

Pitfalls of formalin fixation for determination of antineutrophil cytoplasmic antibodies

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Abstract

Sera can produce nuclear or perinuclear immunofluorescence staining in neutrophils which may be caused by antibodies with differing antigenic specificities. These include perinuclear antineutrophil cytoplasmic antibodies (P-ANCA), granulocyte specific antinuclear antibody (GS-ANA), and antinuclear antibody (ANA). There is controversy over the value of formalin fixation of neutrophils in differentiating antibodies giving selective or preferential reaction with the nuclear or perinuclear area of neutrophils. In a comparative study of 77 sera, formalin fixation caused inconsistency, non-specific effects, and false positivity owing to enhanced fluorescence. If formalin fixed neutrophils are used in the routine diagnostic laboratory, this will add confusion to the interpretation of the ANCA assay.

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Antineutrophil cytoplasmic antibodies (ANCA) constitute a class of autoantibodies directed at various constituents of myeloid cells, which are clinically very useful in the diagnosis of patients with systemic vasculitides. Two important patterns can be identified by indirect immunofluorescence on ethanol fixed neutrophils. The cytoplasmic (C-ANCA) pattern shows a cytoplasmic granular staining with accentuation of fluorescence intensity in the area between the nuclear lobes. C-ANCA is most commonly associated with Wegener's granulomatosis. The perinuclear pattern (P-ANCA) is an artefact of alcohol fixation of the neutrophils which causes cytoplasmic antigens to redistribute around the nucleus. The P-ANCA pattern is less specific than C-ANCA because of its association with a wider range of diseases including microscopic polyangiitis and idiopathic necrotising crescentic glomerulonephritis. In vasculitic illness, C-ANCA is most commonly associated with antibodies to the proteinase 3, and P-ANCA may be due to antibodies to a variety of antigens including myeloperoxidase, lactoferrin, and other neutrophil enzymes.

The standard method for the detection of ANCA is indirect immunofluorescence on ethanol fixed neutrophils, as validated by previous international workshops. In 1988, the first workshop defined a standard procedure for ANCA by immunofluorescence using ethanol fixed neutrophils as substrate and this has

subsequently been confirmed by the EEC/BCR group for ANCA assay standardisation.^{1,2} Selective or preferential reaction with the nucleus or perinuclear area of neutrophils is shown by P-ANCA, antinuclear antibody (ANA), and granulocyte specific antinuclear antibody (GS-ANA) which are difficult to differentiate by standard immunofluorescence on ethanol fixed neutrophils. It has been suggested in a broadsheet prepared by the Association of Clinical Pathologists that formalin acetone fixation followed by absolute ethanol is useful in differentiating P-ANCA from ANA.³ This additional step has not been confirmed by international consensus and there is controversy over its potential use in the routine diagnostic ANCA assay. Lee *et al* suggested that formaldehyde vapour fixation converts the perinuclear pattern of immunofluorescence to the cytoplasmic pattern when myeloperoxidase (MPO) antibodies were present, but found that formalin-acetone fixation gave inconsistent results.⁴ In earlier studies, Spickett and Broomhead found that formalin fixation of neutrophils did not add any useful information in differentiating P-ANCA from other autoantibodies.⁵ In the present study, we undertook a larger comparative study to assess the value of different techniques of formalin fixation of human neutrophils in the routine ANCA assay.

Methods

Seventy seven sera which had previously been received for detection of ANCA, ANA, and rheumatoid factors were collected from our routine diagnostic laboratory. These sera were reported previously as C-ANCA positive (15), P-ANCA positive (29), ANA positive (14), negative for ANCA by immunofluorescence (8), and positive for rheumatoid factor by particle agglutination (11). Previous reports of sera on ANCA and ANA were based on ethanol fixed human neutrophils and rat liver tissue, respectively. All sera were blinded and reanalysed by indirect immunofluorescence for ANCA on ethanol fixed neutrophils.

Heparinised venous blood was drawn from a healthy donor and neutrophils were separated by sedimentation with 6% dextran (Sigma). Neutrophil slides were prepared by cytocentrifugation (Shandon Cytospin), and ethanol fixation was carried out following the methodology described by Wiik.¹ Antinuclear antibody was detected by immunofluorescence using HEp2 cells (Biodiagnostics) as substrate. Bound IgG was detected using antihuman IgG fluorescein isothiocyanate (FITC) (Dako) at a dilution of 1 in 40. Sera were diluted 1 in 20 and 1 in 40 both for ANCA and ANA, as a

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Table 1 Relation between immunofluorescence patterns on different fixation methods, antigen specificity, and ANA reactivity

Diagnostic report	Ethanol	Formalin acetone	Formalin vapour	Formalin commercial	PR-3 positive	MPO positive	ANA positive on HEp2 cells
C-ANCA (15)	15-C	15-C	15-C	14-C	11	0	0
P-ANCA (29)	29-P	22-C	28-C	22-C	0	21	3
ANA (14)	12-P/N	8-C	9-C	8-C	0	0	14
RhF (11)	11-Neg	6-C	9-C	8-C	0	0	0
Negative (8)	8-Neg	4-C	8-C	8-C	0	0	0

ANA, antinuclear antibody; C, cytoplasmic; MPO, myeloperoxidase; Neg, negative; P, perinuclear; P/N, perinuclear, nuclear, or mixed; PR-3, proteinase 3; RhF, rheumatoid factor.
Number of samples in brackets.

Table 2 Immunofluorescence pattern by different formalin fixation methods

Diagnostic report	FA	FA	FA	FA	FV	FV	FV	FV	FC	FC	FC	FC
	C	P	Neg	Nucl	C	P	Neg	Nucl	C	P	Neg	Nucl
C (15)	15	0	0	0	15	0	0	0	14	0	1	0
P (29)	22	0	7	0	28	0	1	0	22	7	0	0
ANA (14)	8	0	3	3	9	0	2	3	8	0	1	5
RF (11)	6	0	5	0	9	0	2	0	8	0	3	0
Neg (8)	4	0	4	0	8	0	0	0	8	0	0	0

ANA, antinuclear antibody; C, cytoplasmic; FA, formalin acetone; FC, formalin commercial; FV, formalin vapour; Neg, negative; Nucl, nuclear; P, perinuclear; RF, rheumatoid factor.
Number of samples in brackets.

dilution less than 1:20 would not be considered clinically important in adults.

The presence of proteinase 3 and myeloperoxidase antibody was determined by enzyme linked immunosorbent assay (ELISA) using commercial ELISA kits (Shield Diagnostics). Two different formalin fixation techniques were used. Human neutrophils from the same donor were prepared using (1) formalin-acetone fixation followed by absolute ethanol as described by Lock,³ and (2) formaldehyde vapour fixation at room temperature for four minutes as described by Lee *et al.*⁴ Commercially prepared formalin fixed neutrophils (Biodiagnostics) were also used in the assay for comparison. Interpretation of the immunofluorescence patterns was performed by two different observers independently.

Results

Of the 15 C-ANCA positive samples, 11 were proteinase 3 positive and none was positive for MPO or ANA. All showed the cytoplasmic pattern on all formalin fixed neutrophils except one which became negative on the commercial formalin fixed neutrophil slide (table 1).

The degree of fluorescence was variable in different types of formalin fixed neutrophils and was somewhat reduced as compared with ethanol fixed neutrophils. Of 29 P-ANCA sera, all showed a perinuclear pattern when retested on ethanol fixed neutrophils, 21 were MPO positive, and three were ANA positive. Twenty two of 29 converted to the cytoplasmic pattern with formalin acetone and 28/29 with formalin vapour, although patchy fluorescence (some cells showing positive cytoplasmic pattern and some negative) and high background staining was a common problem. When tested on commercial formalin fixed neutrophils, 22 of 29 P-ANCA sera showed the cytoplasmic pattern, while seven of 29 retained the perinuclear pattern, failing to convert to cytoplasmic pattern. Of 14 ANA positive sera, 12 showed positive nuclear/perinuclear staining on ethanol, but all were positive for ANA on HEp2 cells. A weak

cytoplasmic reaction was observed in eight of 14, nine of 14, and eight of 14 on formalin acetone, formalin vapour, and commercial formalin fixed neutrophils, respectively (table 2). Remaining sera either retained variable nuclear stain or became negative on formalin fixed neutrophils. The remaining 19 sera (11 rheumatoid factor positive and eight negative sera for either pattern of ANCA) were negative on ethanol, HEp2, myeloperoxidase, and proteinase 3 ELISAs, but 10 of 19, 17 of 19, and 16 of 19 showed some cytoplasmic reaction on the different formalin fixed neutrophils, the remaining sera being negative.

Discussion

Cross linking reagents like formaldehyde form intermolecular linkage of free amino groups creating a network of linked antigens. It appears from this study that formalin fixation converts P-ANCA to C-ANCA pattern, irrespective of myeloperoxidase specificity—that is, non-MPO-P-ANCA also shows cytoplasmic staining. Therefore formalin fixation is not specific for myeloperoxidase autoantibodies. There is incomplete conversion of P-ANCA to C-ANCA pattern, as some of our sample retained "P pattern" on commercial fixed neutrophils. Patchy fluorescence (some cells showing positive cytoplasmic pattern and some negative) is also observed in many of our sera with formalin fixation. The fixation time is critical in formaldehyde fixatives as prolonged fixation removes the cytoplasmic and nuclear stain and insufficient fixation, on the other hand, fails to show the conversion.⁴ The reactivity of antibodies with cross linked proteins is commonly diminished or even absent.⁷ We tried formalin fixation with varying fixation conditions (time and temperature of fixation) but did not find any significant improvement (data not shown). The problems encountered with formalin fixation make the method difficult to standardise; hence results are inconsistent, as previously observed by Spickett and Broomhead.⁵ Patchy fluores-

cence, incomplete conversion, and variability of reactivity are well explained by the intrinsic problem associated with a cross linking fixative like formaldehyde. Lock has suggested that formalin fixation abolishes the antinuclear staining,³ but in our study we have found that nuclear reactivity is not completely abolished by formalin fixation, with many sera showing a positive cytoplasmic pattern or a nuclear pattern. Lee *et al* also observed weak cytoplasmic fluorescence using sera from patients with systemic lupus erythematosus, and they explained this as an increase in the background staining associated with formalin fixation.⁴

It is evident that formalin fixation does not effectively differentiate true P-ANCA from ANA. We agree there is a probability of coexistence of both antibodies in some cases, but in our study all the ANA positive samples were negative for proteinase 3 and myeloperoxidase. Most specificities of ANA are evident on ethanol fixed neutrophils, anti-dsDNA antibodies persist on formalin fixed cells, and ANA often coexist with an ANCA.⁸ Interestingly, rheumatoid factor positive, ANCA negative samples may also give a positive cytoplasmic pattern on formalin fixed neutrophils, indicating that formalin fixation may enhance autofluorescence or non-specific binding and give rise to increased false positivity. Although it was not investigated specifically in earlier studies, a positive C-ANCA reaction was seen in negative sera by some investigators on formalin fixed neutrophils.^{5,6} La Cour *et al* have shown that formalin acetone fixation always leads to an increase in autofluorescence in their experience.⁷ These observations are consistent with our present findings. The enhanced fluorescence caused by formalin fixation may result in false positivity not only in negative sera but also in ANA positive sera negative for my-

eloperoxidase antibodies and in the presence of other autoantibodies like rheumatoid factor.

In our experience formalin fixation for human neutrophils for ANCA assay will add diagnostic confusion because of its non-specific effects, inconsistency, and false positivity because of enhanced autofluorescence. Solid phase assays are expensive but they are reliable and complementary to immunofluorescence. We suggest that ANCA assay by immunofluorescence on ethanol fixed neutrophils should be cross checked with solid phase, antigen specific assays and HEP2 cells. This will prevent a misleading interpretation of antibodies that give selective or preferential reaction with the nucleus or perinuclear area of neutrophils. The additional data presented here confirm the unsuitability of formalin fixation for routine diagnostic use.

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