Antineutrophil Cytoplasmic Autoantibodies: A Review of the Antigens Involved, the Assays, and the Clinical and Possible Pathogenetic Consequences

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Since the original description of the clinical relevance of antineutrophil cytoplasmic antibodies (ANCA) testing for patients with vasculitis and glomerulonephritis, antibodies directed against different enzymes in granulocyte granules have been the subject of many ongoing studies. Although the state of the art has been reviewed previously, new findings over the last 2 years reflect the widespread interest in this new class of autoantibodies. The Fourth International Workshop on ANCA held in Lübeck, Germany in May 1992 was a clear demonstration of the recent progress in antigen identification, the development of new ANCA assays, the association of ANCA subtypes with clinical-pathologic syndromes, and the description of in vivo and in vitro models to study the pathogenic role of ANCA in vascular inflammation.

In this review we summarize the present state of the art with emphasis on the most recent findings.

ANCA-RELATED ANTIGENS

The polymorphonuclear granulocyte (PMN) contains two main kinds of granules, the primary or α-granules and the secondary granules. Enzymes within these granules are the targets for ANCA. Therefore, the biochemistry of these granules will be briefly discussed. The α-granules are formed during the promyelocytic differentiation stage of the PMN. They fuse with endosomes to form endolysosomes, cell structures in which microbes are attacked by several granule constituents. The main α-granule proteins are the enzymes myeloperoxidase and a number of serine proteases, including neutrophil-elastase, cathepsin-G, and proteinase-3. Furthermore, lysozyme and other microbicidal enzymes, such as bacterial defensins, have been shown. These granules play an important role in the digestion of both infectious and noninfectious agents. Monocytes possess similar granules that contain myeloperoxidase, neutrophil elastase, and proteinase-3. The secondary granules are formed later during cellular differentiation and contain lactoferrin and vitamin B-12 binding protein. A number of enzymes in the granules are now recognized to be target antigens for ANCA. Depending on the nature, charge, and distribution of the antigen in the ethanol-fixed granulocyte, ANCA of various antigenic specificities can be detected on the basis of different staining patterns in the indirect immune fluorescence test. Two main patterns are distinguished, cytoplasmic and perinuclear or nuclear staining. The latter pattern is caused by an artificial redistribution of proteins to the nucleus, caused by the ethanol fixation and air-drying of the PMNs (the standard technique).

The cytoplasmic ANCA (cANCA) pattern is characterized by diffuse fine granular staining of the cytoplasm with an accentuation of staining in the central area of the cell between the nuclear lobes. The main antigen associated with the cANCA pattern is proteinase-3. These antibodies can be found in patients with Wegener's granulomatosis or other forms of systemic vasculitis. However, not all cANCA sera may react with proteinase-3 in enzyme-linked immunosorbent assay (ELISA). In a large series of Wegener's patients presented during the Fourth International Workshop on ANCA, 80% of the cANCA-positive sera were shown to react with purified proteinase-3. Another investigation showed that 13 of 37 ANCA-positive sera from patients with Wegener's granulomatosis did not react with purified proteinase-3 or myeloperoxidase in ELISA. These findings contrast with earlier data obtained using immune affinity-purified proteinase-3 and a capture ELISA. These discrepancies could be the result of differences in isolation techniques, causing the loss of antigenic epitopes, or due to technical differences in the ELISA systems used. However, on the basis of our experience within the EEC/BCR workgroup on ANCA, we discovered that cANCA-positive sera may contain antibodies directed against antigens other than proteinase-3. One of 12 cANCA-positive sera from vasculitis patients showed a strong cANCA pattern in the IIF-test, but did not react with any of five proteinase-3 preparations. Therefore, recent reports about a lower than 100% correlation between cANCA-positive sera and antibodies to proteinase-3 are probably a reflection of the real situation and are not solely based on technical differences.

The second staining pattern recognized in the indirect immune fluorescence test is the perinuclear ANCA (pANCA) staining. The pANCA pattern was described in 1989 by Falk and Jennette in patients with crescentic necrotizing glomerulonephritis and systemic vasculitis. The antigen most commonly recognized by the sera from these patients was myeloperoxidase. However, the staining pattern described is an artifactual one. The myeloperoxidase, although present in the same granules as proteinase-3, redistributes in the PMN upon ethanol fixation and sticks to the nucleus. This artificial redistribution results in the pANCA pattern observed when antibodies against myeloperoxidase react with these fixed cells. However, when PMNs are fixed with paraformaldehyde, the same sera show cytoplasmic staining. Antinuclear antibodies show a nuclear staining pattern both on ethanol-fixed and on paraformaldehyde-fixed granulocytes. To distinguish pANCA from antinuclear antibodies, it is advised to test the sera either on paraformaldehyde-fixed PMNs.
or to use a different cellular substrate than PMNs, such as liver sections or cultured HEp-2 cells. In addition to myeloperoxidase, antibodies directed against other enzymes of the PMN may give rise to a similar staining pattern. Although rare, antibodies have been described against lactoferrin (in Wegener’s granulomatosis), and cathepsin-G (in patients with arthritis), neutrophil elastase (in patients with vasculitis and liver sections or cultured HEp-2 cells. In addition to myeloperoxidase antibodies using commercial antigen preparations” gave comparable results. For the detection of ANCA, various antigen preparations were used. The antigen, in a complexed form with al-antitrypsin can also be obtained in large quantities from purulent sputum preparations” gave comparable results. For the detection of ANCA, various antigen preparations were used. The antigen, in a complexed form with al-antitrypsin can also be obtained in large quantities from purulent sputum or nasopharyngeal secretions. ANCA may give rise to a similar staining pattern. Although rare, antibodies have been described against lactoferrin (in Wegener’s granulomatosis), and cathepsin-G (in patients with arthritis), and neutrophil elastase (in patients with vasculitis and liver sections or cultured HEp-2 cells). The staining pattern(s) caused by these antibodies may be perinuclear, or present an entirely different staining pattern. During the Fourth International ANCA Workshop, it was decided that staining patterns other than cANCA and pANCA were to be noted as atypical until the antibodies responsible for such staining and their antigenic targets are further characterized and their clinical relevance shown.

ANCA ASSAYS

Although the indirect immune fluorescence test is of proven value for clinical application, solid-phase assays to allow large-scale testing, better quantification, and determination of the ANCA specificity are necessary for further improvement of clinical diagnosis. In a previous review we have given an outline of the evolution of ANCA solid-phase assays until 1991. Currently, to determine or confirm seropositivity for cANCA, either an α-granule extract or purified proteinase-3 are used. Proteinase-3 may be purified by several isolation methods, including affinity chromatography using anti-proteinase-3 monoclonal antibodies, affinity chromatography on an Orange A column, reverse-phase high-pressure liquid chromatography, gel filtration, and cation exchange chromatography. The antigen, in a complexed form with αl-antitrypsin can also be obtained in large quantities from purulent sputum and may be used successfully in ELISA systems in this form. It is the aim of the EC/BCR Study Group for ANCA Assay Standardization to standardize ANCA assays within Europe. This group of 14 laboratories in 12 countries is working cooperatively to develop well-standardized ANCA assays and to study both the sensitivity and specificity of such assays. In the first phase of this study, solid-phase assays for antimi- loperoxidase antibodies using commercial antigen preparations gave comparable results. For the detection of anti-proteinase-3 antibodies, various antigen preparations were compared. There were discrepancies between the various assays. When a simialx antigen (α-granules) was used by different laboratories, the results were discrepant as well. In the next phases of the EEC study, proteinase-3 and myeloperoxidase preparations from selected sources will be provided to all participating centers. The use of these defined antigens for ANCA detection will also be evaluated in a large-scale clinical study.

### Table 1. ANCA-Disease Association

<table>
<thead>
<tr>
<th>ANCA</th>
<th>Target Antigen</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>PR-3, rarely MPO</td>
<td><strong>High sensitivity and specificity of cANCA for active disease, association with disease activity</strong></td>
<td></td>
</tr>
<tr>
<td>PR-3, MPO</td>
<td><strong>High sensitivity for active disease</strong></td>
<td></td>
</tr>
<tr>
<td>Rarely PR-3 or MPO</td>
<td>No PR-3 or MPO</td>
<td></td>
</tr>
<tr>
<td>Unknown, ANA, rarely MPO, LF</td>
<td><strong>ANA can cause pANCA pattern in indirect immune fluorescence test for ANCA</strong></td>
<td></td>
</tr>
<tr>
<td>Cath-G, LF, unknown</td>
<td><strong>Lower frequency than ulcerative colitis</strong></td>
<td></td>
</tr>
<tr>
<td>Cath-G, LF, unknown</td>
<td><strong>Low sensitivity or specificity</strong></td>
<td></td>
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<tr>
<td>Cath-G, LF, unknown</td>
<td>Unknown</td>
<td></td>
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<td>Unknown</td>
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**Other diseases**

<table>
<thead>
<tr>
<th>ANCA</th>
<th>Target Antigen</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>pANCA/typical ANCA</td>
<td>Cath-G, LF, unknown</td>
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<tr>
<td>pANCA/typical ANCA</td>
<td>Cath-G, LF, unknown</td>
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<tr>
<td>pANCA/typical ANCA</td>
<td>Cath-G, LF, unknown</td>
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<tr>
<td>ANCA?</td>
<td>Unknown</td>
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<tr>
<td>cANCA</td>
<td>Unknown</td>
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**Abbreviations:** PR-3, proteinase-3; MPO, myeloperoxidase; LF, lactoferrin; Cath-G, cathepsin-G.
parrable with Wegener's granulomatosis or other forms of systemic vasculitis, the ANCA assay should not be regarded as diagnostic proof for Wegener's granulomatosis, and efforts should always be undertaken to obtain histologic evidence for the disease. Immunosuppressive treatment should not be started solely on the presence of a positive ANCA test. However, during active Wegener's granulomatosis, it is rare to have a negative ANCA test when using immunofluorescence.

It has been claimed that the ANCA test may be of value for monitoring disease activity during patient follow-up. The ANCA titer levels may increase before clinical relapse of disease occurs.17,18 The differentiation between intercurrent infection under immune suppressive therapy and relapse of the disease may be facilitated by an increase in the ANCA titer level. Cohen Tervaert et al19 closely monitored the ANCA titers in patients with Wegener's granulomatosis in a prospective study. They randomized patients with more than a twofold increase in titer levels between a group receiving early immunosuppressive treatment and a group receiving treatment when clinical symptoms occurred.17 The group treated early developed fewer relapses and overall used less immunosuppressive drugs than the group treated when symptoms of disease occurred. However, Kerr et al19 investigated serial sera obtained from a group of 53 patients. They could demonstrate a titer level increase before disease relapse in only 24% of patients. Other patients remained ANCA positive while in complete remission, or ANCA negative while still having active disease. These data and our own experience warrant great caution in initiation of treatment based solely on ANCA titers. The risk of overtreatment with cyclophosphamide and steroids is clearly present.

Other forms of systemic vasculitis. ANCA has been associated with microscopic polyarteritis as well.14 This form of systemic vasculitis, defined by crescentic necrotizing glomerulonephritis in association with systemic small vessel vasculitis, but without granulomas, is associated with both cANCA and pANCA. Anti–proteinase-3 antibodies were reported in 46% of patients with microscopic polyarteritis, and antimonyeloperoxidase in 50%.21 Microscopic polyarteritis is not uniformly accepted as a diagnostic entity. Therefore, the frequency of pANCA and cANCA in these patients varies with the definition used. In the series of Hauschild et al,2 for example, the presence of pANCA was 4 times more frequent than cANCA.

ANCA have not been studied in large series of patients with the Churg-Strauss syndrome, a clinical entity defined as the combination of asthma, eosinophilia, and necrotizing vasculitis, possibly with crescentic necrotizing glomerulonephritis or necrotizing granulomas. In small series of patients, the presence of antimonyeloperoxidase antibodies has been described.1,22

In some cases of classical polyarteritis nodosa, the presence of ANCA has been shown. O'Donoghue et al22a reported ANCA in 27% of patients with polyarteritis nodosa; Hauschild et al21 showed data from 36 patients, 4 having cANCA (1 with anti–proteinase-3), 3 with pANCA (2 anti–cathepsin-G, 1 antielastase). ANCA, therefore, seems to be an infrequent feature in classical polyarteritis nodosa.

ANCA have only rarely been described in patients with giant cell arteritis. In a recent report,23 giant cell arteritis and, to a lesser extent, polymyalgia rheumatica were associated with cytoplasmic staining of formalin-fixed neutrophils, but not with ethanol-fixed neutrophils. The antigen involved in these cases is not known.

ANCA have not been described in patients with other forms of systemic vasculitis, such as thromboangiitis obliterans, Takayasu's disease, M. Behçet, or mixed cryoglobulinemia. ANCA in rheumatic diseases. The first description of antineutrophil antibodies by Wilk et al24 concerned patients with rheumatoid arthritis (RA) and the Felty’s syndrome. These antibodies were described as granulocyte-specific antinuclear antibodies (GS-ANA) and were directed against the nucleus of the PMN. The antigen recognized by GS-ANA is still unknown. However, several studies have shown the presence of ANCA with different staining patterns in sera from RA patients, with various antigenic specificities. Braun et al25 found a pANCA pattern in 20% of RA cases, and 50% of cases of Felty's syndrome. Antibodies against other neutrophil constituents were found as well. Other investigators have shown pANCA in RA patients with varying frequency. We have described pANCA and atypical ANCA in 36% of patients with active seropositive RA, and in 43% of patients with RA complicated by vasculitis. In contrast to the cytoplasmic or perinuclear pattern, the sera showed an atypical staining, with both perinuclear and diffuse cytoplasmic fluorescence. In the RA plus vasculitis patients, there was a significant correlation between a positive atypical ANCA test by indirect immune fluorescence and the presence of antilactoferrin antibodies in ELISA.26

In systemic lupus erythematosus, antinuclear antibodies may be hard to distinguish from pANCA when ethanol-fixed PMNs are used. However, in lupus patients, a number of sera were reported to be pANCA positive and antinuclear antibody negative (HEp-2 cells).27 Some of these sera reacted with myeloperoxidase,28 whereas other sera reacted with lactoferrin.28 The correlation of the titer of these antibodies to the activity of the disease has not been established.

Inflammatory bowel disease. An increasing amount of scientific data has accumulated since the description of ANCA in patients with ulcerative colitis (UC) and Crohn’s disease (CD).29 The original report showed that pANCA were present mainly in UC and not in CD. During the Fourth International Workshop, new data on these clinical entities were presented.

Positivity for pANCA can be found in 40% to 70% of sera from patients with UC. The staining pattern of ethanol fixed neutrophils differs from the perinuclear pattern of antimonyeloperoxidase ANCA. The staining is cytoplasmic when formalin-fixed neutrophils are used.30 A similar staining pattern can be observed in 5% to 35% of patients with CD. There is conflicting data about the relation between disease activity and the presence or absence of ANCA. Most investigators did not find a relation,30,31 whereas Rump et al32 showed that ANCA disappeared after steroid treatment. Several studies were performed to identify the antigen(s) related to the inflammatory bowel disease ANCA. In general, only few sera reacted with proteinase-3 or myeloperoxidase in ELISA. Antibodies against lactoferrin were more frequent (10% to
25%). Lesavre et al found anti–cathepsin-G antibodies in 68% of patients with UC, but reactivity was also observed with a wide variety of chronic liver diseases. Mulder et al described as yet unknown antigens with a molecular size of 67 Kd and 63/54 Kd.

From these data we can conclude that pANCA, directed against both known and unknown antigens, can be found in CD and UC, but more frequently in the latter. During the Workshop, it was decided that the ANCA found in inflammatory bowel disease should not yet be nominated as a special ANCA entity, but instead that more work needs to be performed to identify and define the target antigens involved.

Other diseases. pANCA have also been described in several kinds of chronic liver disease. The antigens involved remain unclear, although Lesavre et al found anti–cathepsin-G in 57% to 88% of sera.

Two reports have shown ANCA staining in patients with human immunodeficiency virus (HIV) infection. Klaassen et al described both cANCA and pANCA in a cross-sectional study of HIV-infected individuals. The antigens recognized could not be identified. Savige et al confirmed these data, and found reactivity against proteinase-3 and myeloperoxidase in some of the positive sera.

ANCAs in patients with acute infection (2 of 22) were found by Mege et al. An earlier report showed ANCA in patients with chronic airway infections. Similar findings have been reported in patients with cystic fibrosis and purulent airway disease or in patients with pneumonia or empyema. These findings have not been confirmed by other centers. The antigenic targets for such ANCA are unknown currently.

PATHOGENESIS AND ANCA

Although we have speculated previously on the pathogenetic role of ANCA, it should be made clear that there is no definitive proof as yet for a causative role of ANCA in any disease. In rare instances, patients with Wegener’s granulomatosis (the disease in which the occurrence of ANCA has been documented most convincingly) may present with all known manifestations of the disease without ever developing detectable cANCA titers. The association of pANCA with active disease of whatever nature is less firm.

Although the pathogenesis of Wegener’s granulomatosis remains unclear, infections of the upper airways are thought to precipitate disease activity. As a result of the respiratory tract infection, large quantities of proteinase-3 may occur in the sputum, leading to an anti–proteinase-3 immune response in susceptible individuals. Further, cytokines such as tumor necrosis factor-α (TNF-α) are now known to translocate ANCA-reactive proteins from the primary granules to the neutrophil cell surface, thereby exposing the antigen to the patient’s immune system. Moreover, there may be an association between certain HLA class allotypes and the susceptibility for ANCA-associated diseases, suggesting that only susceptible individuals may develop ANCA after an infectious or inflammatory stimulus.

There are now five independent lines of evidence generated from in vitro experiments that suggest that ANCA or ANCA-antigen related immunity may directly play a role in the pathogenesis of the histologic lesions encountered in Wegener’s granulomatosis and related diseases:

1. Myeloperoxidase may directly bind to cultured human endothelial cells and remain antigenic. Savage et al showed that myeloperoxidase bound to endothelial cells is recognized by pANCA-containing sera. Seven pANCA-positive sera led to complement-mediated cytotoxicity after the addition of rabbit complement. It is thought that the initial binding of (positively charged) myeloperoxidase to the (negatively charged) endothelial cell surface can be explained by charge interactions.

2. Proteinase-3 is possibly produced by endothelial cells; Mayet et al reported that proteinase-3 could be shown to be present in the cytoplasm of untreated cultured endothelial cells using both monoclonal mouse and polyclonal human antibodies in confocal laser scanning microscopy. Stimulation with TNF-α led to a markedly increased antigen expression in the Golgi region and a time-dependent translocation to the cell surface. Incubation of endothelial cells stimulated with TNF-α with cANCA-positive sera led to enhanced procoagulatory activity and adhesion of neutrophils. The increased neutrophil adhesion of granulocytes to cultured endothelium induced by ANCA can be inhibited by monoclonal antibodies to CD18, suggesting that integrin molecules play an important role in this phenomenon.

3. ANCA may activate neutrophils and thereby act as proinflammatory mediators. In 1973, Dale et al reported that total blood pools and turnover rates were significantly higher in patients with active Wegener’s granulomatosis as compared with normals or patients on therapy. Falk et al showed that ANCA-positive sera, ANCA-IgG, heterologous antemyeloperoxidase, and myeloperoxidase-ANCA–positive F(ab)2 fragments are able to stimulate the release of reactive oxygen species. This stimulation is facilitated by priming the neutrophils with TNF-α and is also reflected by degranulation and chemotaxis.

4. Anti–proteinase-3 antibodies prevent the inactivation of proteinase-3 by its natural inhibitor α1-antitrypsin. On the other hand, these antibodies directly decrease the proteolytic activity of proteinase-3 towards large substrates. The net effect in vivo of these antibodies remains uncertain, although it is conceivable that anti–proteinase-3 antibodies may play a role in the widespread tissue necrosis observed in Wegener’s granulomatosis, through the interference with complex formation of proteinase-3 with α1-antitrypsin.

5. T lymphocytes of Wegener’s granulomatosis patients may proliferate in response to proteinase-3. We have reported that T lymphocytes from patients with Wegener’s granulomatosis proliferate in response to proteins derived from the azurophilic granule and proteinase-3 isolated from sputum. This proliferative effect was not seen in normal patient control cells. These results were later confirmed by others. It has not been elucidated how T lymphocytes are involved...
in the production of autoantibodies and/or how tissue destruction with granuloma formation occurs in Wegener's granulomatosis patients. There is little data on in vivo studies of the pathogenic effect of ANCA. Mathiesen et al. reported on the occurrence of antimyeloperoxidase antibodies after the administration of HgCl₂ to Brown Norway rats; however, these animals develop autoantibodies against several antigens and it is therefore hard to attribute a specific role of the antimyeloperoxidase antibodies in the pathogenesis of the disease manifestations in this animal model. Kiser et al. succeeded in increasing vascular permeability in the dermal vasculature of the anesthesized and spontaneously hypertensive rat by intradermal injection of polyclonal rabbit antimyeloperoxidase antibodies. It is not clear if this is a direct effect or one mediated by antimyeloperoxidase-induced leukocyte activation.

Finally, Brouwer et al. reported on a renal perfusion model with myeloperoxidase in Brown Norway rats immunized with myeloperoxidase. After 10 days, giant cell formation and vasculitis could be observed in the glomeruli after perfusion with myeloperoxidase, H₂O₂ and antimyeloperoxidase antibodies. Damage to the glomerular basement membrane by the myeloperoxidase enzymatic activity possibly explains these findings: this model might reflect a pathogenetic mechanism relevant for ANCA-related diseases in humans.

CONCLUSION

Several antigens recognized by ANCA in sera from patients with systemic vasculitis or glomerulonephritis have now been identified. However, new classes of ANCA in diseases such as inflammatory bowel disease and chronic liver disease have recently been identified. The nature of the target antigens in these diseases is poorly understood and their clinical relevance is thus far unclear. The clinical spectrum of ANCA has become more extensive, and sometimes more confusing. In the near future, antigen-specific ELISAs may clarify the issue of disease specificity of ANCA.

ACKNOWLEDGMENT

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