

Papers

University
Department of
Medicine, Austin and
Repatriation Medical
Centre, Heidelberg,
Victoria, Australia
J A Savige

Department of
Biochemistry, St
Vincent's Hospital,
Fitzroy, Victoria,
Australia
B Paspaliaris

Department of
Immunopathology,
Westmead Hospital,
Westmead, New South
Wales, Australia
R Silvestrini

South West Area
Pathology Service,
Liverpool, New South
Wales, Australia
D Davies

SouthPath, Flinders
Medical Centre, South
Australia, Australia
T Nikoloutsopoulos

Department of
Rheumatology, St
George Hospital,
Kogarah, New South
Wales, Australia
A Sturgess

Department of
Immunology, Royal
Brisbane Hospital,
Herston, Queensland,
Australia
J Neil

Immunology
Laboratory, Gribble's
Pathology, South
Yarra, Victoria,
Australia
W Pollock

Department of
Immunology, Alfred
Hospital, Prahran,
Victoria, Australia
K Dunster

Immunology
Laboratory,
Queensland Medical
Laboratories, West
End, Queensland,
Australia
M Hendle

Correspondence to:
Dr Judy Savige, Department
of Medicine, The University
of Melbourne, Austin and
Repatriation Medical Centre
(Austin Campus),
Heidelberg, Victoria,
Australia 3084.

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A review of immunofluorescent patterns associated with antineutrophil cytoplasmic antibodies (ANCA) and their differentiation from other antibodies

J A Savige, B Paspaliaris, R Silvestrini, D Davies, T Nikoloutsopoulos, A Sturgess, J Neil, W Pollock, K Dunster, M Hendle

Abstract

Aim—To describe the neutrophil fluorescent patterns produced by antineutrophil cytoplasmic antibodies (ANCA) with different antigen specificities, and by other auto- and alloantibodies.

Background—Most sera from patients with active generalised Wegener's granulomatosis result in diffusely granular cytoplasmic neutrophil fluorescence with internuclear accentuation (cANCA) and proteinase 3 (PR3) specificity. About 80% of the sera from patients with microscopic polyangiitis result in perinuclear neutrophil fluorescence with nuclear extension (pANCA) and myeloperoxidase (MPO) specificity, or a cANCA pattern with PR3 specificity. However, many different neutrophil fluorescence patterns are noted on testing for ANCA in routine immunodiagnostic laboratories.

Methods—Sera sent for ANCA testing, or containing a variety of auto- and alloantibodies, were studied. They were examined by indirect immunofluorescence according to the recommendations of the first international ANCA workshop, and for PR3 and MPO specificity in commercial and in-house enzyme linked immunosorbent assays (ELISA).

Results—Sera with typical cANCA accounted for only half of all neutrophil cytoplasmic fluorescence. Other sera had "flatter" fluorescence without internuclear accentuation, and the corresponding antigens included MPO and bactericidal/permeability increasing protein (BPI), but were usually unknown. Peripheral nuclear fluorescence without nuclear extension occurred typically when the antigens were BPI, lactoferrin, lysozyme, elastase, or cathepsin G. Most types of ANA were evident on ethanol fixed neutrophil nuclei. AntidsDNA, antiRo, and antilamin antibodies resembled pANCA. Antimicrobial and antiribosomal antibodies produced cytoplasmic fluorescence, and antiGolgi antibodies, a pANCA. Sera

from patients with anti-smooth muscle antibodies were associated with cytoplasmic fluorescence. There was no neutrophil fluorescence with anti-skeletal muscle and anti-heart muscle antibodies, anti-liver/kidney microsomal, antithyroid microsomal, or antiadrenal antibodies. Alloantibodies such as antiNB1 typically resulted in cytoplasmic fluorescence of only a subpopulation of the neutrophils.

Conclusions—The ability to distinguish between different neutrophil fluorescence patterns, and the patterns seen with other auto- and alloantibodies is helpful diagnostically. However, the demonstration of MPO or PR3 specificity by ELISA will indicate that the neutrophil fluorescence is probably clinically significant, and that the diagnosis is likely to be Wegener's granulomatosis or microscopic polyangiitis.

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A test for antineutrophil cytoplasmic antibodies (ANCA) is usually requested because the diagnosis of microscopic polyangiitis or Wegener's granulomatosis is suspected or needs to be excluded.^{1 2} Serum is first screened by indirect immunofluorescence (IIF) on normal ethanol fixed peripheral blood neutrophils and antigen specificities are then confirmed in enzyme linked immunosorbent assays (ELISA) for antibodies against myeloperoxidase (MPO) and proteinase 3 (PR3).^{1 3} About 90% of sera from patients with active generalised Wegener's granulomatosis result in a diffusely cytoplasmic neutrophil fluorescence with internuclear accentuation (cANCA), where the target antigen is PR3.⁴ The sera from 80% of patients with microscopic polyangiitis result in perinuclear neutrophil fluorescence with some nuclear extension (pANCA) and MPO specificity, or with a cANCA and PR3 specificity.⁴

However, ANCA are also present in patients with inflammatory bowel disease, rheumatoid

arthritis, systemic lupus erythematosus (SLE), cystic fibrosis, and other diseases.⁵⁻⁸ The associated fluorescence is often a pANCA without the nuclear extension seen with MPO-ANCA.⁹ This occurs when the target antigens are lactoferrin, lysozyme, elastase, cathepsin G, or bactericidal/permeability increasing protein (BPI).¹⁰⁻¹⁴ This pattern can, however, be difficult to distinguish from the pANCA associated with MPO-ANCA. A granulocyte specific antinuclear antibody (ANA), where there is only neutrophil nuclear fluorescence,¹⁵⁻¹⁷ probably represents a form of pANCA; and the corresponding antigen specificities are still not clear. All other neutrophil fluorescent patterns are described as "atypical" ANCA.

The aims of this study were to review the fluorescent patterns associated with ANCA of different antigen specificities, and to determine the auto- and alloantibodies that produced fluorescence that could be confused with these patterns. The patterns were presented and discussed at the Second Australasian ANCA Workshop, and at the ANCA and Vasculitis Symposium, held recently in Melbourne.

Methods

All sera were tested for ANCA by IIF according to the recommendations of the First International ANCA Workshop¹⁸, and sera were also tested for PR3-ANCA and MPO-ANCA in commercial and in-house ELISAs. These examinations were performed in the laboratories of the contributors, whose initials are indicated in brackets. Each laboratory used slightly different conditions to test for ANCA: the screening serum dilutions varied from 1/10 to 1/40; both in-house and commercial neutrophil preparations were used; and the cell magnifications varied. Sera were tested for ANA by IIF on Hep2 cells; and for other autoantibodies using the appropriate substrates.

Fluorescent patterns

(1) ANCA

cANCA, PR3-ANCA positive

Figure 1, panel 1: In-house neutrophil cytospin preparation, serum tested at 1/10 dilution, magnification $\times 400$, PR3-ELISA in-house, MPO-ELISA in-house (DD). This serum was from a patient with active generalised Wegener's granulomatosis. This is the classical cANCA pattern, with finely granular fluorescence present diffusely throughout the cytoplasm and with accentuation between the nuclear lobes. More than 90% of these sera react with PR3.⁴ Antibody levels shown either by fluorescence or in ELISA are typically high at presentation in patients with Wegener's granulomatosis, fall with treatment, and usually recur at relapse. About half of the patients with ANCA recurring after remission will relapse.^{19, 20}

cANCA, PR3-ANCA negative, MPO-ANCA positive

Figure 1, panel 2: In-house neutrophil cytospin preparation, serum tested at 1/40, magnification $\times 500$, PR3-ELISA Eurodiagnostica, MPO dot-blot in-house (AS). This serum is from an elderly female with a peripheral neuropathy secondary to systemic vasculitis. The fluorescence is cytoplasmic without the internuclear accentuation, and has been described as more coarsely granular than the classical cANCA.²¹ The cytoplasmic fluorescence probably arises from a subpopulation of epitopes on MPO molecules that do not migrate to the nuclear membrane, rather than from cross reactivity between PR3 and MPO, or contamination of PR3 with MPO. MPO-ANCA account for 5-10% of all cANCA,²¹ and other antigen specificities include BPI,²² cathepsin G, and further undefined antigens.

cANCA (> 1/2560), BPI-ANCA positive

Figure 1, panel 3: PR3-ANCA negative, MPO-ANCA negative. In-house neutrophil preparation, serum tested at 1/10 dilution, magnification $\times 400$, BPI-ELISA in-house, PR3-ELISA INOVA, MPO-ELISA in-house (JN). This serum was from a 10 year old female with cystic fibrosis. BPI-ANCA in these patients are associated with worse disease, lung infections with pseudomonas, and systemic vasculitis.^{8, 23} The erythrocytes are red because of the Evan's blue counterstain.

cANCA (>1/640), PR3-ANCA negative

Figure 1, panel 4: MPO-ANCA negative, ANA negative, antidsDNA negative. Antigen specificity unknown. In-house cytospin neutrophil preparation, serum tested at 1/10, magnification $\times 600$, PR3-ELISA ORGentec, MPO-ELISA ORGentec (RS). This was from a 54 year old female with a three month history of purulent sputum and no evidence of any systemic vasculitis. The fluorescence is flat and there is no internuclear accentuation. This pattern probably accounts for about half all the cANCA seen in a routine immunopathology laboratory.²⁴ Many laboratories would not distinguish it from a classical cANCA, and would rely on the lack of specificity for PR3 to indicate that the diagnosis of Wegener's granulomatosis was unlikely.

cANCA (1/640), both PR3-ANCA positive and MPO-ANCA positive

Figure 1, panel 5: In-house cytospin neutrophil preparation, serum was tested at 1/10, magnification $\times 600$, PR3-ELISA ORGentec, MPO-ELISA ORGentec (RS).²⁵ This serum was from a 65 year old male with seropositive rheumatoid arthritis. Four years earlier he had had a cANCA (1/640) with PR3 specificity only. There is diffuse cytoplasmic fluorescence with internuclear accentuation; this pattern cannot be differentiated from that seen with PR3-ANCA.²⁶ Dual antigen specificities are common in patients with a propylthiouracil or hydralazine induced systemic vasculitis^{27, 28}; however, binding in ELISAs for both MPO and PR3 usually indicates non-specific binding.

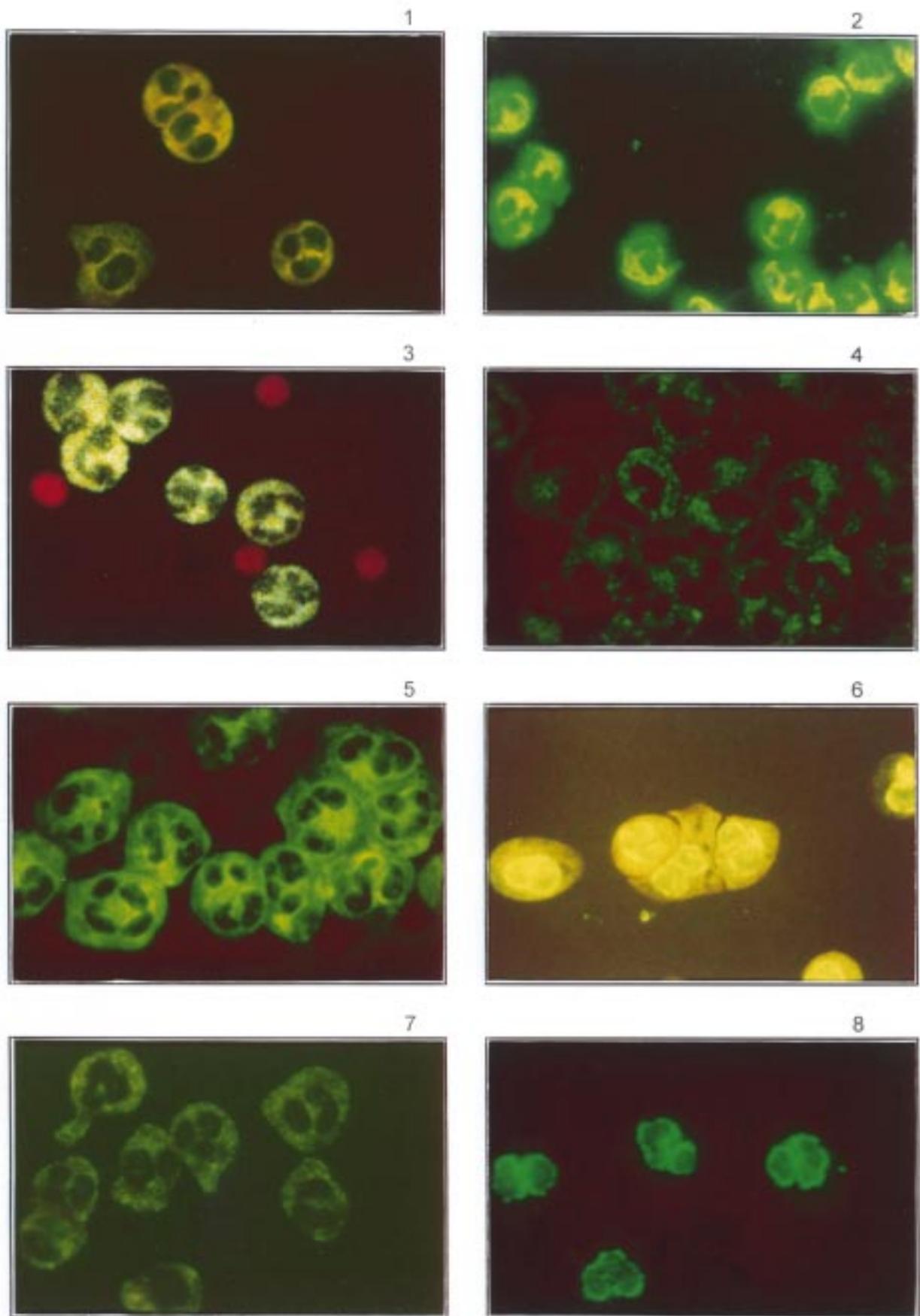


Figure 1

pANCA (1/640), MPO-ANCA positive

Figure 1, panel 6: ANA negative. In-house neutrophil preparation, serum tested at 1/10, magnification $\times 400$, PR3-ELISA in-house, MPO-ELISA in-house (DD). This was from a 24 year old female with segmental necrotising glomerulonephritis. The fluorescence produced by pANCA due to MPO-ANCA is typically perinuclear with some nuclear extension. This pattern is a useful artefact which occurs because ethanol results in the redistribution of positively charged antigens to the negatively charged nuclear membrane. Occasionally MPO also binds to the nuclear membranes of nearby lymphocytes. MPO-ANCA produce cytoplasmic fluorescence on formalin fixed neutrophils (but not on formalin fixed HL60 cells²⁸). Formalin is a cross linking fixative which causes MPO and other pANCA associated antigens to remain in their native location—the primary granules. About 80% of patients with microscopic polyangiitis have pANCA directed against MPO.⁴

Panel 7 shows the cytoplasmic fluorescence seen when serum was tested on formalin fixed neutrophils (DD).

pANCA, PR3-ANCA positive

Figure 1, panel 8: MPO-ANCA negative, ANA negative. INOVA neutrophil preparation, serum tested at 1/20, magnification $\times 400$, PR3-ELISA INOVA, MPO-ELISA INOVA, (TN). There were no clinical details for this patient. There is a pronounced nuclear rim fluorescence but minimal nuclear extension, in contrast to the pattern seen with MPO-ANCA. Probably about 5% of all sera with pANCA are specific for PR3. It has been suggested that these patients have a systemic vasculitis that resembles Wegener's granulomatosis with more lung, ear, nose, and throat involvement, and a higher rate of relapses.²⁹

Other antigens that produce a sharply defined pANCA without the nuclear extension include BPI, cathepsin G, elastase, and lysozyme.^{10–14} These antibodies can often be differentiated from true pANCA by their non-reactivity with formalin fixed neutrophils. This occurs either because the epitopes are destroyed by formalin or because the molecules leak from the cells because they are highly soluble in the fixative.³⁰ These ANCA occur most often in patients with inflammatory bowel disease, primary sclerosing cholangitis, and rheumatoid arthritis.

BPI-ANCA can be associated with either cytoplasmic or perinuclear fluorescence, probably because different epitopes are targeted in different diseases. In addition we have noted perinuclear fluorescence when the smears are examined immediately; this becomes cytoplasmic when the smears are examined after 24 hours, presumably because the BPI diffuses away from the nuclear membrane.

pANCA, antilactoferrin antibodies

Figure 2, panel 9: PR3-ANCA negative, MPO-ANCA negative, ANA negative. In-house neutrophil preparation, serum tested at 1/10, magnification $\times 600$, PR3-ELISA ORGentec,

MPO-ELISA ORGentec (RS). This was from a patient after thyroidectomy. It is not known if she had been treated with carbimazole, which can be associated with these antibodies. Again there is a very defined perinuclear fluorescence with minimal nuclear extension. This is identical to the pattern seen most often with BPI-ANCA in patients with inflammatory bowel disease.

pANCA ("granulocyte specific ANA") (1/640)

Figure 2, panel 10: PR3-ANCA negative, MPO-ANCA negative, ANA negative. In-house neutrophil cytospin, serum tested at 1/10, magnification $\times 400$, PR3-ELISA in-house, MPO-ELISA in-house (DD). This serum was from a 33 year old male with ulcerative colitis. There is diffuse homogeneous nuclear staining with some perinuclear accentuation, but no fluorescence of contaminating lymphocytes. This fluorescence is not usually evident on formalin fixed neutrophils.^{31–33} A granulocyte specific ANA can occasionally be confused with an ANA or high titre pANCA. It is considered to be a pANCA³¹ although the antigens have not been identified. This pattern occurs in perhaps 5% of all patients with rheumatoid arthritis or inflammatory bowel disease, and more commonly in those with Felty's syndrome.

Different fluorescent patterns at different serum dilutions

Figure 2, panels 11 and 12: cANCA (1/160)/pANCA (1/40), PR3-ANCA negative, MPO-ANCA negative, ANA positive (speckled, 1/160, nucleolar, 1/40). This serum was from a 68 year old female, clinical details unknown. Panel 12 is an in-house neutrophil preparation, serum tested at 1/10 dilution, magnification $\times 1000$, PR3-ELISA ORGentec, MPO-ELISA ORGentec (JN). The pattern is predominantly perinuclear. In panel 13, the serum was tested at a 1/160 dilution, and examined at $\times 400$ magnification. The fluorescence is more cytoplasmic. The reason for this is not clear.

(2) ANA

Most specificities of ANA are evident on ethanol fixed neutrophil nuclei. Sera with ANA are usually negative on formalin fixed cells since formalin denatures most nuclear antigens. However, antidsDNA antibodies persist. An ANA often coexists with an ANCA.

Speckled ANA (1/160, ENA negative)

Figure 2, panel 13: ANA evident on Hep2 cells and on neutrophil nuclei. This serum also contains a cANCA and is PR3-ANCA negative and MPO-ANCA negative. In-house cytospin neutrophils, serum tested at 1/10, magnification $\times 600$ (RS). There are fine speckles over the nucleus, and diffusely cytoplasmic neutrophil fluorescence.

cANCA (1/320), PR3-ANCA positive, ANA positive (homogeneous, 1/40)

Figure 2, panel 14: In-house cytospin neutrophil smears, serum was tested at 1/10, magnification $\times 400$, PR3-ELISA in-house, MPO-

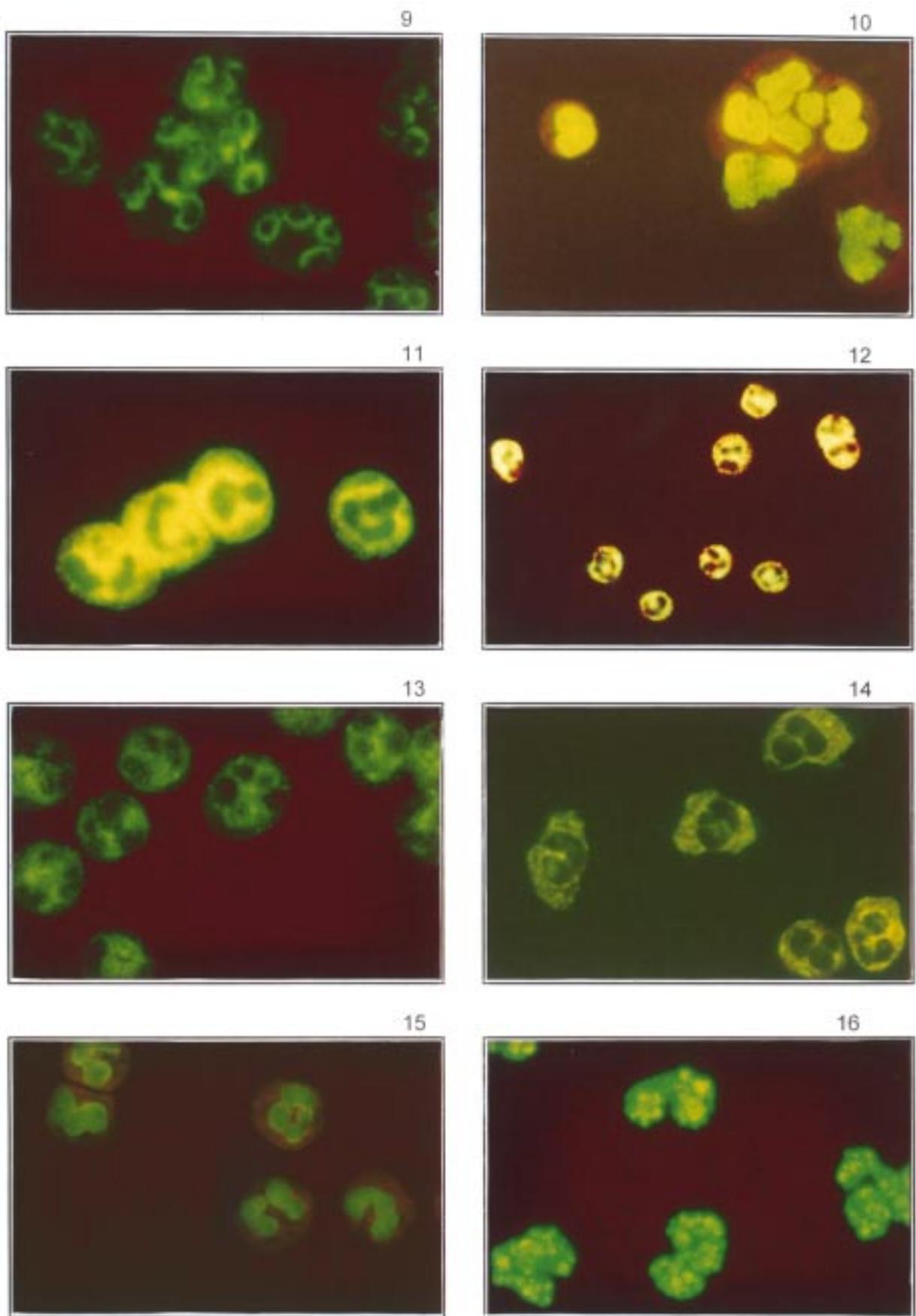


Figure 2

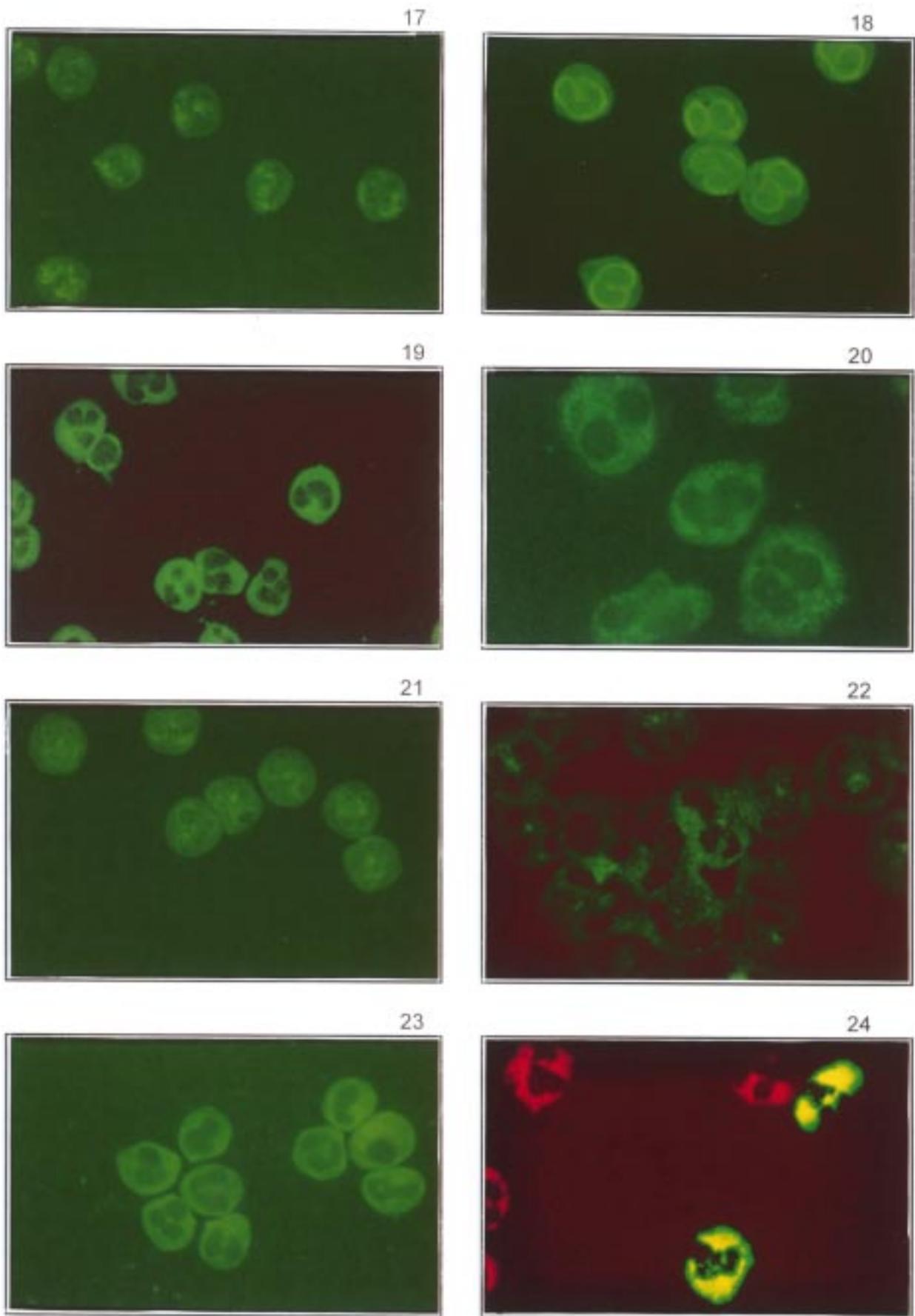


Figure 3

ELISA in-house (DD). This was from a 53 year old man with a lung infiltrate and segmental necrotising glomerulonephritis. There is diffuse cytoplasmic fluorescence, but the ANA was not evident.

pANCA (1/160) MPO-ANCA positive, PR3-ANCA negative, ANA positive (homogeneous, 1/80)

Figure 2, panel 15: INOVA neutrophil preparation, serum was tested at 1/20, magnification $\times 400$, PR3-ELISA in-house, MPO-ELISA in-house (DD). There were no clinical details. There is perinuclear and nuclear fluorescence.

AntidsDNA antibodies (91 IU/ml, normal <7 IU/ml)

Figure 2, panel 16: PR3-ANCA negative, MPO-ANCA negative. In-house cytospin neutrophil smears, serum tested at 1/10, magnification $\times 600$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (BP). The neutrophil fluorescence is nuclear and there is also binding to the lymphocyte nuclei.

AntiRo antibodies (titre 1/640)

Figure 3, panel 17: PR3-ANCA negative, MPO-ANCA negative, ANA speckled. In-house neutrophil smear, serum tested at 1/10, magnification $\times 400$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (BP). Faint speckled perinuclear fluorescence.

Antilamin antibodies

Figure 3, panel 18: PR3-ANCA negative, MPO-ANCA negative, ANA negative. In-house neutrophil smear, serum tested at 1/10, magnification $\times 400$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (BP). Nuclear fluorescence with perinuclear accentuation.

(3) Anticytoplasmic antibodies

Antimitochondrial antibodies (titre >1/640)

Figure 3, panel 19: PR3-ANCA negative, MPO-ANCA negative, ANA negative. In-house cytospin neutrophils, serum tested at 1/40, magnification $\times 500$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (AS). Dull cytoplasmic fluorescence.

Antiribosomal antibodies (titre 1/320)

Figure 3, panel 20: PR3-ANCA negative, MPO-ANCA negative. INOVA neutrophil preparation, serum tested at 1/20, magnification $\times 400$, PR3-ELISA in-house, MPO-ELISA in-house (JS). Granular cytoplasmic fluorescence.

Antigolgi antibodies (titre 1/320)

Figure 3, panel 21: PR3-ANCA negative, MPO-ANCA negative. In-house neutrophil smear, serum tested at 1/10, magnification $\times 400$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (BP). Faint perinuclear accentuation.

(4) Tissue specific autoantibodies

With anti-liver/kidney microsomal antibodies, there was background neutrophil fluorescence only.

Anti-smooth muscle antibodies (titre 1/640)

Figure 3, panel 22: PR3-ANCA negative, MPO-ANCA negative, ANA negative. In-house neutrophil cytospin, serum tested at 1/10, magnification $\times 600$, PR3-ELISA ORGentec, MPO-ELISA ORGentec (RS). ANCA are common in patients with chronic active hepatitis, and the antigen is believed to be actin.³⁴ It is not clear whether the "ANCA" are due to antineutrophil actin antibodies or to coincidental ANCA and anti-smooth muscle antibodies.

Anti-smooth muscle antibodies (titre 1/640)

Figure 3, panel 23: PR3-ANCA negative, MPO-ANCA strongly positive, ANA negative. In-house neutrophil smears, serum tested at 1/10, magnification $\times 400$, PR3-ELISA Eurodiagnostica, MPO-ELISA in-house (BP). Cytoplasmic fluorescence.

With *anti-skeletal muscle, anti-heart muscle, antithyroid microsomal, and antiadrenal antibodies*, there was background fluorescence only.

(5) Alloantibodies

Figure 3, panel 24: cANCA, PR3-ANCA negative, MPO-ANCA negative, ANA speckled. INOVA neutrophil slides, serum tested at 1/20, magnification $\times 500$, PR3-ELISA in-house, MPO-ELISA in-house (KD, WP). Cytoplasmic fluorescence. This is probably from a patient with antiNB1 alloantibodies. Alloantibodies occur in fewer than 1% of the population, especially in multiparous females and after multiple blood transfusions.³⁵ The most common target is NB1, and antiNB1 antibodies typically react with 50–90% of all neutrophils, producing a fine granular cytoplasmic fluorescence.³⁶ AntiMart antibodies produce the same pattern.

Controls

"False positive" ANCA fluorescence occurs when too high a concentration of serum is used in screening, when a polyspecific antiglobulin is used in detection, and when aggregated immunoglobulin binds to neutrophil Fc receptors (after multiple thawings or after heating to inactivate HIV^{37 38}).

Conclusions

The ability to distinguish between different neutrophil fluorescence patterns and to differentiate these from the patterns seen with other auto- and alloantibodies is helpful. However, the demonstration of PR3-ANCA or MPO-ANCA by ELISA will indicate that the neutrophil fluorescence is clinically significant, and that the diagnosis is Wegener's granulomatosis or microscopic polyangiitis. The diagnostic and clinical significance of ANCA in other autoimmune diseases is less clear.

Registrants at the Second Australasian ANCA Workshop, held in Melbourne, Australia, April, 1996, were invited to present unusual ANCA fluorescent patterns that they had seen. These were discussed then, and subsequently at the "ANCA and vasculitis symposium" satellite to the XIV International Congress of Nephrology held in June 1997. We would also like to thank Allan Wiik for his helpful comments.

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