Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against antigens found in the cytoplasmic granules of neutrophils and monocytes. These antibodies occur at presentation in most patients with active Wegener's granulomatosis or microscopic polyangiitis (reviewed in Savige and colleagues). The “International consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)” advocates screening by indirect immunofluorescence (IIF), but external quality assessment programmes often demonstrate different IIF patterns for a single serum. The consensus statement describes two fluorescence patterns that occur in patients with Wegener’s granulomatosis and microscopic polyangiitis. Cytoplasmic fluorescence (C-ANCA) is diffuse cytoplasmic staining with coarse granular and central interlobular accentuation. It is seen in most patients with microscopic polyangiitis, whereas perinuclear fluorescence (P-ANCA) is seen in patients with Wegener’s granulomatosis and is usually the result of PR3 specificity. With perinuclear fluorescence (P-ANCA), the nuclear lobes are outlined, and there is some nuclear extension when the specificity is MPO. MPO-ANCA occur in about 60% of patients with microscopic polyangiitis, but sometimes patients with microscopic polyangiitis have P-ANCA with PR3 specificity, or C-ANCA with PR3 or MPO specificity.

**Abbreviations:** ANCA, antineutrophil cytoplasmic antibodies; C-ANCA, cytoplasmic antineutrophil cytoplasmic antibodies; ELISA, enzyme linked immunoassay; IIF, indirect immunofluorescence; MPO, myeloperoxidase; P-ANCA, perinuclear antineutrophil cytoplasmic antibodies; PR3, proteinase 3
Immunofluorescent patterns produced by ANCA

C-ANCA have to be distinguished from C-ANCA (atypical), where the fluorescence is “flatter”, and there is no accentuation of the interlobular fluorescence or granular staining. This pattern occurs as often as C-ANCA in a routine testing laboratory, but is not present in the systemic vasculitides. It is found mainly in rheumatoid arthritis, inflammatory bowel disease, cystic fibrosis, and in infections.

The other category of atypical ANCA is uncommon, and usually caused by a combination of both cytoplasmic and perinuclear fluorescence. These sera have multiple antigenic specificities including PR3, MPO, bactericidal permeability increasing protein, cathepsin G, elastase, lactoferrin, and lysozyme. Atypical ANCA occur in inflammatory bowel disease, some autoimmune forms of arthritis, and drug induced vasculitides. The consensus statement recommends that sera with C-ANCA (atypical) and atypical ANCA should be tested in ELISAs for both PR3-ANCA and MPO-ANCA.

ANCA testing is widespread in Australia, and external quality assessment programmes have demonstrated an unexplained variation in the IIF patterns for individual test sera. The aim of our study was to determine whether this variation is caused by errors in the interpretation of ANCA patterns.

METHODS

Sera with C-ANCA and PR3 specificity (n = 2) (producing 3+ or 2+ fluorescence), P-ANCA with MPO specificity (3+) (n = 1), and P-ANCA without MPO specificity (3+) (n = 1) were selected by the reference laboratory that coordinates external quality assessment testing for ANCA in Australia. Sera were chosen that were typical of the C-ANCA found in Wegener’s granulomatosis and the P-ANCA found in microscopic polyangiitis or inflammatory bowel disease. None of the sera contained antinuclear antibodies when tested on HEp2 cells.

Each serum was distributed to 11 laboratories and tested with the neutrophil substrates, serum dilutions, and conjugate type and dilutions used routinely in that laboratory (table 1). Substrates and conjugates have been coded, and P1 and C1, P2 and C2, etc are from the same manufacturer. All in house neutrophil preparations were made according to the published method.5 Slides were then examined using ultraviolet microscopy; and the IIF patterns described and strength of fluorescence graded from 0 (negative) to 3+ (strong). The process was repeated, the fluorescence patterns were photographed, and the patterns and intensity of staining were subsequently characterised by members of the Australian ANCA study group at a face to face meeting. The “consensus” pattern was the pattern seen in most laboratories.

RESULTS

All test sera were positive for ANCA by IIF in each of the 11 laboratories. All fluorescence patterns were consistent between the first and second testing in each laboratory, and with the description at the meeting of the Australian ANCA study group. Thus, variations in IIF patterns cannot be attributed solely to errors in interpretation.

The observed IIF patterns were inconsistent with the consensus patterns in eight assays, each from a different laboratory (eight of 43) (table 1; fig 1). One of these produced a C-ANCA (atypical) rather than a C-ANCA pattern. The other seven resulted in at least some cytoplasmic fluorescence when the consensus pattern was P-ANCA with (n = 4) or without (n = 3) MPO specificity. The intensity of fluorescence for all sera varied by 1+ at most between assays and laboratories. Neutrophil substrates varied in their ability to demonstrate the consensus IIF patterns. In general, C-ANCA was demonstrated well but substrates differed in their ability to distinguish between a C-ANCA and C-ANCA (atypical). P1 neutrophils demonstrated C-ANCA in all four laboratories and the cytoplasmic granularity and central accentuation were
more prominent and more consistent than with other neutrophil preparations. P2 neutrophils were satisfactory where there was strong cytoplasmic fluorescence, but the central accentuation and granularity of weaker cytoplasmic staining were not easily demonstrated, resulting in a C-ANCA being interpreted as a C-ANCA (atypical). Both the P3 and in house neutrophils were too small for cytoplasmic fluorescence to be demonstrated clearly.

All three commercial and three in house neutrophil substrates consistently demonstrated nuclear extension of perinuclear fluorescence with sera containing P-ANCA with MPO specificity. However seven assays of sera containing P-ANCA demonstrated (mostly) additional cytoplasmic fluorescence with each of the three different commercial substrates and one in house assay. In one case only cytoplasmic fluorescence was present. Of the eight assays that resulted in IIF patterns that differed from the consensus, seven used commercial neutrophil substrates and one an in house substrate. Conjugates included anti-IgG, anti-F(ab’), anti-IgG (heavy and light chains), and anti-IgG, A, and M. All six conjugates produced an abnormal IIF pattern on at least one occasion. Of the seven assays tested on commercial substrates, four had been performed using the manufacturer’s conjugates, and three had not.

DISCUSSION

The 11 laboratories participating in our study used different patient serum dilutions, neutrophil substrates, conjugates, and microscope magnifications to examine the test sera. All four sera were considered positive for ANCA by IIF in all the laboratories, most patterns were correctly identified as C-ANCA or P-ANCA, and the fluorescence intensities were generally consistent between assays and laboratories. All descriptions of IIF patterns were confirmed on retesting in individual laboratories and on review by the Australian ANCA study group. The participating laboratories comprised the ANCA study group, members of which have a particular interest and expertise in ANCA testing. This may have biased our findings away from those obtained in inexperienced laboratories, where variations in IIF patterns may result from observer error. However, our study demonstrates that variations in ANCA IIF patterns do not necessarily result from the misinterpretation of patterns, but may result from other, probably technical, factors.

In our study, neutrophil substrates from different manufacturers varied in their ability to demonstrate interlobular fluorescence and cytoplasmic granularity, and thus to differentiate between a C-ANCA and C-ANCA (atypical) pattern. However, most laboratories distinguished successfully between these patterns. Although all three commercial and three in house neutrophil substrates demonstrated the nuclear extension of P-ANCA that correlates with MPO specificity, there were, in general, more problems testing sera containing P-ANCA.

Eight assays from eight different laboratories resulted in IIF descriptions that differed from the consensus patterns. These were mostly caused by additional cytoplasmic fluorescence.
Immunofluorescent patterns produced by ANCA

Take home messages

- All four sera were positive for antineutrophil cytoplasm antibody (ANCA) by indirect immunofluorescence (IIF) in all the laboratories, most patterns were correctly identified as cytoplasmic or perinuclear ANCA, and the fluorescence intensities were generally consistent between assays and laboratories.

- The variations that did occur were mostly caused by additional cytoplasmic fluorescence and were found in sera with perinuclear ANCA, with or without myeloperoxidase specificity.

- Variations in ANCA IIF patterns do not necessarily result from the misinterpretation of patterns, but may result from other, probably technical, factors.

- Such variations cannot be attributed solely to the use of a particular neutrophil substrate or conjugate, or to the use of substrate from one manufacturer and conjugate from another.

“The explanation for the development of cytoplasmic fluorescence is unclear but may relate to the use of alcohol in neutrophil fixation because this permeabilises the membranes”

and were found in sera with P-ANCA with or without MPO specificity. However, this preliminary study suggests that the development of cytoplasmic fluorescence in sera containing P-ANCA cannot be attributed solely to the use of a particular neutrophil substrate or conjugate, or to the combination of substrate from one manufacturer and conjugate from another. The explanation for the development of cytoplasmic fluorescence is unclear but may relate to the use of alcohol in neutrophil fixation because this permeabilises the membranes and allows the normally cytoplasmic MPO to diffuse around the nucleus to produce the perinuclear pattern. Cytoplasmic fluorescence may also result from delays in examining the substrate, which allows the perinuclear MPO to diffuse back into the cytoplasm. Finally, cytoplasmic fluorescence may result from the use of polyclonal antiserum that detects additional IgA or IgM ANCA.

The international consensus statement emphasises that IIF is a screening test for ANCA, and that assays for both PR3-ANCA and MPO-ANCA should be performed on all IIF positive sera. However, some laboratories test for ANCA by IIF alone, and about 10% of patients with systemic vasculitis are positive only by IIF. In our study, the interlaboratory variation in the character of fluorescence patterns and intensity was surprisingly small considering the differences in substrates, conjugates, and ultraviolet microscopy techniques. Our results indicate that the variation in interpretation of ANCA fluorescence patterns seen with individual sera cannot be attributed solely to errors in observer interpretation.

Authors’ affiliations

J Savage, The University Department of Medicine, Austin and Repatriation Medical Centre, Heidelberg, VIC 3084, Australia

W Pollock, K Clarke, K Gallagher, J Hall, E Luckhurst, R McEvoy, J Melny, J Neil, A Nikoloutsopoulos, T Thompson, M Trevisin, Members of the Australian ANCA study group

This work represents a collaboration of the Australian ANCA study group.

REFERENCES


Immunofluorescent patterns produced by antineutrophil cytoplasmic antibodies (ANCA) vary depending on neutrophil substrate and conjugate

W Pollock, K Clarke, K Gallagher, J Hall, E Luckhurst, R McEvoy, J Melny, J Neil, A Nikoloutsopoulos, T Thompson, M Trevisin and J Savige

doi: 10.1136/jcp.55.9.680

Updated information and services can be found at:
http://jcp.bmj.com/content/55/9/680

These include:

References
This article cites 8 articles, 2 of which you can access for free at:
http://jcp.bmj.com/content/55/9/680#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Immunology (including allergy) (1664)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/