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 clinico-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 microbial enzymes, such as bacterial permeability increasing protein or cationic protein 57, and 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-3.3 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 with a capture ELISA.8 These reports showed that almost all cANCA-positive sera reacted with proteinase-3 in 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 artificial 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. Anti-nuclear 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.
Table 1. ANCA-Disease Association

<table>
<thead>
<tr>
<th>Systemic vasculitis</th>
<th>ANCA</th>
<th>Target Antigen</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Wegener’s granulomatosis</td>
<td>cANCA, rarely pANCA</td>
<td>PR-3, rarely MPO</td>
<td>High sensitivity and specificity of cANCA for active disease, association with disease activity</td>
</tr>
<tr>
<td>2. Microscopic polyarteritis</td>
<td>cANCA, pANCA</td>
<td>PR-3, MPO</td>
<td>High sensitivity for active disease</td>
</tr>
<tr>
<td>3. Churg Strauss syndrome</td>
<td>pANCA</td>
<td>MPO</td>
<td></td>
</tr>
<tr>
<td>4. Classic polyarteritis nodosa</td>
<td>Low percentage of ANCA</td>
<td>Rarely PR-3 or MPO</td>
<td></td>
</tr>
<tr>
<td>5. Other forms of systemic vasculitis</td>
<td>Rarely</td>
<td>No PR-3 or MPO</td>
<td></td>
</tr>
</tbody>
</table>

Rheumatic diseases

| 1. Rheumatoid arthritis | GS-ANA/pANCA/atypical ANCA | Unknown, ANA, rarely MPO, LF | When complicated by vasculitis often anti-LF antibodies |
| 2. Systemic lupus erythematosi | pANCA | Rarely MPO, LF | ANA can cause pANCA pattern in indirect immune fluorescence test for ANCA |

Inflammatory bowel disease

| 1. Ulcerative colitis | pANCA/atypical ANCA | Cath-G, LF, unknown | Low sensitivity or specificity |
| 2. Crohn’s disease | pANCA/atypical ANCA | Cath-G, LF, unknown | Lower frequency than ulcerative colitis |

Other diseases

| 1. Chronic liver diseases | pANCA/atypical ANCA | Cath-G, LF, unknown | |
| 2. Acute/chronic infection | ANCA? | Unknown | |
| 3. HIV infection | cANCA | Unknown | |

Abbreviations: PR-3, proteinase-3; MPO, myeloperoxidase; LF, lactoferrin; Cath-G, cathepsin-G.

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 patients with systemic lupus erythematosus and rheumatoid arthritis), neutrophil elastase (in patients with vasculitis and Wegener’s granulomatosis), and cathepsin-G (in patients with inflammatory bowel disease and chronic hepatic disorders). 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 1991.4 Currently, to determine or confirm seropositivity for cANCA, either an α-granule extract or purified proteinase-3 are used.10 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.11 The antigen, in a complexed form with α1-antitrypsin can also be obtained in large quantities from purulent sputum and may be used successfully in ELISA systems in this form.12 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 antimeyloperoxidase antibodies using commercial antigen preparations10 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 similar antigen (α-granules) was used by different laboratories, the results were discrepant as well.10 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.

ANCA-DISEASE ASSOCIATION (TABLE 1)

Wegener’s granulomatosis. ANCA were first described in patients with glomerulonephritis in association with a viral infection.13 The original report on the association of cANCA with Wegener’s granulomatosis1 has since been confirmed by numerous investigators.7,8,14 At present, the sensitivity for generalized, histologically proven Wegener’s granulomatosis is believed to be around 80%, with the specificity of these tests reaching 97%. In a recent study, values obtained for sensitivity and specificity using consecutive laboratory samples were not higher than 71% and 84%,15 respectively. Moreover, there were 95 (57%) confirmed false-positive results (54 pANCA and 41 cANCA). These latter results are in line with those obtained in an earlier study.16 Although the ANCA indirect immune fluorescence test is still useful in the diagnostic workup of patients with symptoms and signs com-
patible 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 al17 closely monitored the ANCA titer 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 granulomas50 is associated with both cANCA and pANCA. Anti-proteinase-3 antibodies were reported in 46% of patients with microscopic polyarteritis, and antemyeloperoxidase 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 antomyeloperoxidase antibodies has been described.1,4,22

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, Takayasus’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 al23 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,22 whereas other sera reacted with lactoferrin.22 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).28 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 antityperoxidase ANCA. The staining is cytoplasmic when formalin-fixed neutrophils are used. 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
tumor necrosis factor-α (TNF-α) are now known to trans-
tract infection, large quantities of proteinase-3 may occur in
proteinase-3 antibodies may play a role in the
In the pathogenesis of the histologic lesions encountered in Weg-
proteinase-3 with anti-proteinase-3 immune re-
proteinase-3 antibodies may play a role in the
proinflammatory mediators. In 1973, Dale et al^72^ 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^72^ showed that ANCA-positive sera, ANCA-IgG, heterologous antimyeloperoxidase, 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.~^72,73^ 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.~^14^ 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.

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.~^35^ This proliferative effect was not seen in normal patient control cells. These results were later confirmed by others.~^36^ 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\(^\text{57}\) reported on the occurrence of antimyeloperoxidase antibodies after the administration of HgCl\(_2\) 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\(^\text{38}\) succeeded in increasing vascular permeability in the dermal vasculature of the anesthetized 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\(^\text{39}\) 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. H\(_2\)O\(_2\) 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 defined. 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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**REFERENCES**


2002 HAGEN ET AL.


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