The role of flow cytometric ANCA detection in screening for acute pauci-immune crescentic glomerulonephritis

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Abstract

**Background.** Most cases of pauci-immune crescentic glomerulonephritis (PICGN) are associated with serum anti-neutrophil cytoplasmic antibodies (ANCA). This article studied the sensitivity and specificity of serum ANCA, determined by flow cytometry and indirect immunofluorescence (IIF), to identify patients with acute PICGN.

**Methods.** 577 adults presenting for first biopsy of their native kidneys with serum taken for ANCA (flow cytometry and IIF) determination were studied. A positive ANCA was defined using a flow cytometric ANCA assay as a screening test, followed by a slide-based indirect IIF technique. Pathological confirmation of acute PICGN was used to assess the sensitivity and specificity of this combined approach and its positive predictive value (PPV) and negative predictive value (NPV) in patients presenting for renal biopsy due to abnormal urinary sediment.

**Results.** Forty-nine patients were found to have acute PICGN on renal biopsy. Of these 47 were ANCA positive (sensitivity 95.9%). Overall 93 of the renal biopsy patients were ANCA positive, (specificity 91.3%). A further seven patients (two ANCA positive) had advanced sclerosing disease consistent with PICGN but without evidence of current disease activity. The PPV and NPV of ANCA, assessed by flow cytometry and slide IIF, in predicting that patients presenting with undifferentiated renal disease would have acute PICGN was 50.5 and 99.8%, respectively.

**Conclusions.** Flow cytometric screening of serum for ANCA in patients undergoing renal biopsy has a high NPV for determining those with acute PICGN. It may provide a rapid, simple screening test for this lesion in laboratories using diagnostic flow cytometry and may complement IIF/ELISA in evaluating ANCA positive patients.

**Keywords:** ANCA; ANCA associated glomerulonephritis; crescentic glomerulonephritis; diagnosis; flow cytometry

Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCA) react with extra-nuclear cytoplasmic constituents of neutrophils and are found in the circulation of patients with necrotizing vasculitis [1–3]. In renal disease, the presence of ANCA correlates strongly with pauci-immune focal and segmental necrotising crescentic glomerulonephritis (PICGN), reviewed in [4] and [5] and evidence continues to accumulate as to their pathogenicity [6]. The evaluation of the presence of serum ANCA is used to assist in the diagnosis of this disease. The treatment of ANCA positive patients with acute PICGN involves urgent immunosuppression. However, a minority of patients with this disease have advanced, inactive and sclerosing glomerular lesions on presentation.

Current recommendations arising from the International Consensus Statement on testing and Reporting of ANCA are that at all patients undergo at least IIF for ANCA and if practical also ELISA for anti-proteinase 3 (PR3-ANCA) and anti-myeloperoxidase antibodies (MPO-ANCA) [7]. Since describing the flow cytometric method of screening samples for ANCA [8] the Department of Clinical Immunology at Monash Medical Centre, Melbourne, Australia has used this flow cytometric assay to screen samples as part of the assessment of adult patients presenting for a first native kidney biopsy at Monash Medical Centre. Samples that are positive by this assay are then tested...
by the standard slide-based indirect immunofluorescence assay (IIF) to confirm and characterize positively. More recently, ELISA for PR3-ANCA and MPO-ANCA has been performed to confirm ANCA positivity and characterize the antigen involved. From 1995 to May 2001, 577 patients at Monash Medical Centre had a first native kidney biopsy and ANCA test performed concurrently by both flow cytometry and IIF. The ANCA and renal biopsy results of these patients were assessed to determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of this assay in detecting acute PICGN. As the PPV and NPV of a test depends on the prevalence of the disease in the population studied [9], this population of patients presenting with for renal biopsy was chosen for its relevance to the practice of nephrology.

Subjects and methods

Patients

All adult (≥ 18 years) patients undergoing a first renal biopsy of their native kidneys at Monash Medical Centre (a secondary and tertiary referral centre for renal medicine in southeast Melbourne, Australia) from January 1, 1995 to May 31, 2001 were considered for this study. The range of histopathological diagnoses was typical of a large tertiary referral centre. Direct immunofluorescence was performed on sections to detect IgG, IgA, IgM, C1q, C3 C4 and fibrin. An independent pathologist viewed all biopsies. Acute PICGN was diagnosed when at least one cellular or fibrocellular glomerular crescent was observed in the biopsy specimen, focal and/or segmental glomerular necrosis was present, there was a maximum of 1+ (light, scale 0–3+) staining for immunoglobulin in glomeruli and no electron dense deposits were present on electron microscopy (Table 1). Biopsies that were consistent with PICGN but had no active necrotizing lesions and no cellular/fibrocellular crescents were considered to represent inactive or ‘burnt out’ disease and were categorized as ‘sclerosed’ PICGN (scl-PICGN). A total of 1071 closed renal biopsies were performed in patients 18 years or over during the study period. 174 transplant biopsies and 41 repeat biopsies were excluded. An additional 21 cases were excluded due to insufficient data, leaving 835 first native kidney biopsies. 624 out of 835 patients (74.7%) had ANCA estimations within 1 month of their biopsy (the majority within 3 days) at the Department of Clinical Immunology, Monash Medical Centre. Of these 624 patients, 46 had had IIF without flow cytometric evaluation and one patient had had flow cytometry followed by anti-PR3 and anti-MPO ELISA without IIF. These were excluded from further analysis. Therefore, this study focuses on the 577 adult patients with a first closed native renal biopsy who had both flow cytometric evaluation of ANCA and IIF for ANCA.

Detection of anti-neutrophil cytoplasmic antibodies and anti-nuclear antibodies

ANCA was detected by flow cytometry as described previously [8]. Normal human neutrophils were isolated from EDTA blood using Polymorphprep liquid media (Nycodenz, Axis-Shield, Oslo, Norway) density gradient separation media. The polymorphonuclear interface was removed, washed in 0.45% NaCl, residual red blood cells were lysed and cells washed twice in 0.9% NaCl. Neutrophils were resuspended (1.5 × 10⁶ cells/ml) in 2% paraformaldehyde (4°C, 30 min), then 0.2% Tween 20 in PBS (37°C, 15 min), then 1% BSA/0.01% Azide buffer in PBS at a concentration of 1.5 × 10⁶ cells/ml. Neutrophils were labelled by adding 100 μl of neutrophil cell suspension to 20 μl serum preparation, diluted 1 in 5 in 0.001% saponin (4°C, 20 min), buffer added and samples spun. After spinning, supernatant was aspirated, cells resuspended and 20 μl anti-human IgG FITC (1 in 50, Silenus, Hawthorn, Victoria, Australia) added, diluted in 0.01% saponin and incubated (4°C, 20 min). Samples were washed and centrifuged twice in buffer, then assayed on a flow cytometer (MoFlo, Dako Cytomation, Ft Collins, CO, USA). Forward and right angle light scatter (FALS) and FITC fluorescence signals were collected. A gate was set on the neutrophils using the FALS vs 90% light scatter scattergram and FITC fluorescence measured. Pooled normal human serum (negative control) was used to establish a baseline (Figure 1A). If > 20% of the population of duplicate sample cells were above the cutoff point for normal pooled sera (i.e. greater than the fluorescence intensity for 97% of normal cells) the sample was deemed positive (Figure 1B and C).

<table>
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<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>ANCA positive</td>
<td>Positive by flow cytometry and by IIF</td>
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<tr>
<td>Acute pauci-immune crescent GN (PICGN)</td>
<td>Cellular/fibrocellular crescent(s); necrotizing active lesion(s); maximum of (light) 1+ staining for immunoglobulin</td>
</tr>
<tr>
<td>Sclerosed (scl)-PICGN</td>
<td>Fibrous crescent(s); no active necrotizing lesions; maximum of (light) 1+ staining for immunoglobulin</td>
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<tr>
<td>Sensitivity</td>
<td>True positive ANCA result/all patients with the disease</td>
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<tr>
<td>Specificity</td>
<td>True negative ANCA result/all patients without the disease</td>
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<tr>
<td>PPV (True positive ANCA result/all patients with positive results) × 100 NPV</td>
<td>Percentage of positive patients with the disease [9]</td>
</tr>
<tr>
<td>ANA positive</td>
<td>Titer &gt; 1:160</td>
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Table 1. Definitions used in this study
For detection of ANCA by IIF, 50 μl of sera diluted 1 in 30 in PBS was added to an ethanol-fixed human neutrophil ANCA assay slide (INOVA Diagnostics, San Diego, CA, USA), then incubated (20 min, room temperature). Slides were rinsed, then incubated with 50 μl of anti-human IgG FITC (1 in 100, 20 min, room temperature) and rinsed again. Slides were assessed using a fluorescent microscope to determine whether the assay was positive or negative and to determine the pattern of staining (cytoplasmic (C-ANCA) or perinuclear (P-ANCA)). ELISA to detect the presence of PR3-ANCA and MPO-ANCA was performed on positive samples from 1999 onwards using Immunoscan (Euro-Diagnostica, Malmo, Sweden) kits according to the manufacturer’s instructions. Patient samples were diluted 1 in 50, 100 μl of sample added in duplicate and incubated (60 min, room temperature) with a standard curve and known positive and negative patient samples. After washing, conjugate reagent was added (60 min, room temperature). Substrate was added and absorbance at 405 nm read. A positive result for PR3-ANCA or MPO-ANCA was considered at >10 EU/ml.

Results

Flow cytometric assay of ANCA with IIF has a high sensitivity and NPV for acute PICGN

577 patients underwent first closed renal biopsy of their native kidney and had ANCA assessment by both flow cytometry and IIF. Forty-nine (8.5%) of these patients had acute PICGN. A further seven patients with advanced, ‘immune negative’, sclerosing disease with sclerosis of glomerular tufts and sclerosed crescents but no cellular crescents or necrosis patients were considered to have inactive or ‘burnt out’ sclerosed (scl)-PICGN. One ANCA positive (i.e. positive by both flow cytometry and IIF) patient with acute PICGN (included in analyses) had coexistent anti-GBM antibodies in serum and faint linear IgG deposits on the basement membrane.

Results of the combined flow cytometric and IIF assessment of ANCA are presented in Table 2. All samples were assayed for ANCA by flow cytometry. Samples that were positive were tested by IIF for confirmation and ANCA pattern. This protocol resulted in a positive ANCA result (i.e. positive by flow cytometry and by IIF) in 47 of the 49 of the patients with acute PICGN (on renal biopsy). Two patients were ANCA negative. These results equate to a sensitivity of 95.9% and a NPV of 99.8% for detection of this glomerular lesion (Table 3). Flow cytometric/IIF analysis of ANCA was positive in a further 46 patients that did not have acute PICGN, equating to a specificity of 91.3% and PPV of 50.5%.

When ANCA results from patients with scl-PICGN were analysed, two of seven patients had a positive ANCA, while the other five patients were ANCA negative. Inclusion of these patients into the full analysis resulted in a reduction of the sensitivity of combined flow cytometry/IIF ANCA to 87.5%, but little change in the NPV (98.6%) specificity (91.6%), and a modest increase in the PPV to 52.7% (Table 3).

False positive results: patients with ANCA positive tests, but not acute PICGN on biopsy

The histological diagnoses of the 46 patients who were ANCA positive by flow cytometry/IIF but who did not have acute PICGN these patients are presented in
Four of these 46 patients could be considered to have ANCA associated disease, which was not acute PICGN. Seventeen of the remaining 42 patients had lupus nephritis. The majority of the ‘false positive’ ANCA results were P-ANCA (37/46). Many (60%) of these P-ANCA positive, acute PICGN negative patients were associated with a clear positive ANA (>1:160). Of patients with a biopsy diagnosis of lupus nephritis and a positive ANCA, 15 out 17 had a positive ANA (>1:160) and in one patient the ANA was 1:160.

As expected, patients with a positive ANCA without PICGN were significantly more likely to have a positive ANA than ANCA positive PICGN positive patients (52 vs 17%, Table 5). Only a minority (33%, 9/27) of patients with a positive ANCA and a diagnosis other than acute PICGN or lupus nephritis had a positive ANA (seven had an ANA titre of 1:160 and 11 an ANA <1:160).

Proportion of positive cells and mean fluorescence intensity in true and false positive results

Flow cytometry for ANCA detects both the proportion of cells (%) positive and the mean fluorescence intensity (MFI) for positive samples. However, there was no difference in these flow cytometry parameters between the ANCA positive, acute PICGN positive group and the ANCA positive, acute PICGN negative group [(mean ± SD) acute PICGN positive 72.6 ± 22.1% positive cells, MFI 226 ± 152 units; acute PICGN negative 65.6 ± 22.5% positive cells, MFI 205 ± 178 units].

Use of anti-PR3 and anti-MPO ELISA with flow cytometry ANCA assay

Following the international consensus statements on testing for ANCA in 1999 [7], patients later in the current series of patients underwent ELISA following a positive ANCA result by flow cytometry/IIF. Of 19 ANCA positive patients with acute PICGN who was tested for ANCA by ELISA, 15 were positive. All four C-ANCA positive patients were PR3-ANCA positive and nine of 14 P-ANCA positive patients were MPO-ANCA positive. One P-ANCA positive patient was PR3-ANCA positive, and one patient with a mixed pattern was both PR3-ANCA and MPO-ANCA positive. Twenty-one ANCA positive patients who did not have PICGN underwent PR3 and MPO ELISA. Seven patients were positive (three PR3-ANCA, four MPO-ANCA). Two PR3-ANCA positive patients had extrarenal vasculitis but minimal renal lesions and of eight patients with lupus nephritis, three were MPO-ANCA positive.
Flow cytometric detection of ANCA in GN

Discussion

Acute PICGN is strongly associated with ANCA, which may be pathogenetic [6]. There are two traditional ways of detecting ANCA, by fluorescent evaluation of IIF slides and by ELISA for anti-PR3 and MPO antibodies. The current study demonstrates that screening samples for ANCA by flow cytometry has a NPV value (99.8%) for acute PICGN and for all cases of PICGN (98.6%). The patients were representative of a typical population presenting from renal biopsy in Australasia. A reasonably high proportion (69%) of patients presenting for first closed renal biopsy were tested under this protocol. Some patients who presented either as a tertiary referral, with known diseases (such as lupus nephritis), or with relatively minor urinary abnormalities may have undergone ANCA estimation elsewhere or not at all, and as such we cannot exclude the possibility that there could be some selection bias in this study.

In addition to the use of flow cytometry, our series differs from some others, for example that of Hagen et al. [10] and Harris et al. [11] in that a narrow focus was chosen, that is to limit the diseases studied to only PICGN. The disease control patients were those with renal lesions other than acute PICGN. This approach has the advantage of high, near gold-standard diagnostic accuracy, of being explicitly relevant to nephrological practice and being able to distinguish active from inactive disease. However, its disadvantages include including as ‘false positives’ at least two patients with biopsy proven systemic vasculitis but no significant renal lesion, and not being as relevant to everyday practice in the clinical immunology/pathology laboratory. The sensitivity and specificity of this approach was similar to that reported in one recent series [10] and at the upper level of that expected [7], which may be due to the patient population studied, the more narrow definition of positive disease and the use of ‘disease controls’ that are less likely (with the exception of lupus nephritis) to produce false positives.

The low sensitivity (2/7 cases) of flow cytometric and IIF ANCA estimation for advanced sclerosing and inactive crescentic GN is interesting and is likely to reflect the fall in rates of ANCA positivity in inactive disease. It is possible, though we believe unlikely that the use of ELISA for anti-PR3 and anti-MPO in these cases may have identified these patients as ANCA positive. While the use of flow cytometry as a screening test for ANCA had a high NPV in this whole population, it does not entirely rule out acute PICGN. Furthermore, in a clinical presentation of rapidly progressive GN, the NPV of this approach could fall considerably, as the pre-test probability of acute PICGN would be much higher than that for the entire spectrum of patients presenting for renal biopsy in this study. The PPV would however, rise in this subpopulation.

In addition to its NPV value in this population, potential advantages of flow cytometry estimation in ANCA include its rapid execution, the modest reagent cost, and low staff costs in laboratories set up for and proficient in flow cytometry. Flow cytometric assessment of ANCA also has the ability to report and assess intensity by MFI, though its practical value remains to be determined. A confident diagnosis of an ANCA positive sample should not be made by flow cytometric analysis alone as (i) flow cytometry does not distinguish ANCA pattern and (ii) patients with positive ANA tests may also test positive for ANCA by flow cytometry. Supporting the argument that flow cytometry alone has a relatively low specificity is data from a subset of our study patients—of 110 patients that were positive by flow cytometry, 32% were negative by IIF. We believe that flow cytometric estimation of ANCA could be of value in screening samples, before embarking on IIF and ELISA tests for samples that are positive by flow cytometry. Of course, the use of both IIF and anti-PR3 and anti-MPO to assess all samples remains the gold standard for ANCA estimation [7]. However, the cost of ELISA estimations in some laboratories means that some laboratories screen for ANCA only with IIF, a practice currently acceptable according to the latest guidelines [7]. While this study does not address the potential role of flow cytometric ANCA estimation in conjunction with ELISA, the use of both flow cytometry and IIF might conceivably provide a practical and cheaper alternative, with ELISA being performed only on samples positive by either flow cytometry or IIF. Such studies are currently underway.

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Conflict of interest statement. None declared.

References


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