Rapid screening assay for anti-GBM antibody and ANCA; an important tool for the differential diagnosis of pulmonary renal syndromes

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Abstract

Background. Pulmonary renal syndrome is encountered in several diseases such as Goodpasture's syndrome, antineutrophil cytoplasmic antibody (ANCA) associated systemic vasculitis, systemic lupus erythematosus (SLE) and infection-associated or drug-induced glomerulonephritis. To preserve organ function it is of vital importance to make the correct diagnosis and institute adequate therapy early, in the acute phase.

Method. An enzyme-linked immunosorbent assay (ELISA), specially designed as a rapid screening assay for antiglomerular basement membrane (anti-GBM) antibody, proteinase-3 (PR3-) ANCA and myeloperoxidase-(MPO-) ANCA were evaluated from 1060 serum samples drawn from patients with clinically suspected pulmonary renal syndrome or rapidly progressive glomerulonephritis (RPGN).

Results. Of the 1060 serum samples, 142 were positive for anti-GBM antibody (n = 19), PR3-ANCA (n = 60), or MPO-ANCA (n = 73). Of the 142 samples, 10 were double positive. Reanalysis of positive sera with a quantitative ELISA yielded results manifesting good correlation with those of the rapid screening assay. Of 918 sera found to be negative in the rapid assay, 105 were also tested with IIF, 11 being found to be positive. However, these 11 sera manifested no specificity for PR3 or MPO, but some were specific for bactericidal/permeability-increasing proteins, lactoferrin or elastase ANCA. Two of the patients whose sera yielded negative results in the rapid assay had systemic vasculitis.

Conclusion. The ELISA thus detects the true antibodies to PR3, MPO, and GBM, whereas IIF detects additional specificities. The findings suggest the rapid assay results to be of high positive predictive value, and the assay to be of high diagnostic specificity and sensitivity and thus useful in the diagnostic workup in suspected cases of RPGN or pulmonary renal syndrome.

Key words: pulmonary renal syndrome; rapidly progressive glomerulonephritis; ANCA; anti-GBM antibody

Introduction

Patients presenting with haemoptysis or pulmonary infiltrates together with glomerulonephritis, particularly the rapidly progressive cases, are considered to be cases of pulmonary renal syndrome. In many instances such patients may initially be investigated extensively for malignancy or therapy-resistant infectious disease. The relative degrees of respiratory tract versus renal involvement vary, and in some patients the initial symptoms may be confined to one or the other of these organ systems. Pulmonary renal syndrome is encountered in several diseases such as Goodpasture's syndrome, antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis (Wegener's granulomatosis, microscopic polyangiitis), systemic lupus erythematosus (SLE), and infection-associated or drug-induced glomerulonephritis [1]. To preserve both renal and respiratory function, it is of vital importance to institute therapy early, in the acute phase.

The majority of patients with pulmonary renal syndrome or rapidly progressive glomerulonephritis (RPGN) manifest one of three types of antibodies: antiglomerular basement membrane (anti-GBM) antibody, proteinase 3 (PR3) ANCA, or myeloperoxidase (MPO) ANCA. The prevalence of one of these types of antibodies among patients with pulmonary renal syndrome was recently reported to be more than 80% [2].

The standard method for detecting ANCA has been indirect immunofluorescence (IIF), which enables cytoplasmic ANCA (c-ANCA) and perinuclear ANCA (p-ANCA) to be distinguished. The major c-ANCA antigen is a 29-kDa protein, proteinase 3 [3]. The p-ANCA antigen is one of several neutrophil enzymes,
mostly myeloperoxidase [4], though human elastase, cathepsin G, and lactoferrin can also yield a p-ANCA staining pattern [5]. In some cases, however, MPO-ANCA can also yield a c-ANCA staining pattern [6].

As the clinically most important antibodies are directed against GBM, PR3, and MPO, and as the progress and outcome of disease differs to some extent according to which of these antibodies are present [2], we considered it of great value to test specifically for these three antibodies in the acute phase of disease. It is well known that both rapidly progressive glomerulonephritis and pulmonary renal syndromes associated with anti-GBM are characterized by the gravis prognosis. Conditions associated with PR3-ANCA, which occur predominantly in Wegener’s granulomatosis, have been described in some reports to be characterized by a more serious outcome than those associated with MPO-ANCA which are more commonly found in conjunction with renal limited vasculitis [7,8]. We tested a rapid screening ELISA to ascertain whether it might facilitate early diagnosis during the acute phase of disease.

Subjects and methods

All 1060 serum samples, drawn from Swedish patients with clinically suspected pulmonary renal syndrome or RPGN, and sent to the Wieslab laboratory for analysis during the period, 1989–1994, were analysed routinely with the rapid assay and residual sera stored at −20°C until reanalysed with IIF and quantitative ELISA.

Clinical data were available for anti-GBM antibody-positive patients in 100% (19/19) of cases, for PR3-ANCA-positive patients in 91% (52/57) of cases, and for MPO-ANCA-positive patients in 74% (52/70) of cases.

To determine the false-negative rate of the test, a randomly selected 105 (10%) sera negative in the rapid screening test were further analysed with IIF. The sera found to be positive at IIF were additionally analysed with the respective quantitative ELISAs for anti-GBM ab, PR 3-ANCA, MPO-ANCA, elastase-ANCA, lactoferrin-ANCA, and BPI-ANCA, and with Western blotting to identify any antigen present. Clinical data were obtained for all patients with positive IIF results.

Clinical classification of vasculitis was done according to the ACR criteria [9].

The screening assay

An enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection of autoantibodies against Goodpasture (GP) antigen, proteinase 3, and myeloperoxidase in one assay, which is performed within 30 min, was obtained from Wieslab AB, Lund, Sweden. The rapid ELISA consists of an eight-well strip. Two wells are precoated with PR3, two with MPO, two with Goodpasture antigen, one with HSA (human serum albumin), and one well is empty. Patient serum is diluted 1:4 and added to all eight wells. Incubation time is 10 min at room temperature both for serum and for conjugate. Alkaline phosphatase labelled anti-human IgG is used as the conjugate and para-nitrophenyl phosphate as the substrate. The absorbance, i.e. optical density (OD), is read at 405 nm on a microplate reader, with an inter-assay CV of 9–18% and intra-assay CV of 3–16%.

The cut off-level for a positive sample is three times the value obtained with a negative control serum.

Evaluation of the assay

Statistical methods

Sensitivity was the ability of the test to give a positive finding when the person tested truly had the disease, i.e. the rate (%) positive results obtained in the rapid assay out of all those persons tested who had the disease. ‘Disease’ included the diagnosis of Goodpasture’s syndrome for anti-GBM antibodies, and Wegener’s granulomatosis or microscopic polyangiitis for those with PR3-ANCA or MPO-ANCA. Specificity was the ability of the test to give a negative finding when the person tested was free from the disease, i.e. the rate (%) negative result in the rapid assay out of those without the disease. Positive predictive value (PPV) as the true-positive rate, i.e. those with positive results and disease out of all those with positive results in the test. Negative predictive value (NPV) as the false-negative rate i.e. those with disease but a negative result in the assay out of all those with negative results in the test. Spearman rank correlation, Abacus Concepts, Statview, Abacus Concepts, Inc., Berkeley, CA, 1994, was used for calculation of the correlation coefficient (Rho-value) between the OD values obtained for the rapid test and the corresponding unit values obtained by the quantitative test.

Clinical evaluation

The assay was first tested with 62 pathological sera: 21 with a known content of anti-GBM antibody, 21 with PR3-ANCA, 20 with MPO-ANCA, and 20 disease-control sera from patients with SLE, idiopathic crescentic glomerulonephritis, as well as 40 normal sera from healthy donors. All pathological sera known to be positive reacted as expected and differed clearly from the disease-control and healthy sera. Moreover there was good correlation between the OD values for the rapid test and the corresponding unit values obtained with the quantitative test. Rho values were 0.943 (P<0.0001) for anti-GBM antibody, 0.876 (P=0.0001) for elastase-ANCA, lactoferrin-ANCA, and BPI-ANCA, and 0.905 (P<0.0001) for MPO-ANCA.

Sensitivity of the rapid test was assessed by testing samples from randomly selected patients with confirmed clinical diagnoses, 39 with anti-GBM antibody (ab) mediated nephritis, 46 with Wegener’s granulomatosis (WG), and 46 with microscopic polyangiitis (MP). Test sensitivity for anti-GBM ab was 99% in anti-GBM ab-mediated nephritis sera; that for PR3-ANCA was 91% in WG sera and 54% in MP sera; and that for MPO-ANCA was 11% in WG sera and 46% in MP sera.

Specificity of the rapid assay was further assessed by testing sera from 112 consecutive patients with renal calculi but no sign of small-vessel vasculitis. All 112 sera were negative for anti-GBM ab, PR3-ANCA, and MPO-ANCA both in the rapid assay and in the conventional ELISA.

Quantitative anti-GBM ab ELISA

Microtitre plates (NUNC, Roskilde, Denmark) coated with purified Goodpasture (GP) antigen were obtained from Wieslab AB, Lund, Sweden, and ELISA was performed as described previously [10].
Rapid screening test for anti-GBM and ANCA

Quantitative PR3-ANCA and MPO-ANCA ELISAs

Microtitre plates were coated with isolated human proteinase 3 [11] or human granulocyte myeloperoxidase [12] respectively, were obtained from Wieslab AB, Lund, Sweden, and ELISA was performed as described earlier [13].

Lactoferrin and elastase ANCA ELISAs

Microtitre plates were coated with human milk lactoferrin (LF) (Sigma, St Louis, MO, USA) or human neutrophil elastase (EL) (Calbiochem, La Jolla, CA, USA), at 5 µg ml⁻¹ and 0.5 µg ml⁻¹ respectively, in 0.05 M carbonate buffer, pH 9.6. OD values >0.12 were considered positive. The respective positive controls were monoclonal anti-lactoferrin and anti-elastase (The Binding Site, Birmingham, UK). Mean OD and standard deviation for normal sera was 0.06 ± 0.015.

Bactericidal/permeability-increasing protein (BPI-) ANCA

BPI was purified from neutrophils and ANCA determination done with ELISA, using the method described by Zhao and co-workers [14].

Indirect immunofluorescence

IIF was performed on ethanol-fixed leukocytes according to a previously described standard procedure [15]. In short, leukocytes from healthy blood donors were fixed on glass slides in absolute ethanol at 4°C for 5 min. Patient sera were diluted 1:20 in PBS and bound IgG was detected by FITC-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark).

Western blotting

SDS–PAGE was performed with the Laemmli buffer system [15]. An alpha-granule extract from neutrophils containing all the alpha-granule proteins such as elastase, BPI, MPO and PR3 was used. This extract was incubated at room temperature in sample buffer containing only 0.1% SDS for 30 min, to avoid denaturation of the protein. Several lanes with identical amounts of extract were electrophoresed on a 6–20% gradient gel. The separated proteins were transferred to nitrocellulose sheets by standard electroblotting. The nitrocellulose sheet was blocked with 1% BSA in 0.1 M carbonate buffer pH 10.5 and strips were incubated overnight at 4°C with the respective serum sample diluted 1:100 in 0.05 M Tris–HCl, pH 7.5, 0.15 M sodium chloride, 0.05% Tween 20, with 2g/l BSA. The strips were washed in 0.15 M sodium chloride, 0.05% Tween 20. Bound antibody was detected with alkaline phosphatase-conjugated anti-IgG (Dako) and stained with nitroblue tetrazolium.

Results

Of the 1060 serum samples, 142 were positive in the rapid screening assay, 19 for anti-GBM ab, 60 for PR3-ANCA, and 73 for MPO-ANCA. Ten sera were double positive: eight for anti-GBM ab + MPO-ANCA, one for anti-GBM ab + PR3-ANCA, and one for PR3-ANCA + MPO-ANCA. Seven samples manifested non-specific reactivity (i.e. reacted with all three antigens and human serum albumin) and were considered to be negative.

Anti-GBM antibodies

All 19 sera found to be anti-GBM ab positive in the rapid test were confirmed by quantitative analysis.

Clinical data were obtained for all the 19 anti-GBM ab-positive patients. In 16 of the 19 patients the clinical diagnosis was Goodpasture’s syndrome, while the remaining three, with low or borderline antibody titres, were found to have acute tubulointerstitial nephritis, septicemia, or Wegener’s granulomatosis respectively. The positive predictive value (PPV) of anti-GBM antibody positivity vis-à-vis Goodpasture’s syndrome was thus 84.2% (16/19). For three of the 1060 sera the test yielded false-positive results; thus specificity was 99.7% (Table 1).

PR3-ANCA

Of the 1060 sera tested, 60 (from 57 patients) were PR3-ANCA positive and confirmed by quantitative analysis.

Clinical data were obtained for 91% of the PR3-ANCA-positive patients. The clinical diagnosis was systemic vasculitis in 98% (51/52) of cases. The majority, 67%, had Wegener’s granulomatosis (WG), and 31% had microscopic polyangiitis (MP). The patient positive for PR3-ANCA but without systemic vasculitis was found to have pulmonary aspergillosis. The PPV of PR3-ANCA positivity vis-à-vis WG and MP was 98%. Two per cent of the results (one of the 52 cases) were false-positive; thus specificity was 99.8% (Table 1).

MPO-ANCA

Of the 1060 sera, 75 (from 70 patients) were MPO-ANCA positive, confirmed by quantitative analysis.

Clinical data were obtained for 74% (52/70) of the MPO-ANCA-positive patients. Small-vessel vasculitis, including patients with concomitant Goodpasture’s syndrome, was diagnosed in 90% (47/52) of cases. Eight patients, all positive both for anti-GBM antibody and MPO-ANCA, had Goodpasture’s syndrome. Thirty-eight patients, 74%, had microscopic polyangiitis.

Table 1. Positive predictive value and specificity of the rapid screening test for anti-GBM ab, PR3-ANCA, and MPO-ANCA; results calculated from analyses of 1060 consecutively tested sera

<table>
<thead>
<tr>
<th></th>
<th>Positive predictive value (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-GBM ab</td>
<td>84.2</td>
<td>99.7</td>
</tr>
<tr>
<td>PR3-ANCA</td>
<td>98</td>
<td>99.8</td>
</tr>
<tr>
<td>MPO-ANCA</td>
<td>90</td>
<td>99.3</td>
</tr>
</tbody>
</table>
itis and one patient had Wegener’s granulomatosis. In patients without vasculitis, the diagnosis was septicemia, membranous nephropathy, thrombotic microangiopathy, light-chain deposit disease, or general atherosclerosis. The PPV of MPO-ANCA positivity vis-à-vis microscopic polyangiitis and WG was 90% (47/52). In 10% (5/52) of cases, the results were false-positive; thus specificity was 99.3% (Table 1).

False-negative rate

To determine the false-negative rate for the rapid assay, approximately 11% (105/918) of sera found to be negative in the rapid assay were randomly subjected to further analysis with IIF on ethanol-fixed neutrophils. Eighty per cent (84/105) of the sera were still negative at IIF analysis. Ten sera (9.5%) contained antinuclear antibodies (ANA).

Eleven (10%) of the 105 sera found to be negative in the rapid assay were ANCA positive at IIF, three for c-ANCA, seven for p-ANCA, and one yielding both c- and p-ANCA staining patterns. These 11 samples were further tested for anti-elastase, anti-lactoferrin, and anti-BPI ANCAs, and with Western blotting on alpha granule extracts. On Western blotting none of these 11 samples yielded bands migrating as PR3 or MPO. Three samples contained BPI-ANCA, two elastase-ANCA, and one lactoferrin-ANCA. One sample yielded several bands. The clinical data of the 11 patients are shown in Table 2. Of the patients whose sera were positive at IIF but negative in the rapid ELISA, nine manifested no clinical signs or symptoms of systemic vasculitis. In two patients the clinical picture was compatible with systemic vasculitis; Patient no. 8 was a 67-year-old man with Wegener’s granulomatosis and involvement of the upper respiratory tract, kidneys, skin, and joints; Patient no. 11 was a 79-year-old man with anaemia and rapidly progressive glomerulonephritis culminating in renal failure and chronic haemodialysis despite immunosuppressive therapy. This patient’s sample gave high background in the rapid ELISA, and the test was therefore considered as non-specific.

Two of the 105 samples were from patients with systemic vasculitis, and thus the negative predictive value (NPV) of the rapid test was 98%, which is high.

Discussion

The occurrence of pulmonary renal syndrome, in fact an aetologically heterogeneous group of diseases, constitutes a medical emergency associated with a high risk of fatal outcome unless the patient is treated with immunosuppression and corticosteroids. Accordingly, it is of paramount importance to identify the patients with GP or small-vessel vasculitis as early as possible. To this end, and to be able to provide patients with optimal therapy, it would be of great clinical value to have a reliable rapid test of high sensitivity and high positive predictive value. Figures of 60–90% have been reported for the prevalence of anti-GBM ab, PR3-ANCA and MPO-ANCA among patients with pulmonary renal syndrome and RPGN [2,5,16–19]. The entity with the worst prognosis is Goodpasture’s syndrome [20,21]. Accordingly, the occurrence of anti-GBM ab with or without other antibodies has been reported to be associated with significantly poor outcome in patients with pulmonary–renal syndrome [2]. SLE is one of the differential diagnoses of renopulmonary syndromes and RPGN, and of the present series, 10% manifested ANA.

In pulmonary–renal syndrome the clinical picture is often miscellaneous and may be misdiagnosed as a viral infection. If the clinician is inexperienced in systemic vasculitis, this may delay correct diagnosis, thus putting the patient at risk of irreversible impairment of renal or respiratory function. To avoid this, we suggest that patients presenting with persistent influenza-like illness or respiratory symptoms should routinely undergo a dipstick test for proteinuria and haematuria, as earlier proposed by others [22]. If

Table 2. ANCA pattern at IIF, ELISA for elastase-, lactoferrin- and bactericidal/permeability increasing protein-ANCA, Western blot analysis and clinical data on 11 patients with no antibodies detectable in the rapid screening assay for anti-GBM and ANCAs but with findings of ANCA at IIF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Screening test</th>
<th>IIF</th>
<th>EL-ANCA</th>
<th>LF-ANCA</th>
<th>BPI-ANCA</th>
<th>Western blotting</th>
<th>Clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Neg.</td>
<td>c</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Wegener’s granulomatosis</td>
</tr>
<tr>
<td>9</td>
<td>Neg.</td>
<td>c</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Pos.</td>
<td>30–100 kDa</td>
<td>Myeloma</td>
</tr>
<tr>
<td>10</td>
<td>Neg.</td>
<td>c</td>
<td>Pos</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Neg.</td>
<td>Pulmonary embolism, coronary heart disease</td>
</tr>
<tr>
<td>11</td>
<td>Non-spec</td>
<td>p/c</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Neg.</td>
<td>GN, renal limited vasculitis</td>
</tr>
</tbody>
</table>

Neg., negative; Pos., positive; p, perinuclear ANCA; c, cytoplasmic ANCA.
haematuria or proteinuria is detected, investigation should be extended to include serum-creatinine analysis, a rapid assay for anti-GBM ab, PR3 and MPO ANCs, and a test for ANA.

The IIF technique has been the gold standard for ANCA testing, but requires skilled and experienced personnel. Not only is the technique time consuming but it is also complicated by difficulty in determining the true antibody titres. An advantage of IIF is that it detects antibodies with several different specificities, including ANA. However, in pulmonary renal syndrome PR3 and MPO are most important. Of sera found to be negative in the rapid screening test, 10% (n = 11) were positive at IIF, though in none of these cases was PR3 or MPO ANCs detected by Western blotting. Instead, we found elastase, lactoferrin or BPI ANCs in some of the cases. The occurrence of these autoantibodies has not previously been described in patients with an aggressive clinical course, as has that of anti-GBM ab or PR3 or MPO ANCs [10,14]. Moreover, nine of these 11 patients had no signs of clinical vasculitis, but manifested such entities as multiple myeloma, pulmonary embolism and ornithosis, thus further contributing to the differential diagnosis. These latter cases yielded no positive results in the rapid ELISA.

However, 2% of cases were missed in the rapid screening assay: one patient with clinically verified Wegener’s granulomatosis and a typical c-ANCA pattern in IIF; the other patient had rapidly progressive glomerulonephritis and clinically suspected microscopic polyangitis. Sera from the latter patient, collected several months after presentation, manifested MPO-ANCA. Although his original serum sample at presentation had been found to manifest non-specific reactivity in the rapid assay, when retrospectively re-analysed the OD value for MPO-ANCA was in fact found to be considerably higher than the other OD values (for anti-GBM ab, PR3-ANCA, and HSA).

The advantages of the rapid screening test are that it is easily performed and that all three antibodies are tested at the same time, yielding results within 30 min. The rapid screening test does not demand highly specialised staff, but is suitable for use at smaller hospitals as well. It would then be possible for patients to be referred much earlier to central hospitals for further investigation, therapy and follow-up. Since the earlier the diagnosis the better the clinical outcome, the rapid test seems to have a potential usefulness in improving clinical outcome. For example, Merkel et al. [23] have shown that in patients with anti-basement membrane antibody-mediated disease, the patients presenting with serum creatinine below 200 μmol/l had a better response to therapy and a significantly improved clinical outcome.

Since differential diagnosis in pulmonary renal syndrome and RPGN is not only confined to vasculitic diseases, but also includes infectious diseases, malignancy, and thromboembolism, a rapid screening test must have a high positive predictive value to avoid inappropriate institution of immunosuppression.

Whilst it is naturally of clinical interest also to analyse the prevalence of other autoantibodies such as elastase-ANCA, lactoferrin-ANCA, and BPI-ANCA, it is not imperative to test for these antibodies in the acute phase as it is to test for anti-GBM ab, PR3-ANCA, MPO-ANCA, and ANA. Thus we conclude that the rapid screening test is a very useful tool in the preliminary clinical evaluation of patients presenting with pulmonary–renal syndrome. The advantage of the rapid screening ELISA is that it detects the anti-GBM ab, PR3-ANCA, and MPO-ANCA, whereas IIF detects additional antibodies.

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