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MINIREVIEWS

Clinical Utility of Testing for Antineutrophil Cytoplasmic Antibodies

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Autoantibodies against various "self" intra- or extracellular, cell surface-bound or soluble antigens are frequently present in the sera of patients with autoimmune diseases. Regardless of their precise role in the pathogenesis of these diseases, testing for autoantibodies constitutes an integral part of the initial diagnostic workup of patients with organ-specific or systemic autoimmune diseases. Diseases that affect specific organs—autoimmune thyroiditis (thyroid), myasthenia gravis (neuromuscular system), autoimmune cytopenias (hematopoietic system)—or multiple organs—systemic lupus erythematosus (SLE), scleroderma, Sjögren's syndrome—are typical examples of diseases for which determination of different autoantibodies aids the clinician in establishing the correct diagnosis.

Since the introduction of rheumatoid factor and antinuclear antibody (ANA) testing in the clinical practice, clinicians have increasingly used these tests to aid in the diagnosis of systemic connective-tissue diseases. Following the initial enthusiasm, it was later realized that their sensitivity and specificity varied significantly among different rheumatic diseases and that occasionally these autoantibodies could be detected in normal individuals. Furthermore, substantial variation in the reported results between different laboratories has been observed due to the lack of generally accepted standardized methods for their detection. Despite these limitations, the discovery of new autoantibodies that could aid in the understanding of pathogenesis and noninvasive diagnosis of complex autoimmune diseases should be viewed as a positive development towards the ultimate goal of providing the best of care to patients suffering from these potentially lethal diseases.

This review will focus on the potential role and characteristics of the antineutrophil cytoplasmic antibodies (ANCA) in the pathogenesis and diagnosis of certain diseases.

METHODS FOR DETECTION AND ANTIGENIC TARGETS OF ANCA

Davies et al. (9) were the first to report the presence of antibodies that produced diffuse cytoplasmic staining of neutrophils by indirect immunofluorescence (IIF) techniques in patients with segmental necrotizing glomerulonephritis (GN). Several years later, the same antibody reaction pattern was recognized to occur in sera of patients with Wegener's granulomatosis (WG) (63). A neutrophil perinuclear cytoplasmic staining pattern was subsequently noted to be associated with antibody reactivity in patients with microscopic polyangiitis (MPA) and idiopathic crescentic necrotizing GN (12). This led to a large number of studies that have examined the presence and pattern of ANCA in various disease states.

Routinely, ANCA are detected by IIF on ethanol-fixed neutrophils (30). According to guidelines established at the 1st workshop on ANCA in 1988, neutrophils isolated from heparinized blood are cytocentrifuged, fixed in absolute ethanol on glass slides, and then incubated with dilutions of patient's serum (67). Slides are stained with fluorescein-labeled anti-human immunoglobulin, and the fluorescence pattern is read with a fluorescence microscope (66, 67).

With this technique three distinct patterns of IIF can be observed (see Table 1). Cytoplasmic ANCA (c-ANCA) staining refers to diffuse coarse granular, centrally accentuated, cytoplasmic staining, whereas perinuclear ANCA (p-ANCA) staining indicates a perinuclear or nuclear fluorescence staining of the ethanol-fixed neutrophils. An atypical ANCA (a-ANCA) pattern refers to either a fine-speckled, linear, or other cytoplasmic staining pattern of ethanol-fixed neutrophils. The p-ANCA pattern actually represents an artifact of ethanol fixation, leading to rearrangement of positively charged proteins around the negatively charged nuclear membrane (12). Since the simultaneous presence of ANA (with or without granulocyte specificity) can display a similar appearance with p- or a-ANCA in ethanol-fixed neutrophils, fixation of neutrophils with cross-linking fixatives like formalin is recommended. ANA display nuclear staining on formalin-fixed neutrophils, whereas p- or a-ANCA show predominantly cytoplasmic staining (Table 1).

The interpretation of the ANCA fluorescence pattern can vary significantly between laboratories due to differences in the quality of the neutrophil preparation, methods of fixation, serum dilutions utilized, and the experience of the operator (30). A discordance rate of up to 40% has been reported among experienced readers of IIF ANCA patterns (43). Furthermore, IIF does not provide information about the specific antigenic target(s) of the ANCA.

Over the last decade, significant progress has been made in the effort to identify the specific antigenic targets of the ANCA. Proteinase 3 (PR3), a serine protease present in the azurophilic granules of neutrophils, is the major antigen associated with the c-ANCA fluorescence pattern (16, 48). On the other hand, antibodies to multiple antigens in the cytoplasm of neutrophils may be responsible for the p-ANCA pattern (Table 1). The principal p-ANCA antigen is myeloperoxidase (MPO), an enzyme present in the azurophilic granules of neutrophils (12). Other antigens with p-ANCA reactivity include elastase (EL), cathepsin G (CG), azurocidin (AZ), lactoferrin (LF), lysozyme (LZ), and bactericidal/permeability-increasing protein (BPI) (Table 1) (30). In many cases, the targeted antigens of sera with p- or a-ANCA reactivity have not been characterized (45, 62).

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TABLE 1. Immunofluorescence staining pattern and specific antigenic targets of ANCA

Antigen(s) targeted	Formalin fixation of neutrophils		Ethanol fixation of neutrophils	
	Staining	IIF	Staining	IIF
PR3	Diffuse granular cytoplasmic	c-ANCA	Diffuse granular cytoplasmic	c-ANCA
MPO, LF, LZ CG, AZ, BPI, EL, and others	Diffuse granular cytoplasmic	c-ANCA	Perinuclear cytoplasmic/nuclear	p-ANCA
Uncharacterized cytoplasmic proteins and lamins[a]	Fine-speckled or linear cytoplasmic	a-ANCA	Fine-speckled or linear cytoplasmic	a-ANCA
Nuclear proteins or nucleic acids	Nuclear	ANA	Nuclear/perinuclear cytoplasmic	ANA

[a] Lamin A, B1, and C and lamin B receptor.

In order to circumvent the limitations of the IIF technique, several antigen-specific detection assays have been developed. These include enzyme-linked immunosorbent assays (ELISA), radioimmunoassays, immunoprecipitation techniques, and immunoblotting. Currently, the ELISA techniques are the ones that are widely utilized in clinical practice. There are two main ELISA methods used for the detection of specific ANCA: the direct, or standard, ELISA and the capture, or sandwich, ELISA (30) (Fig. 1).

In the standard ELISA the target antigen is directly coated on the plastic well, whereas in the capture ELISA a specific monoclonal antibody fixed on the plastic surface is used to capture the target antigen (Fig. 1). Incubation with the patient's serum followed by the addition of enzyme-labeled anti-human antibodies (immunoglobulin G [IgG], IgM, or IgA) is then used for the detection of the specific ANCA. Limitations of the direct ELISA technique include wide variations in the quality of the antigen preparation. This may vary depending on methods used for isolation and purification of cytoplasmic proteins from human neutrophils (64) and conformational changes of the antigenic epitopes that occur after coating of plastic surfaces with antigen (30). Recent multicenter studies in Europe have attempted to standardize the available ELISA techniques for the detection of anti-PR3 and anti-MPO antibodies. Despite these efforts the intercenter coefficient of variability for these ELISAs ranged between 15 to 35% (20). Although initial studies have shown wide variations in the performance of commercial ELISA kits (64), a recent study in the United States found more than 90% agreement in the results between seven commercially available ELISA kits and a

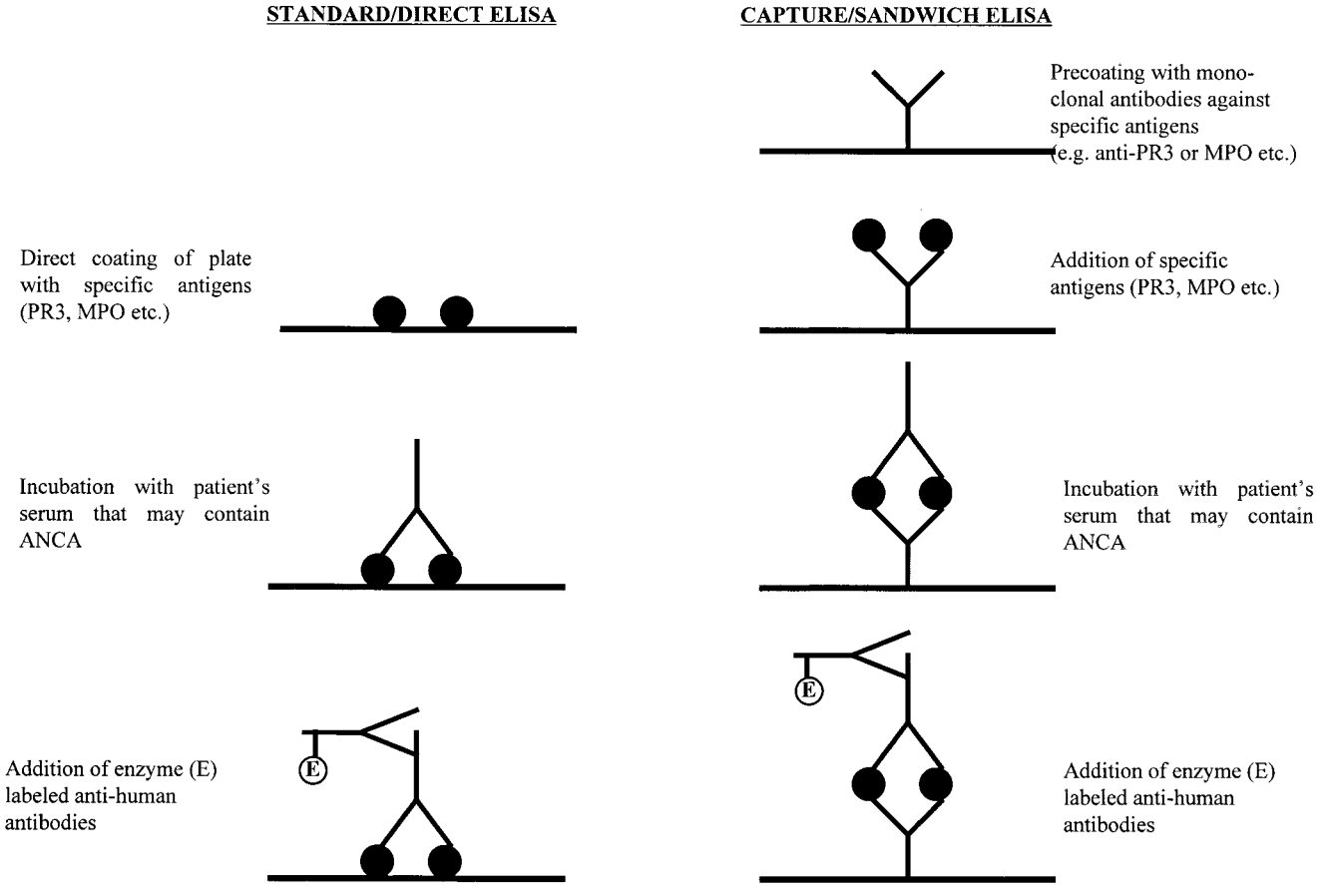

FIG. 1. Two types of ELISAs for detection of specific ANCA.

TABLE 2. ANCA-disease association

Disease	IIF pattern	Main antigenic target(s)	% Positive (IIF or ELISA)
Strong association			
WG	c-ANCA/rarely p-ANCA	PR3/occasionally MPO	65–90
MPA	p-ANCA	MPO/occasionally PR3	50–75
Idiopathic rapidly progressive GN	p-ANCA/c-ANCA	MPO/PR3	45–65
Frequent association			
Rheumatic disease			
CSS	p-ANCA/c-ANCA	MPO/PR3	40–60
RA	p-ANCA/a-ANCA	MPO/LF/LZ	15–30
SLE	p-ANCA	LF/MPO/GC	15–30
IBD/liver diseases			
Ulcerative colitis	a-ANCA/p-ANCA	BPI/CG/lamins	40–80
Crohn's disease	a-ANCA/p-ANCA	BPI/CG/lamins	10–20
PSC	a-ANCA/p-ANCA	BPI/CG/LF/lamins	65–80
AIH	a-ANCA/p-ANCA	Actin/lamins	65–95
Amebiasis	c-ANCA	PR3	90
Anti-GBM disease	p-ANCA/rarely c-ANCA	MPO	20–40
Infrequent association[a]	p-ANCA/a-ANCA	Various	Variable or case reports

[a] Rheumatic diseases: classical polyarteritis nodosa, Sjögren's syndrome, polymyositis/dermatomyositis, reactive arthritis, relapsing polychondritis, juvenile chronic arthritis, and antiphospholipid syndrome. Infections: endocarditis, HIV infection, chromomycosis, tuberculosis, and malaria. Drugs: hydralazine, minocycline, PTU, methimazole, penicillamine, allopurinol. Liver diseases: primary biliary cirrhosis and hepatitis C infection. Miscellaneous conditions: myelitis. Buerger's disease, anterior uveitis, cystic fibrosis, Graves' disease, and Hashimoto's thyroiditis.

study reference standard, indicating continuing progress in the field (40).

The development of the capture ELISA has improved the sensitivity of the assay and has avoided the conformational changes of the antigenic epitopes that may occur during antigen purification or coating of the plastic surface. Recent studies have confirmed its clinical usefulness (3, 43, 61, 65), but additional prospective studies are certainly needed. Occasionally the presence of antibodies in the patient's serum that bind to the monoclonal catching antibody can lower the sensitivity of the assay, so some authors recommend the use of additional controls (43).

Based on the above observations and the results of two large multicenter studies, testing for ANCA should always include IIF and well-standardized antigen-specific ELISAs. The combination has been shown to greatly enhance specificity for certain vasculitides such as WG and MPA (22, 43).

ANCA-DISEASE ASSOCIATION

ANCA have been detected at different frequencies in a number of heterogeneous conditions including rheumatic-autoimmune, liver, bowel, renal, and presumed drug-induced diseases (Table 2).

Rheumatic diseases. (i) WG. A strong association between c-ANCA IIF or PR3-ANCA and WG has been established in numerous studies over the last 15 years (30). Most of the sera from patients with WG are c-ANCA positive by IIF (80 to 95%). The sensitivities of the assays vary significantly depending on the extent (generalized versus limited), severity, and activity (active versus inactive) of the disease. A recent meta-analysis of the reported studies found a pooled sensitivity of the IIF assay ranging from 63% (inactive disease) to 91% (active disease) (52). The overall sensitivity of the assay has been estimated to be around 65%, whereas the specificity ranges between 95 and 99% (22, 52). The use of ELISAs (direct or capture) that detect PR3 ANCA has improved the specificity. Recent studies that utilized combined IIF and PR3 ANCA testing for patients with WG have shown enhanced specificity of approximately 99% (22, 43). Despite this approach the sensitivity of the combined approach did not increase significantly (~73%) (22).

(ii) MPA. Data concerning the sensitivity and specificity of the ANCA testing in patients with MPA are difficult to interpret due to the controversy regarding the exact definition of this vasculitic process (28). Differentiating on clinical and even histological grounds between MPA and classic polyarteritis nodosa (PAN), WG, or Churg-Strauss syndrome (CSS) can be a challenging task even for experienced physicians. Based on the available data, ANCA are detected in 50 to 75% of patients with MPA (19, 30). The predominant IIF pattern is p-ANCA, with most sera showing reactivity against MPO (19). In some studies, PR3 ANCA can be found in approximately 25% of MPA patients (22).

(iii) CSS. ANCA are found in approximately 40 to 60% of patients with this rare form of systemic vasculitis (18). At least half the patients demonstrate a p-ANCA IIF pattern, whereas others may have a c-ANCA fluorescence pattern (18, 21, 24, 56). With ELISA both MPO ANCA and PR3 ANCA have been detected in these patients, although the former are more common.

(iv) Other rheumatic diseases. p- or a-ANCA can be detected in approximately 15 to 30% of patients with SLE or rheumatoid arthritis (RA) and less often in Sjögren's syndrome, polymyositis, relapsing polychondritis, antiphospholipid syndrome, and reactive arthritis (1, 10, 15, 30, 43, 46, 57). In patients with RA, multiple target antigens for ANCA have been detected, including MPO, LF, and LZ, but in the majority the targeted antigens remain unknown (1, 46). Sera from SLE patients display reactivity against LF (15), MPO, and CG (60, 68). PR3 ANCA are rarely detected in patients with rheumatic autoimmune diseases (20, 30, 43).

Renal diseases. (i) Idiopathic rapidly progressive GN (immune complex sparse or negative). In idiopathic rapidly progressive GN, a form of crescentic GN, characterized by the absence or paucity of immune complex deposits by IIF microscopy, ANCA are found in 45 to 65% of the cases (7, 12, 22). Most of the sera display p-ANCA IIF reactivity, with MPO being the target antigen in approximately 65% of the cases (7, 12, 22). Whether this clinical entity represents a limited form of WG or MPA is currently unclear.

(ii) Anti-GBM disease. Patients with anti-glomerular basement membrane (anti-GBM) antibodies against type IV collagen usually develop rapidly progressive GN (anti-GBM disease) that is occasionally associated with pulmonary hemorrhage (Goodpasture's syndrome). Several studies have shown that 20 to 40% of sera positive for anti-GBM antibodies are also positive for ANCA by IIF or ANCA-specific ELISAs (26, 31, 40, 58). p-ANCA are the predominant IIF pattern, with MPO being the major target antigen (26, 40, 58). Carefully performed studies have not revealed any cross-reactivity between ANCA and anti-GBM antibodies (26, 58). The coexistence of these two classes of autoantibodies in patients with rapidly progressive GN raises fascinating questions regarding the pathogenesis and differential diagnosis of the associated diseases.

IBD. ANCA have been found in 40 to 80% of patients with ulcerative colitis and 10 to 20% of patients with Crohn's disease (2, 14, 27, 55, 62). The IIF pattern is usually a-ANCA, with some sera displaying p-ANCA IIF. CG and BPI constitute the main antigenic targets that have been identified so far. The targeted antigen in many cases of inflammatory bowel diseases (IBD) remains undefined. A recent study has identified granulocyte-specific nuclear lamina proteins like lamin A, B1, and C and lamin B receptor as potential targets of ANCA, raising the possibility that these actually represent granulocyte-specific ANA (62).

Liver diseases. A high percentage of sera from patients with autoimmune liver diseases such as primary sclerosing cholangitis (PSC) (65 to 80%) (53) and autoimmune hepatitis type I (AIH) (65 to 95%) (44, 69) display ANCA reactivity. As in patients with IBD, an atypical or p-ANCA IIF pattern is observed. The specific target antigens for PSC are similar to those for IBD, whereas in AIH, actin seems to represent the major antigenic target (49).

Drugs. Clinicians ordering ANCA should be aware of the association between the use of certain drugs and a positive ANCA test, with or without signs of vasculitis (42). Hydralazine and antithyroid drugs like propylthiouracil (PTU) and methimazole have been associated with ANCA-positive vasculitis, presenting either as GN or cutaneous vasculitis. Recently, cases of ANCA-positive vasculitis have been reported for patients receiving penicillamine, minocycline, and allopurinol (5, 42). In most cases, p-ANCA were detected, reactive against MPO or EL (42). c-ANCA with PR3 reactivity have been mainly reported in association with PTU therapy (42). Choi et al. (6) recently reported an interesting case of a patient with WG who demonstrated an alternating ANCA specificity from c-ANCA PR3 to p-ANCA MPO during concomitant therapy with PTU (6). These observations emphasize the need to obtain a detailed drug history in the evaluation of patients with changing patterns of ANCA reactivity.

Miscellaneous conditions. ANCA have been occasionally found in patients with various acute or chronic infections including endocarditis, pneumonia, fungal infections like chromomycosis, tuberculosis, sepsis, amebiasis, malaria, and human immunodeficiency virus (HIV) infection (30). Most of the findings have been reported as case reports, so the true prevalence of ANCA in diseases that are frequently associated with neutrophil activation and that may mimic certain vasculitides is unknown.

Similarly, an association of ANCA with diverse diseases that share clinical manifestations with ANCA-positive vasculitides such as myelitis (47), uveitis (17), cholesterol embolism syndrome (35), thrombangiitis obliterans (Buerger's disease) (23), or mixed cryoglobulinemia (38) is being increasingly reported. p-ANCA IIF is the predominant IIF pattern in these patients with variable target antigen specificity by ELISA. In a recent review of all patients who tested positive for anti-MPO antibodies over a 10-year period, Franssen et al. (13) found that 20% of the positive findings were seen in a variety of nonvasculitic disorders.

PATHOGENETIC ROLE OF ANCA

In parallel to clinical studies examining the prevalence and diagnostic utility of ANCA testing in certain vasculitides like WG and MPA, extensive research has been conducted in an attempt to dissect the exact role that these antibodies may play in the pathogenesis of these diseases. Most of the data supporting a potential pathogenetic role for ANCA are derived from in vitro experiments (34).

The prevailing hypothesis postulates that ANCA mediate directly or indirectly a neutrophil-induced endothelial injury leading to vasculitis (25). ANCA targets like PR3 and MPO are regularly expressed on the cell surface of neutrophils following apoptosis or cytokine-induced priming of neutrophils. Binding of ANCA to their antigenic targets on the cell surface leads to neutrophil oxidative burst, degranulation, and cytokine release. Coengagement of the FcγRIIα and FcγRIIIb on the neutrophil surface by ANCA further enhances ANCA-induced neutrophil activation (37, 50). Activated neutrophils can then bind to activated endothelium, leading to endothelial-cell detachment and lysis. Whether ANCA actively participate in this process by directly binding to endothelial cells expressing PR3 or MPO remains a controversial issue. Furthermore, ANCA induce the release of monocyte chemoattractant protein 1 (MCP-1) and interleukin-8 from monocytes in vitro (51). Both molecules are potent chemokines that recruit monocytes and neutrophils to inflammatory sites and thus could intensify the initial endothelial injury.

These in vitro findings, in conjunction with the clinical observation that ANCA titers tend to correlate with disease activity in two-thirds of patients with WG, led many investigators to suggest a pivotal pathogenetic role for these autoantibodies (34).

On the other hand, several lines of evidence argue against a causal relationship between ANCA and vasculitides like WG and MPA. In vitro studies have failed to identify ANCA or IgG deposition in affected tissues from patients with these diseases (4). The frequent presence of granulomas and activated T cells in vasculitic lesions (59) or the peripheral blood (41) from patients with vasculitides such as WG or CSS points to an important role of cell-mediated immune response to the pathogenesis of these conditions. Similar findings have been noted recently for renal lesions of patients with ANCA-positive, immune-complex-sparse or -negative GN (8).

Although several animal models of "ANCA-associated vasculitis" have been developed, none of these models closely resemble the necrotizing vasculitic lesions or crescentic GN that is observed in WG or MPA (25). Furthermore, passive transfer of anti-MPO antibodies failed to induce vasculitis in naive mice (25). In vivo observations invariably show that 35 to 50% of patients with histologically proven WG or MPA lack

ANCA (Table 2). Moreover, in about one-third of patients with WG no correlation between disease activity and ANCA titers can be found, whereas certain patients with quiescent disease display consistently high ANCA titers (36).

In conclusion, available data suggest a contributing role of ANCA in the pathogenesis of necrotizing vasculitis and crescentic GN for most patients with WG, MPA, or CSS. However, imperfect associations of ANCA with these diseases and disease activity also suggest that ANCA are not absolutely required for the initiation or perpetuation of the inflammatory process in these diseases (54).

CLINICAL UTILITY OF ANCA TESTING

The diagnosis of systemic vasculitides such as WG, MPA, or CSS has been traditionally based on characteristic clinical, laboratory, radiologic, and biopsy findings in an involved organ (18, 19, 29). ANCA testing has been utilized as a promising noninvasive tool that can potentially replace biopsy as the means of supporting the diagnosis of these vasculitides in certain patients (33).

Clinicians ordering tests for ANCA should be aware of the advantages and limitations of these tests. Based on the abovementioned data the sensitivity of the IIF or ELISA tests ranges between 65 and 90% for patients with WG and between 50 and 75% for patients with MPA. In the case of CSS the sensitivity of these tests in various studies ranges from <50 to 70%, raising concerns about the usefulness of the assay in patients with suggestive clinical presentation. The specificity of PR3 ANCA or c-ANCA for WG and MPO or of p-ANCA for MPA is much higher, ranging between 85 and 95% and 80 and 90%, respectively (22). Due to inherent limitations of the IIF technique, a specific ELISA for PR3 MPO should always be performed in addition to IIF. An approach that combines the results of both tests has been shown to improve the specificity of these tests to approximately 99% (22, 43).

Apart from the performance characteristics of ANCA testing, physicians should always keep in mind that vasculitides are rare diseases. Thus, overall the pretest probability of a disease like WG or MPA is very low. Furthermore, the probability that a patient with a positive ANCA test has the disease in question (posttest probability) depends mainly on the patient's clinical manifestations. For example, it has been estimated that for patients presenting only with sinus disease, the posttest probability of a positive anti-PR3 for WG is only 7 to 16%. However, in patients who have combined sinus, pulmonary disease, and GN, a positive test for anti-PR3 has a posttest probability of 98 to 99% (39). It is evident that for patients with a low posttest probability of the disease, clinicians must be very cautious in interpreting a positive test, especially because diagnosis of WG or MPA usually calls for strong immunosuppressive therapy. In such cases, a more aggressive diagnostic approach including biopsy is recommended. On the other hand, in clinical situations where the posttest probability is high, a negative ANCA test should not dissuade the physician from further investigation for establishing the correct diagnosis.

A recent study evaluating the laboratory testing practices of rheumatologists caring for patients with WG showed that ANCA testing is being used by the majority of physicians (>65%) during regular follow-up of these patients (11). Although rising titers of PR3 or MPO ANCA correlate with disease activity in about two-thirds of patients with WG or MPA, intensification of the treatment regimen based solely on a rising ANCA titer is not justified (36). Since the treatment of these vasculitides usually involves a combination of steroids with cytotoxic agents, unwarranted treatment can potentially lead to significant short- or long-term toxicity. Nevertheless, in certain patients in whom a pattern of disease flare-ups associated with rising ANCA titers has been observed, closer monitoring is appropriate after a substantial increase in titer.

The differential diagnosis of patients presenting with rapidly progressive (crescentic) GN includes anti-GBM disease, immune-complex-mediated GN (due to SLE, postinfectious GN, Henoch-Schönlein purpura, IgA nephropathy, etc.) and ANCA-associated GN (32). ANCA-associated GN usually occurs in the setting of systemic vasculitides such as WG, MPA, or CSS or renal-limited disease (idiopathic). Part of the initial laboratory evaluation should include measurement of serum ANCA and anti-GBM antibodies (32). Although ANCA measurement could be helpful in this situation, it is difficult to envision any clinical scenario in which a kidney biopsy would not provide more precise diagnostic information. If the biopsy revealed evidence of active disease with rare or absent immune deposits (pauci-immune GN), aggressive immunosuppressive treatment would be indicated regardless of the ANCA status of the patient.

CONCLUSIONS

The development of the ANCA testing as a potentially useful diagnostic tool for certain vasculitides and pauci-immune crescentic GN over the last 15 years has been met with significant controversy. Most of the controversy arises from the fact that these diseases usually follow an aggressive course with substantial morbidity and mortality. Early aggressive immunosuppressive therapy usually saves vital organ function and prevents mortality. At the same time, the applied therapeutic schemes can be associated with severe toxic side effects. Under these circumstances, there is little room for diagnostic errors based on any imperfect laboratory test.

Physicians should be aware of the many diseases that ANCA has been associated with and recognize that certain patients with WG, MPA, CSS or pauci-immune GN may be ANCA negative. The value of an ANCA assay depends on its operational characteristics (sensitivity, specificity, positive or negative predictive value), as well as on the specific clinical situation to which the test is being applied (prevalence of disease, pre- or posttest probability). The prudent use of ANCA studies in the evaluation of certain clinical events such as pulmonary-renal syndromes, rapidly progressive GN, and multisystem diseases with lung and/or kidney involvement can significantly aid the clinician in narrowing the differential diagnosis. Continuous improvement of the available assays and carefully designed studies examining clinical applications may help clarify the controversies surrounding the clinical usefulness of ANCA in the future.

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