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REVIEW

The current status of neutrophil cytoplasmic antibodies

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SUMMARY

Several studies in the past 10 years have demonstrated the occurrence of autoantibodies against cytoplasmic constituents in patients with vasculitis and glomerulonephritis. In this review the nomenclature of these antibodies is discussed and assays and clinical associations are summarized. Although the antigens involved are not completely identified, antibodies and T cells reactive with myeloid lysozomal enzymes may both play a significant role in pathogenesis.

Keywords anticytoplasmatic antibodies neutrophils vasculitis glomerulonephritis T cells

INTRODUCTION

Studies of autoantibodies directed against components of the neutrophil cytoplasm (ANCA) present in sera from patients with vasculitis or glomerulonephritis have recently received great interest. ANCA may be used as a clinical tool to support the diagnosis of vasculitis or glomerulonephritis or to monitor disease activity. In addition, studies on ANCA clarify the pathogenesis of those diseases. Many new findings over the past 5 years have led to the organization of two International Workshops on ANCA: the first one was held in Copenhagen, Denmark in January, 1988, and the second one in Noordwijkerhout, near Leiden, The Netherlands, in May, 1989. We discuss the nature of ANCA and their possible role in pathogenesis, as well as their clinical application.

NOMENCLATURE

Three major types of autoantibodies that react with cytoplasmatic constituents of neutrophils have been recognized. The first ANCA type is now called cANCA (classic anti-neutrophil cytoplasmatic antibody). It is defined by its diffuse, granular cytoplasmic staining with central accentuation of neutrophils and some monocytes but not lymphocytes, using a standard indirect immunofluorescence technique (IIF) with ethanol fixation of the leucocyte preparation. The standard IIF technique was carefully delineated during the first International Workshop on ANCA by Allan Wiik (Wiik, 1989). The international serum standard for cANCA is available from the Laboratory of Autoimmune Serology, Statens Seruminstitut, Copenhagen, Denmark.

The second ANCA system, characterized by a perinuclear staining pattern with the same technique, is pANCA (perinuclear ANCA). An international standard serum for pANCA is not yet available. The term pANCA was accepted at the Second International ANCA Workshop and probably contains several antibody systems, for example, anti-myeloperoxidase antibodies (Falk & Jennette, 1988). However, antibodies previously described as granulocyte-specific anti-nuclear antibodies (GS-ANA) may cause a similar pattern (Wiik, Jensen & Friis, 1974). The antigens which GS-ANA recognize remain to be determined. Either methods to distinguish true GS-ANA, that react only with nuclear constituents, from other pANCAs will need to be developed, or the nomenclature will need to be redefined.

Another system in which IgA-ANCA react with an acidic extract of neutrophils was discovered in Henoch–Schönlein patients (Van den Wall Bake *et al.*, 1987). However, this IgA-ANCA can not be demonstrated using sera in the standard IIF assay, but IgA isolated from IgA-ANCA positive sera gives a hazy indeterminate pattern different from cANCA or pANCA on IIF; this system has yet to be validated by other groups.

IMMUNOGLOBULIN (SUB) CLASS RESTRICTION

cANCA as originally described were restricted to the IgG class (Davies *et al.*, 1982; Hall *et al.*, 1984; Van Der Woude *et al.*, 1985). Later it was claimed that IgM-ANCA are found in a subgroup of patients with pulmonary haemorrhage and rapidly progressive glomerulonephritis (Penning, Jones & Lockwood, 1988). During the second ANCA Workshop, two groups (Noel *et al.*, 1989; Bleil *et al.*, 1989) found independently that most cANCA are restricted to IgG1 and IgG4 subclasses. In a minority of cases (usually later in the course of the disease) IgG2 may be found as well.

Detailed studies on immunoglobulin subclass restriction of pANCA and IgA ANCA are not yet available.

IDENTIFICATION OF ANTIGENS

In the original studies which proved that cANCA was a true autoantibody, it was demonstrated that $F(ab')_2$ fragments of

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cANCA can penetrate living granulocytes from cANCA-positive patients and from healthy controls (Van der Woude, 1985; Van der Woude *et al.*, 1989). This implies that the intracellular antigen is accessible to the antibody without involvement of Fc-mediated internalization mechanisms. This implies that the antigen is present not only within the granulocyte but also on its cell surface.

An initial report on the identification of the antigens suggested that cANCA-positive sera showed binding activity to human neutrophil alkaline phosphatase (Lockwood *et al.*, 1987); in this paper inhibition experiments were described with antigen preparations or calf-intestine alkaline phosphatase as competitors for the antibody activity in ANCA positive sera in a solid-phase radioimmunoassay. Antigen preparations were always produced using acid extraction of sonicated granulocytes. There was a concentration-dependent effect, with 50% or more inhibition of binding compared with control proteins at equivalent concentrations.

However, the role of alkaline-phosphatase has been disputed by others. Rasmussen, Borregaard & Wiik (1987) pointed out that rabbit anti-human alkaline phosphatase in the IIF technique results in an even, homogenous, 'cytoplasmic' staining of the entire cell membrane, quite dissimilar to the cANCA staining pattern. In addition, they showed with Percoll fractionation of granulocytes after nitrogen-bomb cavitation (Borregaard *et al.*, 1983) that cANCA is directed against a cellular compartment with a density between that of the primary and secondary granules of the neutrophils, again different from alkaline phosphatase. The localization of the cANCA antigen within the primary (or alpha or azurophilic) granules of the neutrophils was extensively confirmed during the First International ANCA Workshop ih Copenhagen.

Goldschmeding *et al.* (1989a) showed with the same method that the ANCA antigen co-purifies with the azurophilic granules, and that it is not significantly expressed in the specific granules, phosphosomes, plasmamembrane, or cytosol of these cells. With immunoprecipitation studies they also showed that, using 1% NP-40 extracts from total purified azurophilic granules after freezing and thawing, the ANCA antigen precipitates as a 29-kD band. Stimulation of cytochalasin B-primed neutrophils with f-met-leu-phe peptide (f MLP) revealed that these cells release cANCA antigen on degranulation. Binding studies with tritiated di-isopropyl-fluorophosphate suggested that the cANCA antigen is a serine proteinase.

These results were confirmed by elegant studies performed by Wieslander, Rasmussen & Bygren (1989). Alpha-granules purified with the Borregaard method were extracted in 1% Triton X-100 and 0·05 M NaAC, pH 5·5. The extract was purified with DEAE-Sephacel and Mono-S cation-exchange gel column chromatography. The mol. wt was then estimated to be 30 kD using a TSK SW3000 column and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE).

We used pooled purulent sputum from patients with various diseases to isolate the cANCA antigen (Daha *et al.*, 1989). The sputum was sonicated, stirred overnight, dialysed and subjected to DEAE-Sephacel, high-power liquid chromatography (HPLC) cation-exchange chromatography and HPLC TSK 3000 column chromatography. A 91-kD glycoprotein antigen was recognized by several cANCA-positive sera. Polyclonal and monoclonal antibodies were made against this antigen. Interestingly, such monoclonal antibodies recognize a 91-kD antigen in sputum or supernatant of neutrophils after phorbol myristate acetate (PMA) stimulation, but a 29-kD antigen in SDS extracts from neutrophils.

Lüdemann, Utecht & Groos (1989) presented data that showed strong similarity between a recently described serine protease from azurofilic granules, proteinase 3 (Kao *et al.*, 1988) and the cANCA antigen. The substrate specificity for both enzymes was identical, and fragments of both substances were 80% homologous in amino acid sequence. It remains to be determined whether cANCA-positive patient sera, or polyclonal and monoclonal ANCA developed in the laboratory, react with the purified proteinase 3 or with proteinase 3 bound to its natural inhibitor.

Anti-myeloperoxidase antibodies (MPO) cause a pANCA pattern in the standard IIF technique. However, the percentage of sera containing pANCA which are in fact anti-MPO is difficult to estimate. In the original report (Falk & Jennette, 1988) from the serum samples that showed anti-MPO specificity on ELISA, 13 out of 22 samples produced a perinuclear pattern on IIF. In the patients with necrotizing and crescentic glomerulonephritis 13 out of 16 pANCA-positive sera were positive in the MPO assay, while nine out of 19 cANCA-positive sera were anti-MPO-positive. Other investigators (Goldschmeding *et al.*, 1988) have suggested that pANCA staining may be caused by antibodies directed against elastase. The first pANCA were originally described in patients with Felty's syndrome and rheumatoid arthritis (Wiik *et al.*, 1974) and were originally called granulocyte-specific antinuclear antibodies. The antigens involved in pANCA staining patterns may differ and depend heavily on the patient population. It cannot be excluded that some pANCA-containing sera react with nuclear instead of cytoplasmic constituents. Finally, the antigens recognized by IgA-ANCA have not yet been identified. Our own preliminary data indicate that IgA-ANCA also recognizes a 91-kD antigen isolated from sputum.

SOLID-PHASE ASSAY SYSTEMS FOR ANCA DETECTION

Although the IIF test remains the gold standard for ANCA detection (Wiik, 1989), various groups have started to develop solid-phase assays to facilitate rapid reading and quantification. The first publication on the application of a solid-phase ANCA assay was a radioimmunoassay with acid extract from sonicated granulocytes as antigen preparation (Savage *et al.*, 1987). To check the specificity of binding, samples were tested for specific inhibition by adding the soluble antigen preparation. In patients without vasculitis non-specific positive reactions have to be excluded with the standard IIF technique. Other methods that have been reported make use of antigen preparations isolated with affinity chromatography (Ludemann *et al.*, 1988; 1989a), Percoll gradient centrifugation of nitrogen bomb cavitated material (Borregaard *et al.*, 1983; Rasmussen *et al.*, 1989b; Van Der Woude *et al.*, 1989) and granulocyte lysis by sucrose (Baugh & Travis, 1976; Van der Woude *et al.*, 1989). Finally, sandwich-ELISA systems using goat anti-mouse and a mouse monoclonal antibody against 29-kD antigen for antigen catching and subsequent incubation with patient serum and anti-human immunoglobulin antibodies have been reported (Goldschmeding *et al.*, 1989a, b). Solid-phase assays using these techniques were all discussed at First International Workshop on ANCA,

and it was concluded that further work was required to compare the available solid-phase assays both with the IIF and with each other. Therefore results obtained 'blind' by 13 laboratories on eight sera were compared during the second workshop. Participants were asked to measure cANCA or pANCA by IIF, to perform solid-phase assays with their putative cANCA antigen, myeloperoxidase, elastase, and to test for rheumatoid factors. IgG and IgM rheumatoid factors are present in most ANCA positive sera (Van Der Woude *et al.*, 1978; 1989) and can be expected to interfere in sandwich-ELISA systems.

The International Standard Serum for cANCA was scored positive by all centres with both IIF and solid-phase techniques for cANCA. A negative control was scored negative in all centres. Problems were encountered with sera from patients with inactive Wegener's disease who had previously had high cANCA titres. Discrepancies between centres both for IIF and solid-phase assays and within centres for IIF and solid-phase assays were observed. Similar discrepancies in this category of sera have been reported earlier (Rasmussen, Ludemann & Utecht, 1989a; Van der Woude *et al.*, 1989). Interestingly, one serum containing cANCA on IIF was also positive in the anti-MPO solid-phase assays and negative in 'cANCA solid-phase assays' except for those groups that worked with acid-extracted antigen preparations. Such sera have also been reported by Falk & Jennette (1988) illustrating that solid-phase assays with more-or-less purified antigens do not always help to distinguish between cANCA and pANCA sera.

More extensive studies are warranted for further standardization of solid-phase ANCA assays. In such studies several antigen preparations should be compared: crude extracts from neutrophil granules, immuno-affinity purified antigens and chemically defined constituents of myeloid granules (e.g. proteinase 3, myeloperoxidase, elastase) both in Western blots and solid-phase assays testing a large number of sera from healthy controls and patients with various diseases.

CLINICAL APPLICATIONS OF ANCA ASSAYS

ANCA were originally detected in sera from patients with viral infections, glomerulonephritis and vasculitis, and more specifically in patients with Wegener's granulomatosis (Davies *et al.*, 1982; Hall *et al.*, 1984; Van der Woude *et al.*, 1985). The value of ANCA measurements with the IIF method in Wegener's granulomatosis has been confirmed by several groups (Savage *et al.*, 1987; Ludemann & Gross, 1987; Parlevliet *et al.*, 1988; Andrassy *et al.*, 1988; Joller-Jemelka & Grob, 1988; Specks *et al.*, 1989). In a retrospective series of 379 sera we found that the positive predictive value for Wegener's granulomatosis of a positive immunofluorescence result for cANCA was 89%. In 23 patients disease activity was correlated with cANCA, assayed by immunofluorescence and by ELISA (Halma *et al.*, 1989). In an earlier study, somewhat biased by the heterogenous selection of sera, we found similar results (Cohen Tervaert *et al.*, 1989). The value of cANCA IIF tests in monitoring disease activity seems to be limited; relapses usually occur when titres are high but increasing titres are not necessarily followed by relapses. To exclude disease activity both a negative IIF cANCA and a normal sedimentation rate are useful.

The occurrence of ANCA in patients with other forms of vasculitis and idiopathic necrotizing and crescentic glomerulonephritis has caused some doubt as to the specificity of ANCA for Wegener's granulomatosis, but has also prompted speculations on similarities in the pathogenesis of these disease entities (Lie, 1989). It is now clear that cANCA can also be found in patients with microscopic polyarteritis (Lockwood *et al.*, 1987; Savage *et al.*, 1987), but there has been some discussion about the distinction of this form of vasculitis from Wegener's disease (Rasmussen *et al.*, 1987), since microscopic polyarteritis is not a term commonly used outside the United Kingdom. It is also known that Wegener's disease may initially present with glomerulonephritis only, while other disease manifestations may show only after several years (Van der Woude *et al.*, 1982). Therefore, a clinical prospective study to investigate the value of cANCA assays by documenting clinical signs and symptoms and histology sequentially in a large group of patients with various forms of vasculitis and crescentic glomerulonephritis is still urgently needed. In order to get a good estimate of sensitivity and specificity of cANCA assays, large groups of healthy and diseased controls should be included in such a study.

The situation for pANCA assays is even more unclear. Since the original studies by Wiik *et al.*, (1974) there have been no new data on the value of pANCA in patients with rheumatoid arthritis and related diseases. Falk & Jennette (1988) have reported the occurrence of pANCA and anti-MPO antibodies in patients with necrotizing and crescentic glomerulonephritis, systemic lupus erythematosus and other renal lesions. During the Second International ANCA Workshop, fourteen papers on the clinical associations of cANCA and pANCA were presented (publication in press). Most papers concluded that the vast majority of patients with Wegener's disease (>90%) have cANCA, and only a minority have pANCA. In polyarteritis nodosa cANCA and pANCA each are present in about half of the patients, while in idiopathic necrotizing and crescentic glomerulonephritis most patients will have pANCA. Falk & Jennette (1988) therefore proposed that these patients form a pathologic continuum ranging from renal limited to widespread systemic vascular injury, including patients with primary crescentic glomerulonephritis, Wegener's granulomatosis and polyarteritis nodosa.

In view of the current knowledge on clinical associations of ANCA, it still seems prudent for the clinician to look at ANCA assays as one of the helpful parameters for diagnosis. For example, to make the diagnosis of Wegener's granulomatosis with certainty, abnormalities in the upper and lower respiratory tract and the kidneys, together with the histological finding of granulomatous vasculitis still have to be demonstrated. A negative ANCA test is a strong argument against active Wegener's disease, while a positive cANCA by IIF and solid-phase techniques only increases the likelihood of this diagnosis.

ANCA AND PATHOGENESIS

The hypothesis has been made (Fauci, 1978) that Wegener's granulomatosis is caused by tissue deposition of circulating immune complexes. However, tissue deposition of immunoglobulins is generally not apparent in this disease (Van der Woude *et al.*, 1978; Ronco *et al.*, 1983). The animal experiments by Germuth & Pollack (1967) that are usually cited in this respect, cannot be regarded as conclusive either; in these experiments a massive amount of antigen has been injected in animals, leading

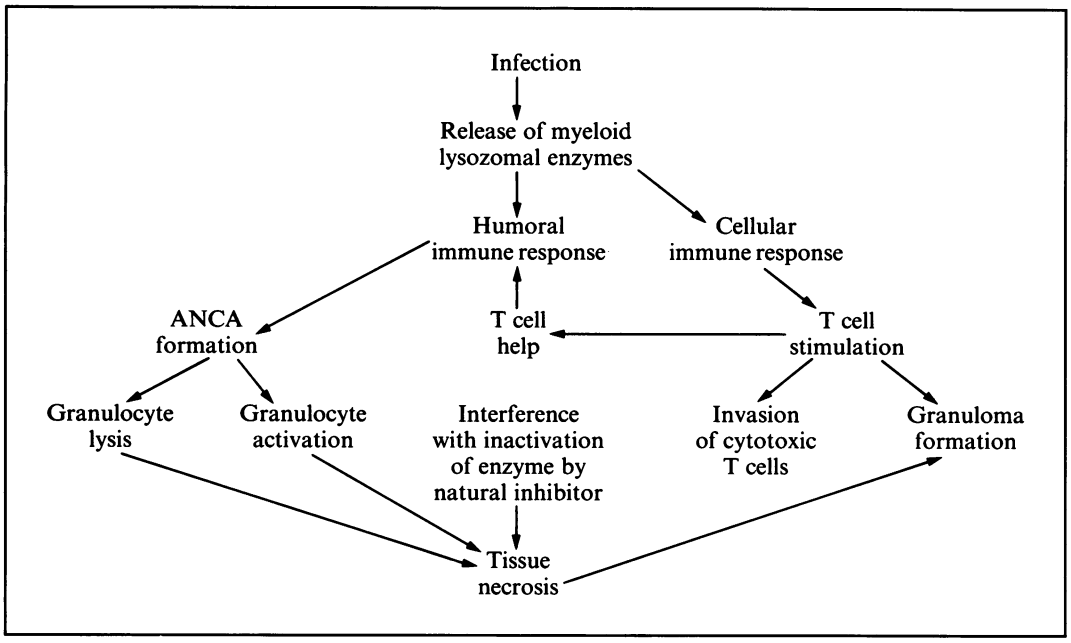

Fig. 1. Hypothesis about the pathogenetic role of classic anti-neutrophil cytoplasmic antibody (cANCA) in Wegener's granulomatosis.

to lesions which are not the same as the histological abnormalities present in Wegener's disease.

On the basis of the current knowledge about the clinical presentation and serological and histological findings in the disease, another hypothesis can be proposed (Fig. 1). Friedrich Wegener himself already postulated that a microbial infection or hyper-reaction to some extrinsic agent was the cause of the disease (Wegener, 1936; 1937). The role of infection in the initiation of relapses in Wegener's disease is suggested by the fact that most relapses occur at the time of infection with common pathogens (Pinching *et al.*, 1980). In addition, several groups claim that antibiotic therapy with sulfomethoxazole-trimethoprim has a beneficial effect on the course of the disease (De Remee, McDonald & Weiland, 1985; Israel, 1988; Yuasa *et al.*, 1988). If infection in the respiratory tract is indeed the trigger for the initiation of relapses, it may do so because it causes the release of myeloid lysosomal enzymes that elicit a humoral and cellular autoimmune response. The individual susceptibility to produce ANCA and T cells reactive with the ANCA antigen may be HLA linked (Katz *et al.*, 1979; Elkon, Sutherland & Rees, 1983). The fact that other autoantibodies are regularly formed in Wegener's disease (Shillitoe *et al.*, 1974) points also to an autoimmune-prone constitution in patients with Wegener's disease. Measurements of IgG production by B cells from untreated patients with Wegener's granulomatosis show normal spontaneous IgG production with occasionally very high values (Rasmussen *et al.*, 1988) but the number of patients is small and such studies need to be confirmed and extended.

Anti-neutrophil antibodies may theoretically play a direct role in pathogenesis by causing granulocyte lysis and subsequent tissue necrosis. Donald, Edwards & McEvoy (1976) found intravascular lysis of polymorphonuclear granulocytes in the vessels of the lesions of the disease; in their report, organelles thought to originate from neutrophilic cytoplasm were found to be coated by electron-dense material, indicating the presence of immunoglobulins on their surface. Another communication (Dale, Fauci & Wolff, 1973) described increased turnover rates of granulocytes during active disease. Widespread tissue necrosis is a major characteristic of the disease. Since cANCA may bind to a 29-kD serine protease from within the neutrophil as well as to a 91-kD antigen from neutrophil supernatant or sputum (Daha *et al.*, 1989), it is attractive to speculate that cANCA may bind to both the proteinase and to the complex formed by the proteinase and its inhibitor. Proteinase–antibody complexes may be protected against inactivation by proteinase inhibitors and transported through the body to other areas, such as the kidneys. If the proteinases in these complexes retain their enzymatic activity, they may then cause local damage.

There is also evidence that ANCA may stimulate neutrophils directly and induce O_2 release, but not degranulation (Falk *et al.*, 1989). Immunohistochemical examinations of biopsies from upper or lower respiratory tract (Gephardt, Ahmad & Tubbs, 1983; ten Berge *et al.*, 1985; Rasmussen *et al.*, 1988) show monocytes and T lymphocytes as the dominating cell types in the cellular infiltrates. We have shown (Daha *et al.*, 1989) that T lymphocytes from several patients with Wegener's granulomatosis are stimulated to proliferate by the cANCA antigen in contrast to lymphocytes from healthy controls. In view of these findings, it is attractive to speculate that T cells directed against the cANCA antigen invade lesions which contain proteinase–antibody complexes. The presence of tissue necrosis and such proteinase–antibody complexes may then induce granuloma formation, together with local lymphokine production by T cells.

CONCLUSION

We have summarized recent data indicating the occurrence of ANCA in vasculitis and glomerulonephritis. Nomenclature and a standard IIF test have been established after two International

Workshops on ANCA. This will undoubtedly stimulate investigators to study clinical associations of cANCA and pANCA; however, the lack of standardization of solid-phase ANCA assays is still an impediment for such studies. At present, the question whether ANCA have any pathogenetic role is most challenging. Therefore, the functional roles of the antigens involved and of ANCA–antigen reactive T cells appear to be the most promising objectives for further studies.

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