Cees G.M.; Brouwer, Elisabeth; Weening, Jan J.; Cohen Tervaert, Jan W.

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Editor's Note:

The scope of nephrology is, happily, continually expanding. Kidney International *is acutely aware of its responsibility, as the official journal of the International Society of Nephrology, to serve the needs of the entire spectrum of renal clinicians and investigators.*

For some years, the invited editorials appearing at the beginning of each month's issue of Kidney International, *together with Nephrology Forum, have served the needs of our constituencies. But clinical nephrology has grown explosively. Moreover, the translation of laboratory information into clinically useful arguments is becoming increasingly short.*

For that reason, beginning with this issue of Kidney International, *we introduce a new series entitled Perspectives in Clinical Nephrology. Both PCN and Nephrology Forum will be directed primarily toward clinicians and/or clinical investigators whose major focus of inquiry is the patient. Like Nephrology Forum, PCN will attempt to translate, as rapidly as possible, advances made in the laboratory into constructs that help analyze the physiology and pathophysiology of disease. In turn, this construct will allow us to orient the Editorial Reviews, to an increasing degree, toward issues focused more on laboratory inquiry.*

The Editors hope that this new series will, to an increasing degree, serve the needs of all of the readership of Kidney International.

T.E. ANDREOLI
FOR THE EDITORS

PERSPECTIVES IN CLINICAL NEPHROLOGY

Anti-neutrophil cytoplasmic antibodies: Current diagnostic and pathophysiological potential

Rapidly progressive glomerulonephritis (RPGN) is a clinical syndrome characterized by rapid deterioration of renal function occurring within days or weeks together with signs of glomerulonephritis, that is, proteinuria and hematuria with cellular casts. The syndrome is, in many cases, histopathologically manifested as fibrinoid necrosis of the capillary wall with extracapillary proliferation and crescent formation [1]. This so-called necrotizing crescentic glomerulonephritis (NCGN) is seen in 5 to 15% of renal biopsies in most series [1–3]. Although it is infrequent, the importance of the condition is illustrated by the fact that most cases of NCGN, if left untreated, develop renal failure within days or weeks [1]. Based on immunohisto-pathology NCGN can be subdivided into three distinct categories. The first one, occurring in 2 to 20% of the cases and characterized by linear staining of the glomerular capillary wall for immunoglobulin and complement, has classically been described as anti-glomerular basement membrane (GBM) disease. It is associated with autoantibodies to structural antigens of the GBM, in particular to the first globular noncollagen domain of collagen type IV [4]. The antibodies are considered of pathogenetic significance. The second category, comprising 15 to 50% of cases, is characterized by granular deposits of immunoglobulin and complement suggesting that immune complexes are pathogenetically involved. This type occurs in conjunction with systemic autoimmune diseases such as lupus erythematosus, in cases of post-infectious glomerulonephritis, IgA nephropathy or Henoch-Schönlein purpura, or as an idiopathic

variety. The third group of NCGN, occurring in 40 to 80%, demonstrates only a few or no immune deposits and is designated as pauci-immune NCGN [1–3, 5, 6]. Pauci-immune NCGN occurs as part of Wegener's granulomatosis (WG) or related conditions, or without systemic vasculitis (idiopathic NCGN). The pathophysiology of this pauci-immune type of NCGN has not been elucidated. Within the last decade, however, it has been recognized that the condition is associated with autoantibodies to cytoplasmic components of neutrophils (anti-neutrophil cytoplasmic antibodies or ANCA).

ANCA were first described in 1982 by Davies et al in a few patients with segmental necrotizing glomerulonephritis [7]. Only in 1985 did it become apparent that ANCA are a sensitive and specific marker for Wegener's granulomatosis (WG) [8]. Later on, ANCA were described in patients with microscopic polyarteritis [9]. Falk and Jennette, in 1988, showed that ANCA are also associated with the idiopathic form of pauci-immune NCGN [10]. These data have now been confirmed by many groups and support the view that ANCA-associated glomerulonephritis and vasculitis is, indeed, a distinct disease category. A number of studies, in addition, have suggested that ANCA are involved in the pathophysiology of the aforementioned disorders. As ANCA, however, have recently also been detected in a wide range of inflammatory and infectious conditions, a critical reappraisal of the diagnostic significance of ANCA-testing seems justified.

In this review we will evaluate the current state of ANCA-testing as well as elaborate on the pathophysiological role of the autoantibodies in necrotizing glomerulonephritis and vasculitis. Data presented recently at the Fifth International Workshop on ANCA, held in Cambridge, United Kingdom, will be included [11]. As such, it adds to previous reviews on ANCA that were published following the Second [12], Third [13], and Fourth [14] Workshops on ANCA.
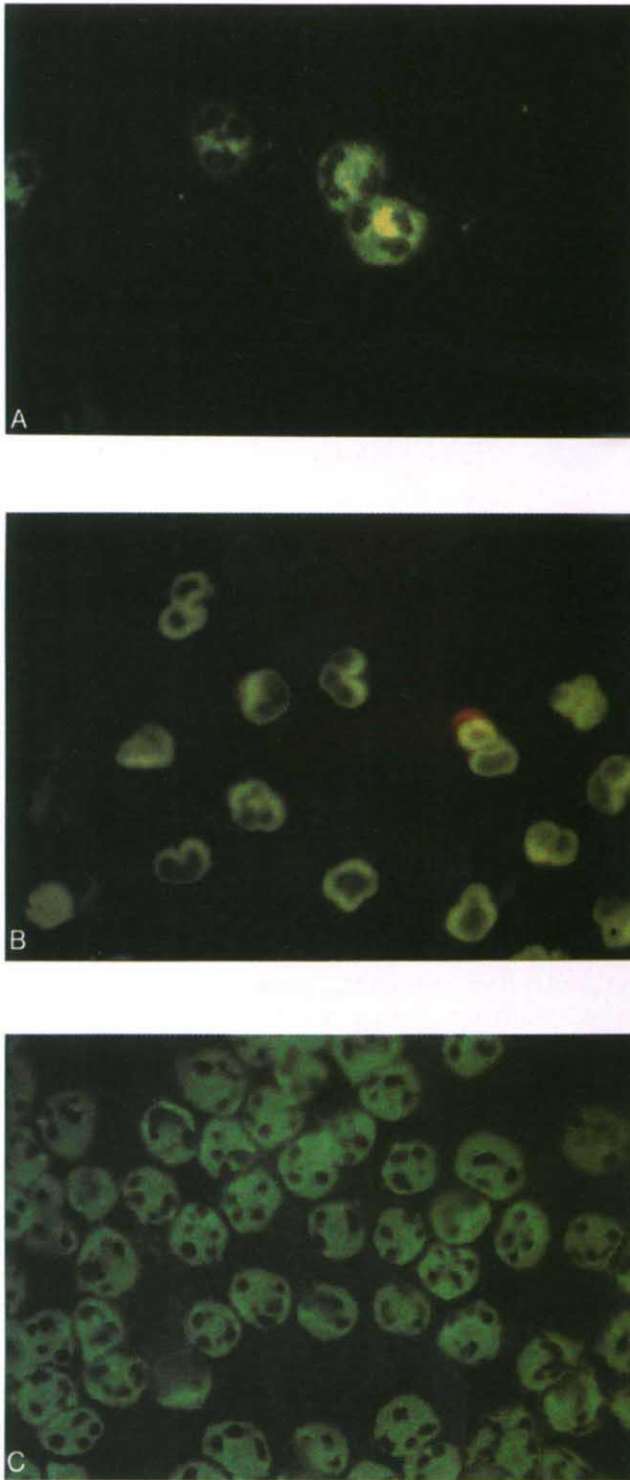
Fig. 1. *Staining of ethanol fixed neutrophils by indirect immunofluorescence by sera producing: (a) a characteristic cytoplasmic staining pattern with accentuation of the fluorescence intensity in the area within the nuclear lobes (c-ANCA, top); (b) a perinuclear staining pattern (p-ANCA, middle); (c) an atypical cytoplasmic staining pattern showing a more diffuse finely speckled fluorescence throughout the cytoplasm (bottom).*

ANCA: A class of different autoantibodies

ANCA as detected by indirect immunofluorescence

ANCA are routinely detected by indirect immunofluorescence (IIF) on ethanol fixed neutrophils [15]. At least three different patterns of fluorescence can be distinguished (Fig. 1): a cytoplasmic pattern with accentuation of the fluorescence intensity in the area within the nuclear lobes (cytoplasmic or c-ANCA), a perinuclear pattern (p-ANCA), and a more diffuse cytoplasmic staining pattern (atypical ANCA). The first pattern has been recognized as a characteristic staining pattern produced by the sera of most patients with WG [8, 16], but also of some 50% of patients with microscopic polyangiitis/arteritis and a minority of patients with other necrotizing vasculitides or idiopathic NCGN [reviewed in 17]. P-ANCA were originally described by Falk and Jennette [10] in patients with idiopathic and/or vasculitis-associated NCGN. Further studies showed that the p-ANCA pattern can be produced also by sera from patients with vasculitis without renal involvement, and patients with inflammatory bowel diseases, autoimmune liver diseases, infectious diseases such as HIV infection, and rheumatic disorders as systemic lupus erythematosus (SLE) and rheumatoid arthritis [17, 18]. The perinuclear pattern cannot always be distinguished from a 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 that of other substrates, have been detected in patients with rheumatoid arthritis long before ANCA had been described, and were named granulocyte-specific antinuclear antibodies (GS-ANA) [19, 20]. Recent studies, however, have shown that the p-ANCA pattern produced by sera from patients with NCGN is an artifact of ethanol fixation [21]. When neutrophils are fixed with a cross-linking fixative such as paraformaldehyde, those sera produce a cytoplasmic staining pattern [21, 22] whereas ANA-positive sera still display a nuclear staining. As will be discussed below, a number of antigens recognized by ANCA, such as myeloperoxidase, are highly cationic proteins. During ethanol fixation those cationic proteins move to the negatively charged nuclear membrane [22] which explains the perinuclear fluorescence pattern. The third pattern, an atypical cytoplasmic fluorescence staining, has not been linked to particular antigens nor to specific disease entities until now.

What do we learn from these data? First, detection of ANCA by IIF should be performed in a standardized way by highly skilled technicians in order to get reproducible results. Cytospins of the buffy coat are preferentially used as a substrate since the simultaneous presence of lymphocytes and neutrophils in the preparation allows the distinction between ANCA, which are myeloid specific, and ANA although the presence of ANCA cannot be excluded in ANA-positive sera. Besides ethanol fixation, paraformaldehyde-fixed preparations may be used since some sera are positive for ANCA on the latter substrate and negative on the former [23]. A recent collaborative European study has shown that ANCA-testing according to a standard protocol using ethanol-fixed leukocytes results in very low inter-laboratory variance [24]. Secondly, a positive

ANCA-test by IIF is in itself not diagnostic for a particular condition but should be followed by antigen-specific assays.

ANCA: Their target antigens

Proteinase 3. The antigen recognized by most c-ANCA positive sera has been identified as proteinase 3, a third serine protease from the azurophilic granules of neutrophils different from elastase and cathepsin G, the other two serine proteases from these granules [25–27].

Proteinase 3 has been cloned and was shown to be a 29 kD glycoprotein of 228 amino acids [28] that is identical to p29b, a recently described antibiotic protein from human neutrophils [29], and myeloblastin, a growth-promoting protein from myeloid cells [30]. The enzyme is slightly cationic, has proteolytic activity as measured on several substrates [31], and is physiologically inhibited by α_1-antitrypsin [32]. Proteinase 3-α_1-antitrypsin complexes can, indeed, be detected in inflammatory fluids [32]. A 47 kD glycoprotein is also recognized by polyclonal and monoclonal antibodies that react with the 29 kD molecule as shown by Goldschmeding et al [25]. It has recently been demonstrated that this 47 kD moiety has serine protease activity and, probably, represents a post-transcriptional modification of the cDNA encoding for myeloblastin/proteinase 3 [33]. Human antibodies to proteinase 3 appear to recognize conformational determinants on the molecule [34]. The epitope(s) in question are localized at or near the catalytic domain of the molecule as human autoantibodies to proteinase 3 interfere with the inactivation of the enzyme by α_1-antitrypsin [35]. In most cases binding of the antibody to proteinase 3 not only inhibits inactivation of the molecule by anti-proteases but also results in inactivation of proteinase 3 itself; in some instances, however, enzymatic activity is retained [35].

Proteinase 3 is localized in the azurophilic or α-granules from myeloid cells. It is present both in monocytes and granulocytes and appears early in mono-myeloid differentiation [36, 37]. Proteinase 3 is released from activated neutrophils by a process involving fusion of the membrane of azurophilic granules with the plasmamembrane [38]. When neutrophils are primed, that is, pre-activated with a low dose of TNFα (100 pg/ml), proteinase 3 appears at the cell membrane being available for interaction with its cognate antibody [39]. *In vivo*, circulating neutrophils showing membrane expression of proteinase 3 have been detected in conditions as septic shock and also in patients with active WG [40]. Interestingly, recent studies by Mayet et al have shown that proteinase 3 can also be expressed in a renal carcinoma cell line [41] and, more importantly, in cultured human endothelial cells from umbilical veins [42]. In cytokine-treated endothelial cells proteinase 3 moves towards the cell membrane suggesting that the antigen may *in vivo* be a direct target for circulating autoantibodies [42]. Proteinase 3 can be purified from neutrophil granules by dye-ligand affinity and ion-exchange chromatography [43], by immunoaffinity chromatography [44], or by high pressure liquid chromatography [45]. Purified protein can be used in ELISA systems for quantitation of specific antibodies [24]. Anti-proteinase 3 antibodies can also be detected by a capture ELISA in which a monoclonal antibody selects the relevant antigen from a crude extract of α-granules [3, 25].

Myeloperoxidase (MPO). Following the description of c-ANCA as a marker for WG several groups observed that a

Table 1. Neutrophil granule constituents

Class of constituent	Azurophilic granules	Specific granules
Microbial enzymes	myeloperoxidase lysozyme	lysozyme
Serine proteinases	elastase cathepsin G proteinase 3 azurocidin	
Metalloproteinases		collagenase gelatinase
Acid hydrolases	*N*-acetyl-β-glucosaminidase cathepsin B cathepsin D β-glucuronidase β-glycerophosphatase α-mannosidase	
Other	defensins	lactoferrin vitamin B12-binding protein

number of sera from patients suspected of vasculitis produced a perinuclear fluorescence pattern on ethanol-fixed neutrophils. During the first international workshop on ANCA in 1988 Goldschmeding et al [46] reported that many of those sera contained antibodies to MPO and, incidentally, to elastase, and Falk and Jennette also mentioned MPO as the relevant antigen [47, 10]. Based on the latter data p-ANCA became almost synonymous for anti-MPO, which, as already has been discussed, is not true. In fact, many p-ANCA positive sera are directed against antigens different from MPO, whereas some sera with anti-MPO antibodies produce a cytoplasmic staining pattern on ethanol-fixed neutrophils [48, 49]. MPO is a highly cationic protein (pI 11.0) with a molecular wt of 146 kD, and consists of two chains. The enzyme plays a critical role in the generation of reactive oxygen species [50]. By immunoprecipitation at least two bands of approximately 60 and 42 kD, representing a complete and a partly degraded single chain, are recognized both by monoclonal and human polyclonal antibodies [25]. Western blotting studies demonstrate that human anti-MPO antibodies recognize native MPO but not denatured MPO suggesting that confirmational epitopes are involved [51–53]. The human autoimmune response to MPO is directed against multiple epitopes as demonstrated by inhibition studies using anti-MPO positive sera and a set of different monoclonal antibodies [54]. The relevant epitopes have not yet been defined but most anti-MPO positive sera do not seem to inactivate the enzymatic activity of the molecule [51, 52]. Studies performed in mice have demonstrated the occurrence of natural anti-MPO antibodies of the IgM-isotype, some of which are polyreactive [55]. Autoantibodies to MPO have also been demonstrated in Brown Norway rats treated with mercuric chloride which induces a T-cell-dependent polyclonal B-cell activation [53]. These data suggest that also in humans low-affinity IgM-class antibodies are present at the germ line which may develop into high-affinity IgG-class anti-MPO by antigen-driven selection in the presence of their cognate T cells. In this respect, cross-reactivity or homology between MPO and other proteins may be relevant. As such, the homology demonstrated between MPO and heat shock proteins is of interest [56].

For the detection of anti-MPO antibodies commercially available MPO can be used in ELISA systems. Those preparations, however, may contain impurities, in particular lactoferrin [57]. Alternatively, a capture ELISA can be used [3, 25, 48].

Other antigens. Many sera that produce a perinuclear or atypical cytoplasmic staining pattern on ethanol fixed neutrophils do not contain antibodies to MPO or proteinase 3 as tested by antigen-specific assays. It has now been recognized that a number of these sera contain antibodies to defined granular constituents of neutrophils (Table 1). In particular, autoantibodies have been demonstrated to lactoferrin [23, 58–63] and lysozyme [64], which are constituents of the specific granules, to elastase [25, 65] and cathepsin G [66], the two other serine proteases that are present in the azurophilic granules, and to β-glucuronidase, another constituent of those granules [67]. In addition, autoantibodies have been detected that are directed against cytosolic components of neutrophils, in particular to alpha-enolase [68]. The presence of these autoantibodies have been demonstrated in most of the cases by ELISA, and not by additional Western blotting or immunoprecipitation. Detection by ELISA did not always parallel detection by IIF on ethanol- or formalin-fixed neutrophils. Their clinical associations will be discussed below.

ANCA: Diagnostic significance

Although ANCA were first described in a few patients with necrotizing glomerulonephritis [7], large interest in the autoantibodies arose from their presence in WG [8]. Numerous studies published in recent years, however, have shown that ANCA are far from specific for WG, and have challenged the diagnostic potential of the autoantibodies. These sometimes conflicting data ask for a critical reappraisal of the clinical value of ANCA testing.

Wegener's granulomatosis

Following the original description of ANCA in WG [8] many studies have confirmed the sensitivity of c-ANCA or anti-proteinase 3 for a diagnosis of WG. Three major studies comprising more than 200 patients have found a sensitivity of 90% for so-called extended WG characterized by the triad of granulomatous inflammation of the respiratory tract, systemic vasculitis, and necrotizing crescentic glomerulonephritis [16, 69, 70]. The sensitivity of anti-proteinase 3 for limited WG, that is, disease manifestations without obvious renal involvement, amounted to 75% [17]. It should be mentioned that these data concern patients with active disease only. During remission c-ANCA were detected in a far lower percentage of the patients. Generally, the presence of c-ANCA as detected by IIF corresponded with their presence as detected by proteinase 3-specific ELISA [3, 69]. Many other studies dealing with smaller numbers of patients have confirmed these findings [reviewed in 71]. The association between anti-proteinase 3 and WG is, however, not absolute: some patients with active (generalized) WG are ANCA-negative. Most of the patients who are negative for anti-proteinase 3 have, however, antibodies to MPO or leukocyte elastase [10, 48, 65, 71].

The specificity of anti-proteinase 3 for active WG has been found as high as 98% [16, 69, 71] when sera were studied from patients with a wide variety of renal, autoimmune, vasculitic, infectious, or lymphoproliferative disorders. It should, how-

Table 2. Traditional classification of systemic idiopathic vasculitides

I.   Affecting predominantly large- and medium-sized blood vessels
     1. Takayasu's arteritis
     2. Giant cell arteritis/temporal arteritis

II. Affecting predominantly medium- and small-sized blood vessels
     1. Classic polyarteritis nodosa
     2. Churg-Strauss syndrome
     3. Wegener's granulomatosis
     4. Polyangiitis overlap syndrome

III. Affecting predominantly small blood vessels
     1. Microscopic polyarteritis
     2. Henoch-Schönlein purpura

ever, be realized that anti-proteinase 3 antibodies are detected in patients with overlapping symptoms of WG and other forms of vasculitis, and in some patients with idiopathic necrotizing glomerulonephritis without systemic involvement [3, 48, 69, 72]. Some of these patients will evolve into definite (extended) WG as classified according to the ACR criteria [73] or according to the definition recently proposed by an international study group on vasculitis [74]. Very recently, anti-proteinase 3 antibodies have been described in patients with invasive amoebiasis [75] and in patients with thyroid disease treated with propylthiouracil [76]. The latter patients simultaneously developed antibodies to other myeloid enzymes such as MPO and elastase together with clinical signs of vasculitis. These findings may suggest that the thiol group of the drug is involved in the induction of this polyclonal autoimmune reaction.

As mentioned before, the prevalence of c-ANCA/anti-proteinase 3 in patients with active WG is far higher than that in inactive disease. Various longitudinal studies have shown that titers of ANCA rise prior to a relapse of WG [16, 77]. The rise proved very sensitive for an ensuing relapse and was detectable a mean of 49 days (range 9 to 106 days) before the moment of clinical relapse [16]. Based on these findings, a prospective study was undertaken on 58 patients with WG to test whether treatment based on changes in c-ANCA titer could prevent the occurrence of relapses [78]. In this study blood samples were tested every month for ANCA titer. Over an observation period of 24 months titers of ANCA rose in 20 patients. None of the nine patients who were randomized for treatment at the time ANCA titer had risen (without having major clinical signs at that moment) relapsed. In contrast, nine of the 11 patients who were not treated at that moment, finally developed a relapse. Those latter patients used more immunosuppressives and corticosteroids than the patients who were treated immediately at the time ANCA titer had risen. These data have been challenged by a recent report from the NIH group [79] demonstrating a rather poor correlation between changes in ANCA titer and disease activity of WG. A closer look at their data [80], however, shows that all 17 patients from the NIH study who were either in remission or had stable disease developed relapses that were preceded (24%) or parallelled (76%) by an increase of c-ANCA titer. Changing titers not corresponding to disease activity mainly occurred in patients with (persistently) active disease. Persistingly or intermittently positive tests for ANCA in patients who come into remission have been shown to be a considerable risk factor for an ensuing relapse (RR 20 compared to patients who are persistently ANCA-negative after

Table 3. Classification of the idiopathic vasculitides as proposed by an international study group at the Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitis (adapted from [74])

I. Large vessel vasculitis
   1. Giant cell (temporal) arteritis
   2. Takayasu arteritis

II. Medium-sized vessel vasculitis
   1. Polyarteritis nodosa
   2. Kawasaki disease

III. Small vessel vasculitis
   1. Wegener's granulomatosis
   2. Churg-Strauss syndrome
   3. Microscopic polyangiitis
   4. Henoch Schönlein purpura
   5. Essential cryoglobulinemic vasculitis
   6. Cutaneous leukocytoclastic angiitis

Table 4. Disease associations of anti-proteinase 3 antibodies and anti-myeloperoxidase antibodies

Disease entity	Sensitivity of	
	Anti-proteinase 3 %	Anti-myeloperoxidase %
Wegener's granulomatosis	85	10
Microscopic polyangiitis	45	45
Idiopathic crescentic glomerulonephritis	25	65
Churg-Strauss syndrome	10	60
Polyarteritis nodosa	5	15

Data are derived from the references cited in the text.

remission) [81]. Interestingly, persistence of ANCA is associated with certain HLA class II genes, in particular the DQw7, DR4 haplotype [82], suggesting that genetic factors are involved in the autoimmune response.

In conclusion, the presence of anti-proteinase 3 strongly suggests a diagnosis of WG although the association is not absolute. Increasing titers of the autoantibodies should alert the clinician to the possibility of an ensuing relapse.

Other idiopathic necrotizing vasculitides including idiopathic pauci-immune NCGN

The necrotizing vasculitides are traditionally grouped according to the size of the vessels involved and the histopathology of the inflammatory process (Table 2). Classification criteria based mainly on clinical signs and symptoms have been published by the American College of Rheumatologists (ACR) [83]. Although useful in clinical practice, these criteria do not strictly define the disease in question. Using these criteria, various degrees of sensitivity and specificity are obtained. As the criteria are not mutually exclusive one particular patient may be classified as having two or more disease entities. Recently, an international study group has newly defined the several vasculitides based, generally, on histopathologic findings (Table 3) [74]. The main difference with the traditional classification (Table 2) lies in the distinction between medium sized and small vessel vasculitis. In medium sized (and large) vessel vasculitis vessels smaller than arteries are not involved, whereas in small vessel vasculitis large vessels may be involved as well. A diagnosis of polyarteritis nodosa requires the presence of necrotizing arteritis but is excluded when arterioles, capillaries or venules are involved as well. In the latter situation the diagnosis probably will be microscopic polyangiitis according to the Chapel Hill conference [74]. Classification criteria based on both clinical and histopathologic findings still have to be established by this group. At present, one should realize that the same name may be used for different diseases and vice versa. In addition, in clinical practice many patients present with overlapping features of different vasculitides and cannot be classified within one category. Data on prevalences of ANCA in the various vasculitides should be valued with these considerations in mind.

Microscopic polyarteritis or angiitis (MPA). This is characterized by (pauci-immune) necrotizing vasculitis involving small sized vessels [84]. A pulmonary-renal syndrome consisting of NCGN in combination with pulmonary capillaritis is not uncommon. Most patients with MPA are ANCA-positive, either with specificity for proteinase 3 or for MPO [85]. There seems to be no clear distinction between patients positive for anti-proteinase 3 or anti-MPO, although a number of anti-proteinase 3 positive patients develop histologically confirmed WG during the course of their disease (own observations). The relation between titers of ANCA and disease activity has not been studied systematically in MPA.

Idiopathic pauci-immune NCGN. This can be considered as a renal limited form of MPA. Most patients with this latter disorder are positive for anti-MPO, the remaining generally being positive for anti-proteinase 3 [3, 10]. At a closer look, however, many patients with idiopathic NCGN have constitutional symptoms together with signs of systemic involvement such as arthralgias, purpura and/or otorhinolaryngologic symptoms. As such, the distinction between WG, MPA, and idiopathic NCGN is far from absolute.

Anti-GBM disease. Interestingly, anti-MPO antibodies also have been detected in 30 to 40% of patients with anti-GBM disease [86–88]. Patients having both anti-MPO and anti-GBM antibodies are considerably older than patients with anti-GBM antibodies only. In some of these patients clinical and pathological data suggest associated systemic vasculitis, and among these patients recovery of renal function has been reported despite their initial need for hemodialysis [87]. Whether ANCA in anti-GBM disease develop concomitantly with anti-GBM antibodies, possibly as a result of polyclonal activation, is presently not clear.

Churg Strauss syndrome. Characterized by (a history of) asthma and hypereosinophilia in conjunction with systemic vasculitis with or without renal involvement, Churg Strauss syndrome is associated with anti-MPO antibodies [89]. Combining data from several studies the prevalence of anti-MPO in this disorder may be estimated as 75% [72, 90]. *Classical polyarteritis nodosa (PAN)* defined as necrotizing arteritis involving medium sized arteries is ANCA-negative in the majority of the cases [72, 90]. In a large series from France [90] 20% of the patients with PAN without hepatitis B virus infection were positive for ANCA, whereas 11% of those with HBV infection were positive.

Kawasaki disease. The occurrence of ANCA in Kawasaki disease, a mucocutaneous lymph node syndrome with, in a

considerable number of cases, arteritis, in particular of the coronary arteries, has not been definitely established. Acute sera demonstrate positive homogeneous cytoplasmic staining in 23% of the patients possibly due to IgM-class antibodies directed to cathepsin G [91]. Large vessel vasculitides such as *Takayasu arteritis* [92] and *temporal arteritis* [65, 93] generally are ANCA-negative. Finally, the presence of ANCA, in particular of the IgA isotype, in *Henoch Schönlein purpura* needs consideration. Although these IgA-ANCA have been reported in earlier studies [94], these data have not been confirmed by other groups [95].

In summary, the presence of anti-proteinase 3 or anti-MPO appears to characterize pauci-immune small vessel vasculitis of different varieties frequently involving the kidney as well (Table 4).

Connective tissue diseases

Systemic lupus erythematosus. The prevalence of ANCA in systemic lupus erythematosus (SLE) still is a matter of debate. Antibodies to MPO [96, 97], elastase [97], and lactoferrin [61] have been reported in this disease. Their prevalences differ widely between several series, and their occurrence does not seem to be associated with any particular disease subset. Interestingly, anti-MPO have been detected in the sera of all six patients with hydralazine induced lupus, in five of them in combination with anti-elastase antibodies [98]. The simultaneous presence of specific ANCAs both in hydralazine induced lupus and propylthiouracil induced vasculitis suggests a more polyclonal immune activation in drug-induced autoimmunity comparable to experimental mercuric chloride induced autoimmune disease in which anti-MPO antibodies are also detected [53].

Rheumatoid arthritis. Granulocyte-specific ANA (GS-ANA) [20, see before] have been described in rheumatoid arthritis (RA) already in 1959 [99]. The detection of ANCA has evoked renewed interest in those antibodies in RA. It has been shown that, at least most of, GS-ANA are actually ANCA directed to different antigens [23]. Among these lactoferrin is most prominent. Anti-lactoferrin antibodies have been specifically associated with vasculitis in RA [58] and with Felty's syndrome [60], although these data have not been confirmed by others [23, 100]. The latter studies actually suggest that ANCA in RA are a secondary phenomenon related to disease duration.

Inflammatory bowel disease (IBD) and autoimmune liver disease

The occurrence of ANCA in IBD, in particular in *ulcerative colitis*, has raised interest in ANCA as a marker of ulcerative colitis useful in the work-up of patients with diarrhea [101]. The prevalence of ANCA in ulcerative colitis may be estimated as 50 to 80% compared to a 10 to 40% prevalence in *Crohn's disease* [59, 102–104]. Titers of ANCA possibly follow disease activity in ulcerative colitis but not in Crohn's disease [103–105]. There is still no agreement on the antigens involved. Cathepsin G as well as β-glucuronidase have been proposed [66, 67], but other studies suggest that the same antigens are involved as in RA including lactoferrin [59]. ANCA have also been described in a considerable number of patients with *primary sclerosing cholangitis*, a hepatic disease of possible

autoimmune origin associated with IBD [106]. ANCA, however, were also found in 60 to 70% of patients with *chronic active hepatitis*, and 35% of those with *primary biliary cirrhosis*. The antigens involved have only partly been characterized [107].

Infectious diseases

ANCA, generally of undefined specificity, have been described in HIV infection and mucoviscidosis with secondary infection [108, 109]. As mentioned before, a recent report described the occurrence of anti-proteinase 3 antibodies in patients with amoebiasis [75]. In addition, incidental case reports have mentioned the occurrence of ANCA in different infectious and neoplastic disorders. These findings, although unconfirmed, underscore that the detection of ANCA by IIF only has, at present, limited diagnostic value. The presence of anti-proteinase 3 or anti-MPO in the relevant clinical context, however, clearly points to one of the idiopathic forms of necrotizing vasculitis or glomerulonephritis.

ANCA: Towards a role in the pathophysiology of NCGN/vasculitis

The close association between anti-MPO and anti-proteinase 3 antibodies on the one hand and pauci-immune NCGN and necrotizing vasculitis on the other hand suggests a pathophysiological role for the autoantibodies. There are, indeed, experimental data that further substantiate a role for ANCA in the pathophysiology of the aforementioned diseases.

Effect of ANCA on their target antigens

As discussed before binding of anti-proteinase 3 to its target enzyme can inhibit the irreversible inactivation of the enzyme by α_1-antitrypsin, its natural inhibitor [35]. The inhibitory activity differs between sera and is not directly related to the amount of specific antibody [110]. 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 the anti-proteinase 3 antibodies [110]. This suggests that escape of the enzyme from its inactivation may contribute to the inflammatory process. Although most anti-proteinase 3 positive sera not only inhibit inactivation of proteinase 3 but also its enzymatic activity [35], the reversible antigen-antibody binding may be dissolved at the site of inflammation allowing the enzyme to display its lytic activity.

The phenotypical expression of α_1-antitrypsin is polymorphic: severely-, medium-, and non-deficient proteinase inhibitor phenotypes can be distinguished. Interestingly, among 14 anti-proteinase 3-positive patients with vasculitis, phenotypes deficient for proteinase inhibitor activity were increased compared to controls. Reversely, the prevalence of ANCA of diverse specificities was remarkable amongst a population with the severely deficient phenotype (20 out of 150) [111]. These data suggest that deficient α_1-antitrypsin activity is involved in the induction of ANCA. Otherwise, this deficiency may contribute to the expression of the lesions in ANCA-associated vasculitis.

Interaction between ANCA and endothelial cells

The antigens recognized by anti-MPO and anti-proteinase 3 are constituents of granules from myeloid cells and are thought

to be specific for cells of the monomyeloid lineage. Recent studies from Mayet et al have demonstrated, however, that proteinase 3 can be expressed in other cell types as well, in particular in renal carcinoma cells [41] and in human endothelial cells [42]. In the latter cells cytokine treatment either with TNFα, IL-1, or IFNγ, resulted in increased expression of proteinase 3 with translocation of the enzyme to the cell membrane. As such the antigen is available for interaction with the autoantibody at sites of cytokine production. MPO and proteinase 3 are cationic proteins with an isoelectric point of 11.0 and 7.8, respectively. These cationic proteins 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 endothelial cells incubated with MPO or proteinase 3 [112] although the interaction of protein-ase 3 with endothelial cells has been disputed [113]. Under these conditions, anti-MPO can, *in vitro*, induce complement dependent endothelial cell lysis [112]. 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 still has to be established.

Potential of ANCA to activate inflammatory cells

Falk et al were the first to demonstrate that ANCA can activate neutrophils that are pre-treated ("primed") with low dosage TNFα to the production of reactive oxygen species and the release of lysosomal enzymes [114]. The mechanisms un-derlying neutrophil activation by ANCA have only partly been elucidated. Priming of neutrophils by TNFα results in the expression of lysosomal enzymes such as MPO and proteinase 3 at the cell membrane [39]. Those pre-activated neutrophils showing surface expression of proteinase 3 can be detected in the peripheral blood in conditions as septic shock and at the time of active disease in WG [40]. Binding of anti-proteinase 3 to primed neutrophils results in further activation. Falk et al originally reported that activation can be induced by F(ab')₂-fragments of the antibodies [114]. Recent studies, however, suggest that both F(ab)-binding and Fc-interaction are involved [115, 116]. Blocking of the FcII receptor on neutrophils inhib-ited activation [115]. The FcII receptor particularly interacts with IgG2 and IgG3 subclasses of antibodies. Interestingly, sera with relatively high levels of IgG3 subclass of ANCA preferen-tially activate neutrophils [115], and renal exacerbations of WG are associated with increases of the IgG3 subclass of ANCA [117] although IgG1 and IgG4 subclasses of ANCA are present as well [117]. The signal transduction pathway involved in ANCA-mediated activation of neutrophils is still a matter of debate [118–120]. Different pathways have been described suggesting that protein kinase C (PKC) as well as Ca²⁺-influx are involved. The translocation of PKC to the cell membrane as observed during ANCA-mediated activation [118] seems to be the initial step, further steps are dependent on intracellular calcium [120]. *In vitro* studies also have shown that primed neutrophils in the presence of ANCA can lyse endothelial cells in culture [121, 122]. In addition, it has been suggested that ANCA can induce adhesion of neutrophils to monolayers of cultured endothelial cells [123]. Considering these experiments

one should realize that isolation of neutrophils in itself may result in some degree of activation depending on the methods of isolation. This, apparently, may influence the results. Taken together, ANCA of defined specificity have been shown to activate primed neutrophils. The relevance of this phenomenon for the pathophysiology of the necrotizing vasculitides needs further study. In this respect, the capacity of ANCA to activate primed neutrophils did not correlate with the amount of *in situ* activated neutrophils present in renal biopsies from patients with active WG [124].

T cell reactivity to ANCA-associated antigens

Besides neutrophils, inflammatory lesions of lung and kidney in WG contain mononuclear infiltrates, frequently also in the form of granulomas, which consist of monocytes and T cells, the majority of which are CD4-positive [125, 126]. The presence of these large cellular infiltrates, contrasting with the scanty deposits of immunoglobulins [5, 6, 124], suggests that cell-mediated immunity is involved in the pathogenesis of the lesions as well. In agreement, elevated levels of soluble IL-2 receptors and increase in those levels preceeding major relapses have been reported in patients with WG indicating the presence of activated T cells [127, 128]. The target antigens of those activated T cells are, however, not known. Microbial antigens, in particular Staphylococcal antigens, might be involved as chronic nasal carriage of *Staphylococcus aureus* has been shown to be an important risk factor for the development of relapses in patients with WG [81]. Otherwise, the target anti-gens of ANCA, that is, proteinase 3 and MPO, are released at the site of inflammation and could be targets for cell-mediated immunity. Preliminary data have shown that lymphocytes iso-lated from patients with WG proliferate in response to crude neutrophil extracts containing proteinase 3 [129, 130]. We recently demonstrated using *in vitro* lymphocyte proliferation assays, that patients with WG positive for anti-proteinase 3 antibodies responded more frequently and stronger to protein-ase 3 than controls [131]. However, 33% of healthy controls also reacted *in vitro* to proteinase 3, whereas 35% of patients with anti-proteinase 3 antibodies did not show *in vitro* T cell reactivity to this autoantigen. Thus, the pathophysiological role of (autoreactive) T cells in systemic vasculitis awaits further studies.

The in vivo role of ANCA in NCGN

The *in vivo* potential of ANCA to aggravate the inflammatory response has recently been demonstrated by Kobayashi, Shi-bata and Sugisaki [132]. They injected rats with rabbit anti-rat MPO antibodies together with rabbit anti-rat GBM antibodies. Control rats were injected with either anti-rat GBM or anti-rat MPO antibodies. In the latter group no lesions were apparent. The most severe lesions were found in rats injected with both antibodies. In this group, MPO was found along the glomerular capillary wall and anti-MPO could be eluted from the kidneys. Thus, besides direct activation of rat neutrophils by anti-MPO, MPO released from activated neutrophils may have bound to the GBM due to charge interaction followed by *in situ* binding of anti-MPO. This local immune complex formation may have aggravated the passive anti-GBM nephritis. In line with these findings are the data of Brouwer et al [133]. They immunized
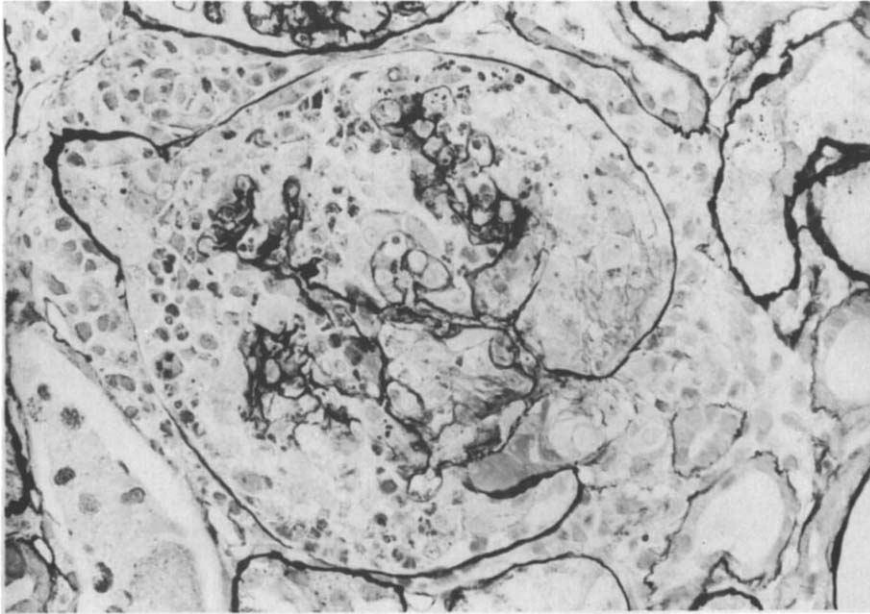
Fig. 2. *Light microscopy of lesions found in Brown Norway rats immunized with myeloperoxidase and perfused with a lysosomal enzyme extract and H_2O_2. Crescent formation, tuft necrosis, and periglomerular infiltration ($\times 560$).*

Brown Norway (BN) rats with human MPO. The rats developed anti-human MPO antibodies, which, in part, showed cross-reactivity with rat MPO, as well as delayed type hypersensitivity to human MPO. Five weeks after immunization the left kidney was perfused with products of activated neutrophils, that is, lytic enzymes, particularly proteinase 3 and elastase, MPO, and its substrate H_2O_2. The rats developed NCGN with interstitial infiltrate and vasculitis (Fig. 2). Whereas MPO, IgG, and C3 could be detected at 24 hours after perfusion along the GBM, immune deposits were generally absent at 4 and 10 days after perfusion, which illustrates the pauci-immune character of the lesions. In control immunized animals perfused with enzymes and H_2O_2 no significant lesions occurred. When MPO-immunized rats were perfused with MPO and H_2O_2 only, more or less comparable lesions developed, but those lesions were accompanied by immune deposits persisting for a longer time along the GBM. From these findings it is suggested that the initial step in the development of NCGN is (focal) immune complex formation. The presence of lytic enzymes, which were concomitantly perfused but may also have been released from neutrophils activated by ANCA, probably is responsible for the degradation of the immune deposits that were detected in the very early stage of the lesions. Yang et al very recently have tried to repeat the experiments of Brouwer et al in both BN rats and spontaneously hypertensive rats [134]. In the latter rats more severe lesions were found demonstrating the additive effects of hypertension. In contrast to Brouwer et al [133] they observed persistence, up to 10 days after perfusion, of immune deposits along the GBM in both rat strains. Differences both in the immunization procedure resulting in different qualities of the antibodies as well as in the composition of the perfusate may explain the discrepancies between both studies.

Anti-MPO antibodies have also been demonstrated in BN rats treated with mercuric chloride [53]. These rats develop a polyclonal autoimmune response comprising, among others, antibodies to MPO. Necrotizing vasculitis, particularly involving the gut, developed in some of the animals, possibly in

conjunction with a microbial infection [135]. The pathophysiological role of anti-MPO in the induction of the vasculitic process is, however, not clear in this particular model. A schematic representation of the possible pathophysiology of ANCA-associated necrotizing vasculitis/glomerulonephritis which summarizes the items discussed before is shown in Figure 3.

Impact of ANCA on treatment

Untreated, the outcome of the systemic necrotizing vasculitides is poor. High doses of corticosteroids have improved prognosis. Although treatment with steroids alone seems to be sufficient in less severe forms of polyarteritis nodosa and Churg Strauss syndrome, many cases of systemic vasculitis treated with corticosteroids alone still progress to renal failure or show a fatal outcome. In WG, for instance, corticosteroids have improved the average survival time from 5 to 12.5 months only. The introduction of cyclophosphamide [136, 137] has been a major advance in the treatment of the necrotizing vasculitides resulting in complete remission in 14 out of 17 patients with severe vasculitis [136] and 79 out of 85 patients with WG [137]. At present, the usual regimen consists of 2 mg/kg/day cyclophosphamide given orally in combination with 1 mg/kg/day prednisolone. Since a considerable number of patients relapse during tapering of immunosuppressives, long-term treatment (1 to 2 years of cyclophosphamide) has been advocated by the NIH group. Conversion to azathioprin after 8 to 12 weeks [138] might result in a higher relapse rate although comparitive studies are not available. A major drawback of long-term cyclophosphamide treatment is the occurrence of severe adverse effects, in particular opportunistic infections [139] and bladder toxicity, that is, hemorrhagic cystitis and carcinoma [140]. In lupus nephritis, the frequency of adverse effects seems to be reduced without loss of efficacy by using intravenous pulse cyclophosphamide instead of oral cyclophosphamide
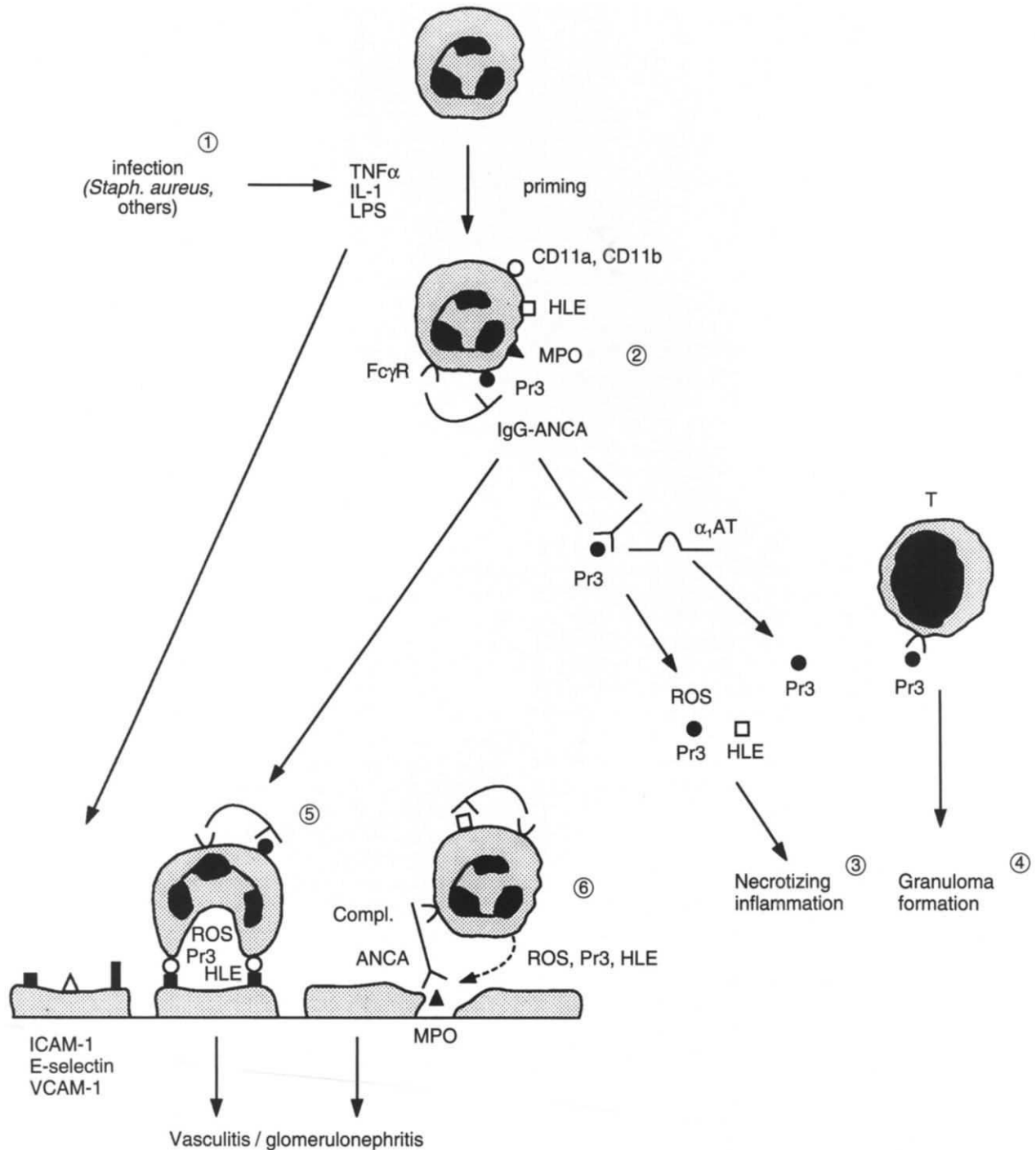
Fig. 3. *Schematic representation of the possible pathophysiology of ANCA-associated necrotizing vasculitis/glomerulonephritis.* 1. Neutrophils (PMN) are primed by low concentrations of cytokines resulting from (local) infection. 2. Primed PMN are further activated by IgG-ANCA to the production of reactive oxygen species (ROS) and the release of lytic enzymes such as proteinase 3 (Pr3) and elastase (HLE). 3. Products of activated PMN induce necrotizing inflammation which might be potentiated by the inhibition by ANCA of the α_1-antitrypsin (α_1AT) induced inactivation of Pr3. 4. CD4-positive T-cells, possibly autoreactive to Pr3, may contribute to granuloma formation. 5. Activated PMN may damage endothelial cells to which they adhere as a result of (focal) up-regulation of adhesion molecules. 6. Binding of cationic MPO to polyanionic (basement) membranes is followed by *in situ* immune complex formation; attracted PMN additionally activated by ANCA evoke the initial step of vasculitis/glomerulonephritis and degrade immune complexes.

[141]. In WG, the NIH group described a less favorable therapeutic effect of intravenous pulse compared to oral cyclophosphamide [142]. In ANCA-positive glomerulonephritis and systemic vasculitis including WG, Falk et al [143], however, found no differences in renal or patient survival between both regimens. A controlled study from the UK also showed that

pulse and oral cyclophosphamide are equally effective in inducing remission in patients with systemic vasculitis [144].

Based on new insights, in part derived from the detection of ANCA, into the possible pathophysiology of the systemic vasculitides, different treatment modalities have been proposed for ANCA-associated glomerulonephritis and systemic vasculitis.

(a) A number of uncontrolled studies have described a favorable effect of trimethoprim/sulfamethoxazole (T/S) in the treatment of WG [145], particularly in limited WG. A prospective study in 17 patients with limited WG recently showed a response rate of 65% [146]. In generalized WG the use of T/S was accompanied by maintenance of remission in 14 out of 32 patients (44%) during a three year period [146]. Prospective controlled studies are, however, not available. The possibly positive effect of T/S might be explained by the observation that relapses of WG are frequently preceeded by infections [147]. Our group recently found that chronic nasal carriage of *Staphylococcus aureus* is frequent in patients with WG, occurring in 36 out of 57 patients (63%), and identifies a subgroup more prone to relapses of the disease. During a 3½ year period 21 of the chronic carriers relapsed, compared to 2 out of 21 who were not considered chronic carriers [81]. Also, chronic nasal carriers had an increased risk of being persistently or intermittently positive for ANCA [81]. The inter-relationship between chronic nasal carriage of *Staphylococcus aureus* and prevention of relapses by T/S treatment has not been studied so far. Although the efficacy of T/S needs to be substantiated, this mode of treatment may be particularly useful in the management of chronic inflammation and superimposed infections of nose and sinuses in patients with WG.

(b) Several newly proposed treatment modalities in systemic vasculitis are based on the assumption that ANCA play a critical role in the pathophysiology of the disease. As stated before, rises of c-ANCA titers seem to preceed the development of relapses of WG [16]. Based on these findings a controlled prospective study was performed in which patients were assigned to treatment or no treatment once a significant rise of ANCA had occurred [78]. Treatment based on rises of ANCA titer appeared to prevent relapses of WG in this study on a small number of patients. These data have to be confirmed by studies on larger groups of patients.

Plasma exchange (PE) has been proposed as a direct approach to remove pathogenic autoantibodies. A controlled study on the additional role of PE in the initial treatment of patients with necrotizing glomerulonephritis without anti-GBM antibodies was published in 1991 [148]. Included were patients with impaired renal function, crescentic necrotizing glomerulonephritis in the biopsy, and a clinical diagnosis of WG, MPA, or idiopathic rapidly progressive glomerulonephritis. Data on ANCA were not consistently available. Forty-eight cases could be analyzed of whom 23 were treated with immunosuppressives only and 25 with additional PE. Only in patients who were dialysis-dependent at presentation ($N = 19$) was an additional benefit of PE observed: 10 out of 11 patients treated with additional PE recovered from renal failure, whereas only 3 patients recovered out of 8 patients treated with immunosuppressives alone. A comparable beneficial effect has been found for the additional use of pulse methylprednisolone in patients with rapidly progressive glomerulonephritis and crescentic glo-

merulonephritis who were dialysis-dependent at presentation [149]. Thus, either PE or pulse methylprednisolone can be added to the oral regimen described before in patients with ANCA-associated vasculitis/glomerulonephritis who present with acute renal failure.

Intravenous gammaglobulin (IVIg) therapy has been tried in an increasing number of autoimmune diseases. Originally used in autoimmune thrombocytopenia in order to block or saturate Fc-receptor mediated clearance of antibody-coated platelets, IVIg might also exert its effect by the presence in the preparation of anti-idiotypic antibodies which interact with the idiotypic network. Anti-idiotypic antibodies reactive to ANCA have, indeed, been detected in pooled human immunoglobulin and in the sera from patients with ANCA-positive vasculitis drawn during remission [150]. Preliminary data suggest that IVIg infused at 0.4 g/kg/day on five consecutive days may have a beneficial effect in patients with ANCA-positive vasculitis resulting not only in clinical improvement but also in some 50% reduction in levels of ANCA [151]. Controlled prospective studies are, however, needed to demonstrate the clinical usefulness of IVIg.

(c) Inflammatory lesions of lung and kidney in WG are characterized by large mononuclear infiltrates containing monocytes and T cells, with a majority of CD4-positive cells [125, 126]. The antigenic specificities of those T cells are not known, but peripheral blood lymphocytes reactive to ANCA-antigens such as proteinase 3 and MPO have been described [129–131]. In a patient with long-standing unclassified vasculitis treatment with the combination of two monoclonal antibodies, one directed against an antigen present on all mononuclear cells (CDw52) and the other against the CD4 molecule, resulted in long-lasting clinical remission whereas injection of the first antibody alone induced a short-lived remission only [152]. Recently, two additional patients with microscopic polyarteritis unresponsive to conventional treatment were treated with a comparable regimen [153]. Long-lasting remission was reached in one patient, the other developed antibodies to the humanized anti-CDw52 monoclonal antibody. These pilot studies await confirmation from a larger scale controlled study.

Conclusion

The diagnostic potential of ANCA is now fairly well established for the target antigens proteinase 3 and MPO. The presence of anti-proteinase 3 strongly suggests a diagnosis of Wegener's granulomatosis (WG), either in its generalized or limited presentation, although the association is not absolute. Changes in levels of anti-proteinase 3 in WG generally reflect disease activity. Anti-MPO is associated with various forms of necrotizing vasculitis including idiopathic necrotizing and crescentic glomerulonephritis (NCGN). Anti-MPO also occurs, incidentally, in other diseases. The diagnostic significance of ANCA of other or still unknown specificities has not yet been definitely established.

The pathophysiological role of ANCA has been studied in *in vitro* and *in vivo* models. *In vitro* anti-proteinase 3 interferes with the inactivation of proteinase 3 by α_1-antitrypsin. ANCA of diverse specificities are able to activate primed neutrophils to the production of reactive oxygen species and the release of

lysosomal enzymes. Both effects may contribute to the inflammatory process. With respect to vasculitis and glomerulonephritis the possible expression of proteinase 3 at the surface of (activated) endothelial cells and the binding of MPO on these cells might allow the respective antibodies to react to their targets *in vivo* and to injure the endothelium. In MPO-immunized animals perfusion of the kidney with the products of activated neutrophils results in the development of pauci-immune NCGN.

Although many questions still remain, the expanding knowledge on ANCA has greatly contributed to the diagnosis and the understanding of the pauci-immune necrotizing vasculitides.

CEES G.M. KALLENBERG, ELISABETH BROUWER, JAN J. WEENING, and JAN W. COHEN TERVAERT
University Hospital, Groningen, and Academic Medical Center, Amsterdam, The Netherlands

Reprint requests to C.G.M. Kallenberg, M.D., Ph.D., Department of Clinical Immunology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

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