Methodological update

How and why should we detect ANCA?

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ABSTRACT

Antineutrophil cytoplasmic antibodies (ANCA) have become an established tool for the diagnosis of systemic vasculitis. The major role for ANCA testing is in diagnosing renal insufficiency of unknown origin, where a positive test indicates whether the patient will benefit from immunosuppressive treatment or not. A negative test result almost completely rules out the presence of systemic vasculitis.

In this clinical setting the major antigens for ANCA are proteinase 3 and myeloperoxidase, and antibodies to these antigens can best be tested by ELISA. In other clinical settings like inflammatory bowel disease, arthritis and so on, several other ANCA specificities have been described and the IIF test is preferred. However, the clinical value of these somewhat more esoteric specificities is doubtful. New developments in assay techniques and better knowledge of specific epitopes will lead to tools for the improved diagnosis as well as follow up of patients during treatment, as has already been seen with the capture assay for PR3-ANCA.

Introduction

ANCA (anti-neutrophil cytoplasmic antibodies) is a family of autoantibodies related to vasculitis and inflammatory disorders. The first reports of antibodies reacting with leukocytes date from 1959 (1) and 1964 (2). Antibodies staining the cytoplasm of neutrophils in necrotizing glomerulonephritis were seen in 1982 (3). These older studies had in large part been forgotten when van der Woude *et al.* (4) in 1985 showed that ANCA was related to Wegener's granulomatosis. Following this study, interest in ANCA has increased each year and today these antibodies are considered major diagnostic tools for systemic vasculitis (5). In this review we will focus on methodological problems, new methods and discuss a strategy for ANCA testing.

Antigens

The granulocyte is full of granules, each with many different proteins, mostly enzymes that are used in the defence against bacteria and therefore the potential number of antigens is very large (6). It was, however, very early shown that when the granules were separated based on their density, all reactivities of sera from systemic vasculitis patients were with the alpha fraction containing the azurophil granules. The proteins from these granules could be isolated and separated and most of the reactivities were found to be with proteins of molecular weights around 29kD and 140kD. Several of the serine proteinases such as elastase, cathepsin G and proteinase 3 have molecular weights around 29kD and it was shown by sequencing that the 29kD antigen actually was proteinase 3 (PR3) (7). The other major antigen was shown to be myeloperoxidase (MPO) (8). These two enzymes take part in the killing of bacteria by cleavage of proteins (PR3) and by the generation of oxygen radicals (MPO) (6).

PR3 is a serine proteinase with a molecular weight of 29kD consisting of a single protein chain that is glycosylated. The sequence is conserved and very similar to other serine proteases like elastase and cathepsin G (9). MPO is a dimer with a molecular weight of 140kD consisting of one heavy (59kD) and one light chain (13.5kD). It is glycosylated with high mannose oligosaccharide chains. The molecule is easily cleaved by heating and has a characteristic green colour (10).

Many other antigens have been described to be associated with ANCA, i.e. elastase, lactoferrin cathepsin G, BPI and more recently molecules like defensins, HMG1/2, catalase etc. (11, 12) as summarized in Figure 1.

Problems and pitfalls in the analysis of ANCA

The first method developed to detect ANCA was indirect immunofluores-

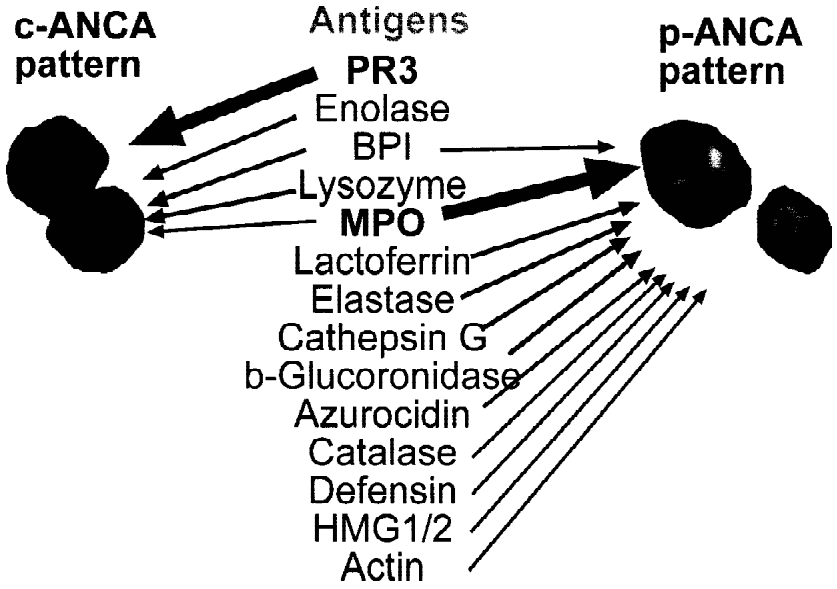

Fig. 1. Assay of ANCA using IIF divides ANCA into two groups, the C-ANCA and the P-ANCA patterns. These patterns can arise from antibodies to many granulocyte proteins and therefore it is impossible to know the specificity of ANCA from the pattern obtained on IIF.

cence (IIF) on ethanol fixed granulocytes (13, 14). By this method two patterns are seen, a cytoplasmic staining of the granulocyte called C-ANCA and a perinuclear staining called P-ANCA. The P-ANCA pattern arises due to a fixation artefact when MPO is redistributed to the negative nucleus due to its positive charge. IIF was followed by RIA and ELISA, first using different extracts of granulocytes (15, 16) and later the purified proteins (14, 17). Thus, antibodies to PR3 are called PR3-ANCA and antibodies to MPO are called MPO-ANCA, when the purified proteins are used in the assay.

The two IIF patterns are presumed to be related to antibody reactivities to defined antigens, i.e. C-ANCA equals PR3-ANCA and P-ANCA equals MPO-ANCA. This is, however, not always the case. A few years ago we studied some patients with a C-ANCA pattern by IIF and MPO-ANCA by ELISA (18). The index patient had an atypical disease with light chain deposition. The antibodies also had an atypical subclass distribution with predominant IgG2 autoantibodies. We could show that the antibodies were directed to an epitope that is different from the regular epitope and probably not redistributed during fixation. The epitope is still on the same molecule and therefore it appears that all MPO do not redistribute during fixation. These sera are rare and probably constitute only around 1-2% of C-ANCA positive sera. The recently discovered BPI-ANCA (antibodies to bactericidal permeability increasing protein), however, can also give a C-ANCA pattern, but only in half of all cases (19).

The problem is much greater when studying P-ANCA since many of the antibodies to cationic proteins of the granulocyte can give rise to this pattern and it is impossible to distinguish P-ANCA from the GS-ANA pattern supposed to be true nuclear staining. Antibodies to MPO, elastase, lactoferrin, BPI etc. can all give this kind of pattern. The clinical selection of patients is important and if the patients have systemic vasculitis the important antigen is MPO, but if the patients have inflammatory bowel diseases, arthritis or autoimmune hepatitis the specificity could be elastase, lactoferrin, BPI, cathepsin G, etc. We still do not know the major dominating antigen in these diseases.

Furthermore, not all antibodies to granule proteins react well by IIF. This has been demonstrated with BPI-ANCA, where about half of sera containing this antibody do not give staining by IIF. Another major problem is ANA that will also react with neutrophils and give a P-ANCA or atypical staining. Therefore it is important to always check for ANA; otherwise it is impossible to be sure that the ANCA staining is not an ANA.

It is well known that the level by ELISA does not correlate to the titre by IIF. Samples can be found that are high by IIF and low by ELISA or the opposite. This is probably a reflection of the nature of the epitopes. It has been shown that the epitopes are conformational, i.e. denaturation of the molecule destroys the epitope (20). The molecule is presented in a different conformation when bound to a plastic surface in the ELISA test than when fixed by ethanol in the granules for the IIF (Fig. 2). Patients react with different epitopes (21) on the molecule

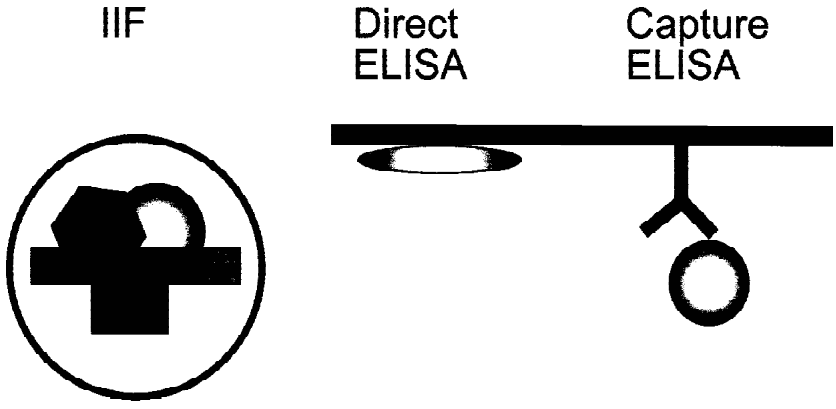

Fig. 2. The antigen (PR3) is presented in different ways in the assay. At IIF PR3 is fixed by ethanol and mixed with several other proteins in the granula and thus epitopes can be destroyed or hidden. In the direct ELISA the isolated protein is bound to a solid phase, generally a plastic surface. The protein will bind to hydrophobic areas and change conformation to a greater or lesser degree. In the capture assay PR3 is held in place by a monoclonal antibody in a more native conformation. This will orient the antigen and the same surface will be accessible.

and thus they will give a low or high reaction in the test depending on how their epitope is presented. This question was recently addressed (22) using a monoclonal antibody to catch the antigen instead of direct binding to the plastic. It was shown that reactivites that were low on antigens directly coated could be high when the antigen was indirectly bound. This highlights how important the characteristics of the assay are when comparing results between laboratories and patients.

A new assay type: The capture ELISA for detection of ANCA

As mentioned above, the three-dimensional structure of the antigen may be crucial for the recognition and binding of antibodies, and thus for the sensitivity of the assay. In an attempt to preserve the native structure of the antigen and hence to increase the sensitivity of the assays, capture ELISAs have been developed. In a capture ELISA the plate is pre-coated with a monoclonal antibody (MoAb) capturing the antigen (Fig. 2). A few different MoAbs have been used for the detection of PR3-ANCA; MoAb 12.8 (23), MoAb 1E8 (24) and MoAb 4A3, 4A5 and 6A6, which recognize different epitopes of PR3 (22). Regarding the latter three, MoAb 4A3 presented the antigen in such a way that 98% of all antibodies that most likely were against PR3 were detected. Recently a capture-ELISA based on recombinant PR3 has been established (25). For MPO-ANCA the MoAb 7.17 has been developed and evaluated (23).

Sensitivity and specificity

When using MoAb 4A3 a high sensitivity could be established. The sensitivity of the capture PR3-ANCA was 85% in c-ANCA positive sera with a specificity of 90% obtained from disease control material comprising patients with various forms of glomerulonephritis. Furthermore, the capture PR3-ANCA assay showed a significantly higher nosographic sensitivity in patients with Wegener's granulomatosis with renal involvement (41/48, 85%) compared to c-ANCA by IIF (28/48, 58%) (26). A capture ELISA using MoAb 12.8 was able to detect PR3-ANCA in 21 out of 24 (79%) patients with Wegener's granulomatosis with renal involvement. The specificity was found to be 96% (23). MoAb 1E8 was evaluated by Merkel *et al*. (24), who reported a somewhat lower sensitivity for the capture ELISA compared to the direct ELISA method. Out of 26 known ANCA-positive sera from patients with Wegener's granulomatosis 23 were positive, while 25 out of 26 were positive with the direct ELISA method (24). However, in this study the same monoclonal anti-PR3 antibody was used as a control for background and for anti-mouse antibodies. Such a procedure may result in a 'false low' level since it has been shown that patients with Wegener's granulomatosis have complexes consisting of PR3 and PR3-ANCA, which can bind to the monoclonal anti-PR3 antibody by themselves (27).

The MoAb 7.17 capture ELISA for detection of MPO-ANCA gave positive results in sera from 11 out of 35 patients with crescentic nephritis. Only serum from one out of 41 disease controls contained MPO-ANCA by this capture ELISA, thus demonstrating the high specificity of the test (28).

Capture PR3-ANCA detects the epitope that is most relevant

In material comprising 123 patients with biopsy-confirmed Wegener's granulomatosis and microscopic polyangiitis with renal involvement, we have previously shown that the level of PR3-ANCA at diagnosis, measured by the capture ELISA, correlates to renal outcome (29). Data revealed that patients with a high level (> 550 U) of PR3-ANCA by the capture ELISA had a 5-year renal survival of 60%, versus 85% for the patients with levels below 550 U, ($p = 0.001$). There was no such correlation to renal survival when PR3-ANCA was measured by direct ELISA, or to MPO-ANCA levels (Fig. 3) (29). We have indications from on-going studies that the capture assay for detection of PR3-ANCA is more sensitive for the detection of relapses.

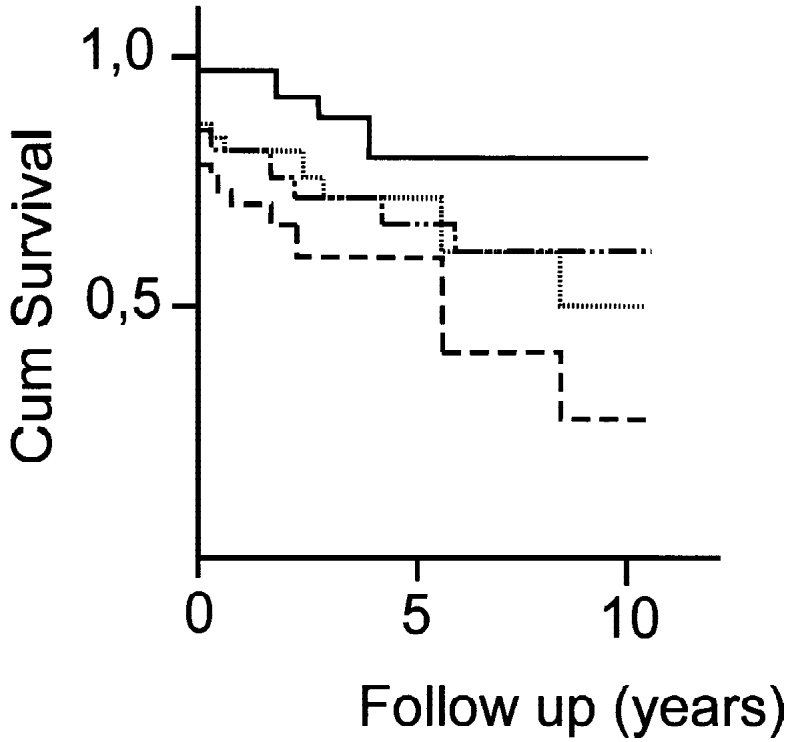

Fig. 3. The capture assay correlates better with renal survival than the direct assay. Patients with a high or low level of PR3-ANCA by capture or direct ELISA at presentation were followed over time.

Low level by capture ELISA ——————————
High level by capture ELISA –
Low level by direct ELISA – · – · – · – · – · – · – · – · – · – · – · – · –
High level by direct ELISA ·

Potential weakness of a capture ELISA

Since the capture assay uses a monoclonal antibody to capture the antigen, there could be a possibility that the epitope that binds to the monoclonal antibody is the same as the one that binds to some of the antibodies in patient sera. However, the results from the clinical evaluations performed do not support such a hypothesis, since the sensitivity is high by the capture method. It is also known that most patients react to several epitopes. Another argument against using a capture ELISA is the risk for non-specific binding and rheumatoid factor binding to the monoclonal antibody. This possibility of a false positive test result could easily be excluded by using a control plate coated with an unrelated monoclonal antibody of the same mouse Ig subclass as that used in the test assay

Therefore one can conclude that a capture ELISA for detection of PR3-ANCA has a higher sensitivity, and an acceptable specificity. Regarding the specificity it is important to set the cut-off level using disease control material comprising patients with, for example, lupus, and not healthy blood-donors.

Release of granule enzymes during coagulation and the level of alpha 1 antitrypsin

A further complication when conducting ANCA assays is the release of granule enzymes during coagulation, which can give rise to a reduction of the test results by binding the antibody and forming an immune complex (22). To complicate the matter further, the proteinase inhibitor alpha 1 antitrypsin (α1AT) can also bind serine proteases and thus compete with the antibody. The level of α1AT can fluctuate in a patient. One can speculate that a patient with a low level of α1AT and a high release of enzymes can be low in the test one day and high another day when centrifugation is done immediately after the blood is drawn.

Correlation of ELISA to IIF

A study was recently carried out to compare IIF and ELISA for the detection of ANCA in systemic vasculitis (30). The study highlights the problem with IIF and the reason for the low specificity of IIF. Three commercial kits for IIF and 7 commercial kits for ELISA were tested and their potential to detect ANCA in systemic vasculitis was compared. Serum samples from 396 patients with various forms of glomerulonephritis, including 146 patients with pauci-immune crescentic glomerulonephritis, were analysed. It was shown that IIF had an overall sensitivity of 88% with a specificity of 71%, while the ELISA had a sensitivity of 84% with a specificity of 94%. Furthermore, the authors could show that the problem with IIF was the presence of samples from patients with SLE that gave a P-ANCA pattern in 68-82%, thus demonstrating what has already been discussed – that IIF detects many specificities while ELISA detects antibodies to the coated antigen only.

By combining IIF and ELISA results a higher specificity can be obtained, as was shown in a European study (31), but in this study it was marginally increased to 96%. The authors also discuss the predictive value of ANCA testing and show that in a patient with strong evidence for rapidly progressive glomerulonephritis a positive test by ELISA provides > 90% confirmation of crescentic glomerulonephritis, while in a patient with weak clinical evidence a positive ANCA is inadequate to confirm a diagnosis of crescentic glomerulonephritis. A negative test, however, rules out the presence of crescentic glomerulonephritis.

Clinical use of ANCA

ANCA in renal disease

The clinical setting where ANCA testing provides the most valuable contribution is probably renal insufficiency of unknown origin. The outcome of an ANCA test dramatically changes the odds as to whether the patient will benefit from treatment with immunosuppressive drugs or not. The likelihood that the patient has such a disease to begin with probably ranges between 5-30%, depending on the symptoms and signs. If antibodies are detected, the likelihood for a disease that normally benefits from immunosuppression increases to > 95%. One reason for this high probability is that some of the diseases that may yield 'false positive results', such as SLE or mesangiocapillary glomerulonephritis with crescents, also benefit from such treatment. Rapid tests are available that can be administered at the bedside and yield results while the patient is still in the emergency room. If the test result is positive, the treatment can be started immediately, but patients (especially those with low levels) should undergo a careful work-up. Thus, in the setting of renal insufficiency of unknown origin it is important to use a test with high sensitivity. On the other hand, a negative result for the ANCA test will drastically decrease the probability of a diagnosis that requires the immediate institution of immunosuppression, especially if anti-GBM antibodies are analyzed simultaneously. Septic endocarditis with renal involvement may yield false positive results and is probably the most important differential diagnosis to be kept in mind.

ANCA in systemic inflammatory disease

Another clinical setting where ANCA testing is often used is in patients with inflammatory conditions of unknown origin. Typical symptoms are malaise and fatigue in combination with myalgia, elevated ESR or CRP and low grade fever. The differential diagnosis includes many types of infections and malignancies, conditions that deteriorate when treated with immunosuppressive drugs. When no life-threatening organ involvement is present, there is time for thorough diagnostic procedures. Under such circumstances the high specificity of the test for systemic vasculitis is more important than high sensitivity. The highest specificity is obtained when the results from ELISA and IIF are combined.

ANCA in inflammatory bowel disease

It is sometimes difficult to distinguish the inflammatory bowel diseases (IBD) ulcerative colitis and Mb Crohn from each other. Both are characterised by an unknown etiology as well as chronic remitting inflammatory processes of the intestine. The established differentiation between Mb Crohn and ulcerative colitis is made by clinical parameters. Serological parameters would be helpful for the clinician in diagnosing and categorising patients with ulcerative colitis

from Mb Crohn. Today the use of ANCA to detect ulcerative colitis is under discussion since ANCA, especially of the P-ANCA (or atypical ANCA) type, is found in 40-60% in these patients. In Mb Crohn, antibodies to the mannan of Saccharomyces cerevisiae (ASCA) can be detected in 60-70% of patients. A patient with a clinical suspicion of IBD who has P-ANCA is more likely to have ulcerative colitis than if the serum contains ASCA (32, 33).

ANCA in cystic fibrosis

Cystic fibrosis (CF) is a genetic disease characterized by hyperviscous mucus and chronic suppurative lung infections. ANCA is a relatively common finding in CF and BPI is the predominant antigen. BPI is active against gram negative bacteria. Recent reports have shown that there is a correlation between chronic colonization with Pseudomonas and BPI-ANCA. Antibodies from CF patients have been shown to react with the C-terminal portion of BPI that is necessary for oppsonization. The autoantibodies were also shown to inhibit the opsonization process. A negative correlation between BPI-ANCA titre and lung function was also detected. Thus it is possible in the future that treatment that would reduce the antibody production may prove beneficial in CF. Today it is too early to conclude that the ANCA test is of clinical value (34).

ANCA for the follow-up of patients with ANCA associated vasculitis

Relapse is a common feature in systemic vasculitis and has been described as late as 15 years after the initial onset of disease. Approximately 50% of the patients who are alive 5 years after a diagnosis of small vessel vasculitis associated with PR3-ANCA have experienced at least one relapse (35); lower figures have been reported in MPO-ANCA associated disease (36). It is widely accepted that relapses occur more often among patients who remain ANCA-positive during remission. However, different views prevail concerning the usefulness of serial ANCA measurements for the follow-up of patients with a systemic vasculitis that was positive at the time of diagnosis. In the original report linking ANCA to Wegener's granulomatosis in 1985, it was stated that ANCA titres are related to disease activity (4). Later it was claimed that treatments based on ANCA titres are more beneficial compared to treatments based only on clinical signs (37). This notion has been challenged, for instance by a report from the NIH group who found changes in ANCA titres to correlate poorly to disease activity (38, 39). Overall there seems to be about a 50% risk of relapse within 6 months after a significant rise in ANCA titre. A high degree of alertness is always indicated when the level is rising. In most published series (4, 36-39) more than 90% of the cases have positive test results at the time of relapse. A negative ANCA result is a good indication that the disease is under control.

ANCA during renal replacement therapy

Uremia necessitating renal replacement therapy is a common sequel of systemic vasculitis. During dialysis treatment relapses can be difficult to diagnose. General symptoms of vasculitis are easily confused with symptoms of uremia, and specific symptoms such as hematuria and raised serum creatinine concentrations cannot be used. Furthermore, ANCA-positivity by IIF have been found in 5% of dialysis patients without any evidence of vasculitis disease (40). Despite the immunosuppressive effect of uremia, a relapse rate close to 10% per year have been reported for dialysis-treated patients (41).

Relapses also occur after renal transplantation, both extra-renal and in the graft. Neither ANCA specificity at diagnosis (PR3 or MPO) nor the ANCA test status at the time of transplantation seem to influence the risk of relapse. In a recent meta-analysis we found the average risk of relapse to be around 20% (42). Many of these were minor extra-renal relapses and the overall graft survival is reported to be similar to other forms of renal disease. It seems as if modern immunosuppression protocols, used to prevent rejection, are also active in preventing relapses.

ANCA strategy

Because of the complications in the analysis of ANCA, an international group of ANCA researchers has published a consensus statement on ANCA testing (43). In this publication guidelines for ANCA testing and reporting are presented. The group states that a minimum requirement for a laboratory that screens with IIF should be to never send out positive results without a confirmation by antigen-specific ELISA, i.e. for PR3 and MPO, since ANA will react with neutrophils and give a positive ANCA. The optimal recommendation is to use both IIF and ELISA on all samples. Results should be reported as C-ANCA, P-ANCA or atypical ANCA for assays carried out by IIF, and as PR3-ANCA, MPO-ANCA etc. for assays done by ELISA.

Our own view is that the tests to be used depends on the clinical question being asked (Fig. 4). If the clinician is interested in diagnosing systemic vasculitis with renal involvement, then ELISA using PR3 and MPO is the method of choice. If the interest is arthritis or inflammatory bowel disease, IIF on granulocytes is the method of choice. When systemic vasculitis is suspected, ELISA for PR3-ANCA and MPO-ANCA is performed and if the sample is positive the result is sent out. If the result is negative a test for anti-GBM antibodies is warranted since patients with systemic vasculitis and Goodpasture syndrome can present in the same way. An IIF test for ANCA can be performed to rule out ANCA since a few samples will be positive by IIF and not by ELISA, but most systemic vasculitis patients will be positive by ELISA. The clinical value of a positive C-ANCA that is negative for antibodies to PR3 or MPO in the context of systemic vasculitis is not known. In systemic vasculitis patients and during follow-up, capture assays may be useful since they seem to detect relapses very well.

ANCA of specificities other than PR3 or MPO are of limited value for patients with systemic vasculitis and occur primarily in patients with other inflammatory disorders such as ulcerative colitis, Mb Crohn, autoimmune hepatitis, etc. It is of little value to know the specificity of these esoteric ANCAs, except perhaps when studying cystic fibrosis.

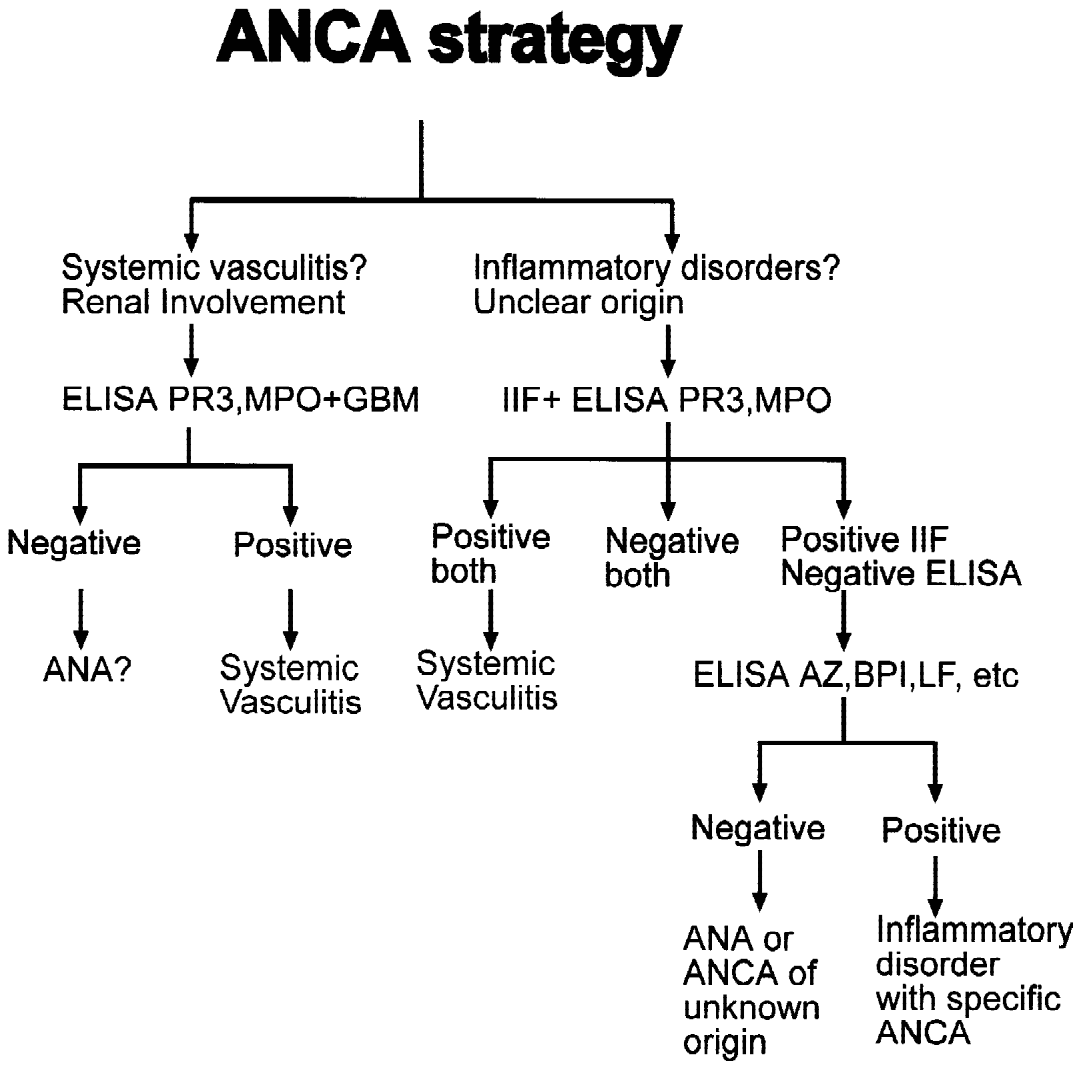

Fig. 4. The optimal way to perform ANCA assays is for the clinician to indicate whether the results are going to be used to diagnose systemic vasculitis or other inflammatory conditions. ELISA for PR3- and MPO-ANCA is the best choice if systemic vasculitis is suspected; if other inflammatory conditions are suspected, IIF is to be preferred.

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