A Dual-Fixed Neutrophil Substrate Improves Interpretation of Antineutrophil Cytoplasmic Antibodies by Indirect Immunofluorescence

Ming-Wei Lin, MBBS,1,2 Roger A. Silvestrini,1 Suzanne Culican,1 David Campbell,1 and David A. Fulcher, MBBS, PhD1,2

From the 1Department of Immunopathology, Pathology West, ICPMR, Westmead Hospital, Westmead, Australia, and 2Discipline of Medicine, Sydney Medical School, University of Sydney, Sydney, Australia.

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ABSTRACT

Objectives: To determine whether the addition of a formalin-fixed neutrophil substrate could improve interpretation and prediction of autoantigenic specificity in antineutrophil cytoplasmic antibody (ANCA) testing.

Methods: Routine diagnostic samples sent for ANCA testing were analyzed prospectively on a dual substrate of both ethanol- and formalin-fixed neutrophils. Positive samples on ethanol-fixed neutrophils were deemed “typical” if formalin-fixed neutrophils also stained, and “atypical” if not. Indirect immunofluorescence (IIF) results were correlated with antimyeloperoxidase (MPO) and anti–proteinase 3 (PR3) results with an enzyme-linked immunosorbent assay (ELISA).

Results: Of 1,426 samples, 201 from unique patients were ANCA-positive (200 on IIF, 1 on ELISA alone). Thirty-two (45%) of 71 typical ANCA staining patterns were positive for either an anti-MPO or anti-PR3 antibodies, whereas only one (0.8%) of 129 atypical patterns was ELISA-positive, in a patient without systemic vasculitis. Only one (3%) of 34 ELISA-positive samples had a negative IIF-ANCA (1/1,426 patients, 0.07%), and this patient did not have vasculitis.

Conclusions: Concomitant staining on formalin fixation of IIF-positive ethanol-fixed ANCA samples improves the interpretation of ANCA testing and is predictive of vasculitis autoantigens MPO and PR3.

Detection of antineutrophil cytoplasmic antibodies (ANCA) is critical for the diagnosis and monitoring of the “ANCA-associated vasculitides” (AAVs), which include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), pauci-immune crescentic glomerulonephritis, and Churg-Strauss syndrome (CSS, also called eosinophilic granulomatosis with polyangiitis or allergic granulomatosis). The test is usually performed with indirect immunofluorescence (IIF) using alcohol-fixed neutrophils as substrate, where the pattern of cytoplasmic staining has distinct diagnostic implications. Traditionally defined, cytoplasmic ANCA (c-ANCA), characterized by diffuse granular cytoplasmic staining with internuclear accentuation [Image 1], is associated with anti–proteinase 3 (PR3) antibodies, a specificity typical of GPA but also found in other AAVs. Similarly, a perinuclear ANCA (p-ANCA) gives rise to homogeneous perinuclear staining with internuclear accentuation [Image 1], is associated with anti–proteinase 3 (PR3) antibodies, a specificity typical of GPA but also found in other AAVs. Subsequent to the description of these “typical” patterns, so-called “atypical” patterns were recognized. Although interpretations vary, a dull, flat, diffuse cytoplasmic staining without central accentuation is often reported as “atypical c-ANCA” (Image 1), whereas a broad “pencil-like” perinuclear staining with no nuclear extension is often termed “atypical p-ANCA” (Image 1). The recognition of atypical patterns is important, because the target autoantigens that give rise to them are different and not associated with...
The four antinuclear cytoplasmic antibody (ANCA) fluorescence patterns based on staining of ethanol- and formalin-fixed neutrophils, as indicated, including cytoplasmic ANCA (c-ANCA), perinuclear ANCA (p-ANCA), atypical c-ANCA, and atypical p-ANCA.
vasculitis. They include lactoferrin, cathespin G, elastase, lysozyme, bacterial permeability increasing protein, catalase, α-enolase, and lamin B1.1 Such specificities are much less useful diagnostically, being found in various conditions including inflammatory bowel disease (IBD),1 rheumatoid arthritis, Felty syndrome, primary sclerosing cholangitis, chronic bacterial infections,2,3 and chronic liver disease.4 However, distinguishing typical from atypical ANCA patterns is not straightforward, a difficulty that gives rise to significant discrepancies among laboratories in regard to interpretive criteria and reporting schemata. Interpretation may also be affected by interference from concomitant homogeneous ANA, which may mimic and therefore mask the presence of an underlying p-ANCA.

To improve interpretation of IIF-ANCA patterns, several groups have proposed that concomitant consideration of the staining pattern on formalin-fixed neutrophils, in addition to the standard ethanol fixation, may be helpful.5-10 Despite initial enthusiasm for this approach, other studies have identified inconsistencies, including false positivity owing to enhanced autofluorescence on the formalin-fixed substrate.11-13 Given these controversies, the selective nature of the cohorts examined in these studies and the paucity of reports correlating IIF-ANCA patterns on a dual substrate with autoantigenic specificity, we examined the latter approach to ANCA testing in the routine diagnostic laboratory.

Materials and Methods

Specimens

Specimens were collected between February and July 2012 from diagnostic samples referred for ANCA testing to the immunopathology laboratory of the Institute of Clinical Pathology and Medical Research, Pathology West, Westmead Hospital, Sydney, Australia, a diagnostic laboratory servicing about one-quarter of public pathology in New South Wales, Australia. For patients with multiple ANCA requests over this time, only the first sample was included in the analysis.

ANCA Interpretation

IIF was performed using a combination of two substrates in a single well (Euroimmun Mosaic ANCA Biochip, ESL Biosciences, Lübeck, Germany), including ethanol- and formalin-fixed neutrophils. Samples were screened at 1/10 dilution, and four patterns were distinguished based on individual staining patterns, as follows:

1. c-ANCA: Finely granular diffuse fluorescence present throughout the cytoplasm with central accentuation, with similar appearance on ethanol- and formalin-fixed neutrophils (Image 1);
2. p-ANCA: Homogeneous perinuclear staining with nuclear extension on ethanol-fixed neutrophils, but a classic c-ANCA pattern (above) on formalin-fixed neutrophils (Image 1);
3. Atypical c-ANCA: Dull homogeneous cytoplasmic fluorescence without central accentuation on ethanol-fixed neutrophils, but no fluorescence on formalin-fixed neutrophils (Image 1);
4. Atypical p-ANCA: Broad rim-like fluorescence of the nuclear periphery without nuclear extension on ethanol-fixed neutrophils, but no fluorescence on formalin-fixed neutrophils (Image 1).

The IIF-ANCA patterns were interpreted by two independent readers (R.A.S. and either S.C. or D.C.), who were blinded to the enzyme-linked immunosorbent assay (ELISA) results.

ELISA

Anti-MPO and -PR3 antibodies were detected using a commercial ELISA (INOVA Diagnostics, Werfen Group, Barcelona, Spain), performed according to the manufacturer’s instructions. An anti-PR3 antibody result greater than 5 U/mL and anti-MPO antibody greater than 10 U/mL was considered positive.

Results

The study included 1,426 unique patient samples, of which 200 were IIF-ANCA positive. Of these, 35 (18%) were c-ANCA, 36 (18%) were p-ANCA, 33 (17%) atypical c-ANCA, and 96 (48%) atypical p-ANCA. Table 1. Positivity for anti-PR3 or anti-MPO by ELISA was almost completely restricted to the samples showing typical ANCA staining patterns on IIF. Thus, of the 35 c-ANCA samples, 17 (49%) were positive for anti-PR3, anti-MPO, or both (Table 1), and of the 36 p-ANCA positive samples, 15 (42%) were positive for one of these two specificities. Importantly, only one (0.8%) of 129 samples with an atypical IIF-ANCA pattern was positive on ELISA. This patient had an atypical p-ANCA on IIF, a low positive anti-PR3 level of 16 U/mL, but no clinical or radiologic evidence of vasculitis.

The close correlation between typical ANCA staining patterns and autoantigenic specificity on ELISA prompted us to question whether IIF screening alone was able to detect all vasculitis-associated specificities, a finding that would allow testing for autoantigenic specificity to be reserved for those showing typical ANCA-IIF patterns. Consistent with this, only one (3%) of 34 ELISA-positive samples (1/1,426 [0.07%]) was negative on fluorescence. This sample was positive for both anti-MPO and anti-PR3 antibodies (both >100 U/mL); the patient had a clinical history of chronic
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typical vs atypical IIF-ANCA pattern, 
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the ethanol-fixed neutrophil substrate, but are negative on 
antinuclear antibodies, which give a p-ANCA pattern on 
neutrophils overcomes the problem of interference from 
fixed neutrophil slide. Furthermore, the use of formalin-fixed 
neutrophils overcomes the problem of interference from 
antinuclear antibodies, which give a p-ANCA pattern on 
formalin-fixed neutrophil substrate, but are negative on 
formalin-fixed neutrophils. 6,16,20 Finally, given the confusion 
surrounding the various definitions of what constitutes a 
typical vs atypical IIF-ANCA pattern, 5,17 an argument could 
be made for abandoning the term “atypical” completely, and 
replacing it with a term such as “nonspecific ANCA,” with the 
potential to cause less confusion in diagnostic interpretation. 
Other researchers have previously examined the 
use of formalin fixation in differentiating the IIF-ANCA 
patterns. 6,8,11-13,18-21 Results have been highly variable, 
possibly because of differences in methodology, such as 
variable incubation times, concomitant use of acetone, 
different types of formalin (vapor vs liquid), and different 
types of alcohol fixative (ethanol vs methanol). Only five 
studies, performed in very selected patient populations, have 
specifically addressed the issue of PR3 and MPO specificity 
in relation to the use of formalin-fixed neutrophils, 6,11,16,20,21 and 
generated results consistent with those reported here in 
our otherwise unselected cohort. Radice et al 8 studied 
serum samples selected on the basis of disease and antigenic 
specificity, and found that samples from patients with vasculitis 
who were positive for anti-PR3 or anti-MPO antibodies were 
the only ones to show ANCA staining on formalin-fixed 
neutrophils, whereas serum samples from patients with 
ulcerative colitis did not. Similarly, studies by Lee et al 20 
and Bang la Cour et al 11 also demonstrated that anti-MPO- 
positive samples could readily be detected on formalin-fixed 
neutrophils. Pollock et al 21 selected patients with MPA and 
compared them with those with IBD or lupus, and found 
perfect correlation between those with active MPA and both 
ANCA staining on formalin-fixed neutrophils and MPO 
positivity; however, in those with vasculitis who received 
treatment, the MPO appeared to be slightly more sensitive. 
Finally, Damoiseaux et al 16 examined the usefulness of the 
Europlus ANCA Biochip mosaic, incorporating a substrate of 
both formalin- and ethanol-fixed neutrophils alongside PR3 
and MPO antigen microdots, in a cohort of patients with AAV. 
Consistent with our results, they found a high correlation 
(>96%) between typical IIF-ANCA patterns and positivity for 
PR3 or MPO; furthermore, all IIF-ANCA samples that were 
negative on a formalin-fixed substrate were also negative for 
anti-MPO and anti-PR3. Although our study lacked clinical 
information on the samples studied, we showed that in the 
diagnostic laboratory environment, processing otherwise 
unselected patient samples, positivity on formalin-fixed 
neutrophils was very predictive of positivity for the two 
major vasculitis-associated antigens. 
Our study has deliberately focused on the detection of 
the vasculitis-associated specificities PR3 and MPO, fully
recognizing that IIF-ANCA is also occasionally used for the diagnosis of nonvasculitic diseases. For example, in IBD, IIF-ANCA patterns can help differentiate ulcerative colitis from Crohn disease, usually in conjunction with anti-

\textit{Saccharomyces cerevisiae} antibodies; thus patients with ulcerative colitis are usually negative for anti-\textit{S cerevisiae} antibodies but have atypical p-ANCA, whereas patients with Crohn disease often have the opposite serologic findings. Although we did not examine patients with IBD specifically, the use of a dual substrate to define an atypical IIF-ANCA pattern in the manner outlined in our study should not detract from the usefulness of IIF-ANCA for this purpose. Indeed, a recent study showed that the distinction between typical and atypical p-ANCA using formalin fixation improved the sensitivity and specificity of ANCA testing in a cohort of 184 patients with IBD\(^1\); MPO specificity was not reported, but seems unlikely to be relevant given the nature of the cohort.

Finally, our study had significant implications for the optimal cost-effective approach for detecting and characterizing serum samples referred for ANCA testing. Although current international guidelines recommend that all serum samples be tested using IIF along with immunoassays for PR3 and MPO,\(^5\) our data support the contention that ELISA testing could be restricted to samples showing a typical ANCA on IIF. Thus, only one ELISA-positive sample of our 1,426 referred samples (or 1/34 ANCA-positive samples) would have been missed with such an approach, and this patient did not have vasculitis. Our findings therefore have significant cost implications for laboratories, particularly given the large number of patients now being screened for ANCA.

We propose that the term “typical” ANCA be used to refer to dual staining on both ethanol- and formalin-fixed neutrophil substrates, divided into c-ANCA and p-ANCA based on the ethanol-fixed slide (Image 1). All other neutrophil staining patterns could be reported as nonspecific ANCA, because they are rarely associated with PR3 or MPO specificity and thus unlikely to convey a diagnosis of vasculitis. Our laboratory now adopts this reporting schema.

Reprint requests to Dr Lin: Dept of Immunopathology, Pathology West, ICPMR, Westmead Hospital, Westmead, New South Wales 2145, Australia; ming-wei.lin@sydney.edu.au.

References


