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, nonspecific 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.

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 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 method of Wiik. 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
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.\(^7\) 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 fluorescence...
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,1 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.1

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.6 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 myeloperoxidase 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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