

# Contribution of immunofluorescence to the identification and characterization of anti-neutrophil cytoplasmic autoantibodies. The role of different fixatives

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## Abstract

### Objective

*To study the sera from selected groups of antineutrophil cytoplasmic antibody (ANCA) positive patients by means of the indirect immunofluorescence test (ANCA-IIF) with different fixatives, in order to better discriminate among the various ANCAs (Ag-specificity and disease associations), especially those for which the antigen targets have not yet been identified.*

### Methods

Eighty pathological serum samples and 15 normal sera were evaluated. Pathological samples included sera from 30 ulcerative colitis (UC) ANCA positive patients, 30 P-ANCA/myeloperoxidase (MPO-ANCA) positive microscopic polyangiitis (MPA) patients, 10 C-ANCA/proteinase 3 (PR3-ANCA) positive Wegener's granulomatosis (WG) patients, and 10 antinuclear antibody (ANA) positive (ANCA negative) systemic lupus erythematosus (SLE) patients. ANCA were detected by IIF on ethanol, methanol and formalin-fixed granulocytes and by ELISAs specific for MPO, PR3, lactoferrin (LF) and bactericidal/permeability-increasing protein (BPI). Additionally, sera were tested for the presence of antinuclear antibodies on IIF.

### Results

96% of serum samples from UC patients, positive by IIF on ethanol-fixed granulocytes, became negative when tested on formalin-fixed neutrophil slides. On the contrary, 95% of sera from vasculitic patients showed a clear diffuse granular cytoplasmic pattern on the same substrate; sera from all 10 SLE patients did not show any reactivity when formalin was used as fixative. On methanol-fixed neutrophils, 100% of UC P-ANCA positive sera were positive with the same pattern versus only 20% of vasculitic P-ANCA positive (MPO positive). Methanol fixation had no effect on PR3-ANCA and ANA positive sera.

### Conclusion

*The comparison of IIF patterns of sera tested on different fixed cells may be useful to distinguish vasculitis-related P-ANCA versus ANA and vasculitis-related P-ANCA versus UC-related P-ANCA.*

### Keywords

ANCA, vasculitis, ulcerative colitis, immunofluorescence, fixatives.

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## Introduction

Antineutrophil cytoplasmic autoantibodies (ANCA) are considered to be sensitive and specific serological markers for Wegener's granulomatosis (WG) and related vasculitic syndromes (1, 2). With the indirect immunofluorescence technique (IIF) on ethanol-fixed human granulocytes, two different staining patterns can be identified: a diffuse granular cytoplasmic (C-ANCA) and a perinuclear/nuclear (P-ANCA) pattern. The former is mainly associated with one of the two major antigenic specificities, proteinase 3 (PR3), and the latter with myeloperoxidase (MPO). The P-ANCA pattern is an artifact of ethanol fixation, giving rise to the redistribution of positively charged antigens to the negatively charged cell nucleus. If indirect immunofluorescence is performed using neutrophils fixed in formalin instead of ethanol, MPO-ANCA produce a C-ANCA pattern.

Other neutrophil granule constituents such as elastase (ELA), cathepsin G (Cat-G), lactoferrin (LF), lysozyme (LYS),  $\beta$ -glucuronidase ( $\beta$ -Glu), azurocidin and bactericidal/permeability increasing protein (BPI) have also been described in some cases as antigenic targets (3-7). A developing new area of investigation concerns variants of ANCA not related to vasculitis (8). ANCA, mainly with a perinuclear/nuclear pattern, have been reported in rheumatic diseases (2, 9-11). Moreover, reports from several groups relate a new class of ANCA to inflammatory bowel diseases (IBD), particularly ulcerative colitis (UC) and primary sclerosing cholangitis (PSC) (12-15). The antigen specificity of such autoantibodies is as yet unknown, despite numerous attempts to identify it (16-18).

In addition to the ones already cited, suggested possible but not confirmed antigen targets in IBD, sclerosing cholangitis and autoimmune hepatitis are the following: actin (19), histones (20, 21) and the high molecular group proteins HMG1 and HMG2 (22, 23). Unfortunately, a diagnostic value has not been demonstrated for any of them, in contrast with the well documented diagnostic role for ANCA directed to PR3 and MPO in vasculitis (4, 5).

The key point concerning this new category of ANCA is that they have the

same or hardly distinguishable perinuclear/nuclear staining patterns on ethanol-fixed neutrophils as MPO-ANCA, but cannot be identified using antigen-specific ELISAs. Conflicting data have been reported concerning the behaviour of P-ANCA sera from patients with UC when granulocytes are formalin-fixed (18, 24-26): some groups have found cytoplasmic staining of the cells (18, 24), while others found that formalin fixation completely abolished staining (25, 26). Additional confusion arises for the following reasons:

- 1) Whereas most groups working on ANCA in vasculitis use ethanol for the alcohol fixation of granulocytes (17, 18, 25, 26), other groups involved in research on ANCA in IBD use methanol as the cell fixative (12, 27-29), which could cause different results and/or patterns.
- 2) Antinuclear antibodies (ANA) are capable of reacting with alcohol-fixed granulocytes and may be hard to distinguish from P-ANCA.

- 3) The presence of granulocyte-specific ANA (GS-ANA) has been described in the past (1, 2).

Preliminary data suggest that ANCA with different Ag specificities and different disease associations may behave differently when granulocytes for ANCA-IIF are prepared using various fixation techniques (26-30). Thus, the combination of different fixatives might help to better discriminate among the different variants of ANCA, especially those for which the antigenic targets have not yet been identified. The aim of our work was to study the behaviour of sera from selected and well characterized groups of ANCA positive patients by means of the ANCA-IIF test using ethanol, methanol and formalin fixation.

## Patients and methods

### Patients

80 pathological serum samples and 15 normal sera were evaluated. Sera were never heated before testing. Pathological samples included sera from 30 UC P-ANCA+ patients, 30 vasculitis P-ANCA+(MPO-ANCA+) MPA patients, 10 vasculitis C-ANCA+(PR3-ANCA+) WG patients, and 10 ANA+ (ANCA-) patients with systemic lupus erythematosus (SLE).

The diagnosis of UC was based on standard clinical, endoscopic and histologic criteria (31, 32). Vasculitis was diagnosed and classified according to the nomenclature of the Chapel Hill Consensus Conference (33). The diagnosis of SLE was made according to the ACR criteria (36).

#### Methods

For the IIF on ethanol-fixed purified normal human granulocytes, cells were obtained as previously described and ANCA were detected according to the standard procedure delineated at the first ANCA Workshop (4, 35).

#### *IIF on ethanol, formalin and methanol-fixed neutrophil substrate slides*

Commercially available slides, generously supplied by the producer (INOVA Diagnostic Inc, CA, USA distributed by MERIDIAN Diagnostic Europe, Villa Cortese, Italy), were used throughout the study. While the commercial preparations generally employed for ANCA-IIF test consist of human purified neutrophils or leukocytes cytocentrifuged onto glass slides, substrates were used where purified human neutrophils had been grown directly on slides for a very short time. In this case, the short-term cell culturing process utilized a cocktail of growth factors, hormones and interleukins to induce the cells to simultaneously adhere to and flatten onto the glass surface. After a specific amount of time in culture, the slides were washed and fixed using ethanol, methanol or a low concentration of formalin. No additional details about the method could be obtained. No apoptotic cells were detectable.

In addition we performed IIF experiments using home-made cytocentrifuged human granulocytes, prepared according to the standard procedure described above, as substrate, fixing them with absolute ethanol, absolute methanol and buffered formalin (9.25% formalin, 45% acetone in 2.2 mM phosphate buffer, pH 7.3) for 5' at -20°C, 10' at 4°C and 10' at RT, respectively.

#### *IIF on methanol-fixed neutrophil substrate slides pre-treated with DNase*

In order to study the effect of DNase on the ANCA-IIF results, the slides with cul-

tured neutrophils (Inova Diagnostic Inc.) were treated with DNase I from bovine pancreas (Sigma, St. Louis, MO, USA) before performing the standard IIF. Lyophilised DNase was dissolved in 1 ml 150 mM NaCl and diluted in 40 mM Tris-HCl pH 7.9, 10 mM NaCl, 6 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub> at a final concentration of 3 µg/ml. Slides were incubated with diluted enzyme or buffer alone for 30' at 37°C in a humid chamber (28). After washing with PBS 3 times for 5' each, the IIF was carried out as usual, using patient's sera or rabbit polyclonal antiserum to human MPO (Dakopatts, Denmark), to LF (SIGMA), to ELA and to CAT-G (Calbiochem-Novabiochem Corp, La Jolla, CA, USA) diluted 1:20, 1:400, 1:500, 1:200 and 1:500, respectively. Rabbit anti-hIgG-FITC (Dakopatts) and goat anti-rabbit IgG-FITC (Sigma) were used to detect the bound antibodies. As a control, in each experiment slides were also incubated with dilution buffer alone in the first step, and then treated as described above.

#### *Antigen-specific ELISAs (MPO, PR3, LF)*

Myeloperoxidase was purchased from Calbiochem and lactoferrin (human milk) from Sigma; PR3 was purified as previously described (5, 36). The same ELISA method with minor modifications was used in the different assays as previously described (4,5). Results were expressed as arbitrary units (AU/ml) by reading off a standard curve and the normal range for each test was established as the mean plus 2 SD calculated from 100 normal blood donors.

#### *Antigen-specific ELISAs using ethanol and methanol as coating diluent*

In order to study the effects of ethanol

and methanol on the binding of sera to purified ANCA Ags, the ELISA method was modified as follows: the different ANCA Ags (PR3, MPO, LF) were coated onto ELISA microtiter plates using ethanol or methanol as the coating diluent. The ethanol and methanol were allowed to evaporate by incubation overnight at 4°C. Apart from that, the ELISA was performed as usual.

The binding of 4 monospecific patient sera and monoclonal Abs to MPO named 9.31, M748, 4.27, 9.21, 13.16 and 4.15 (kindly provided by Dr. R. Goldschmeding) towards ethanol and methanol treated Ags was compared.

#### *ANCA and ANA detection*

BPI-ANCA were detected by ELISA using a commercially available kit (G.E.N.E.S.I.S. Diagnostic, Cambridgeshire, UK). ANA were detected by indirect immunofluorescence using HEp2 cells as substrate (Inova Diagnostics Inc.).

## Results

#### *Indirect IF*

The results of IIF using the different fixatives in comparison with the standard procedure are summarized in Table I. Similar results were obtained using either "home-made" cytocentrifuged human granulocytes or commercially available slides.

Out of the 30 (96%) serum samples from UC patients positive on ethanol-fixed granulocytes, 29 became negative when tested on formalin-fixed neutrophil slides. On the contrary 38/40 (95%) sera from vasculitis patients showed a clear diffuse granular cytoplasmic pattern on the same substrate; sera from SLE patients did not show any reactivity when formalin was used as fixative. On methanol-fixed neu-

**Table I.** Results of indirect immunofluorescence using different fixatives (sera from patients with different diseases).

	Ethanol	Formalin	Methanol
Ulcerative colitis	30/30 P	1/30 C	30/30 P
Vasculitis MPO+	30/30 P	29/30 C	6/30 P
Vasculitis PR3+	10/10 C	9/10 C	9/10 C
Systemic lupus erythematosus	10/10 P	0/10	10/10 P
Normals	0/15	0/15	0/15

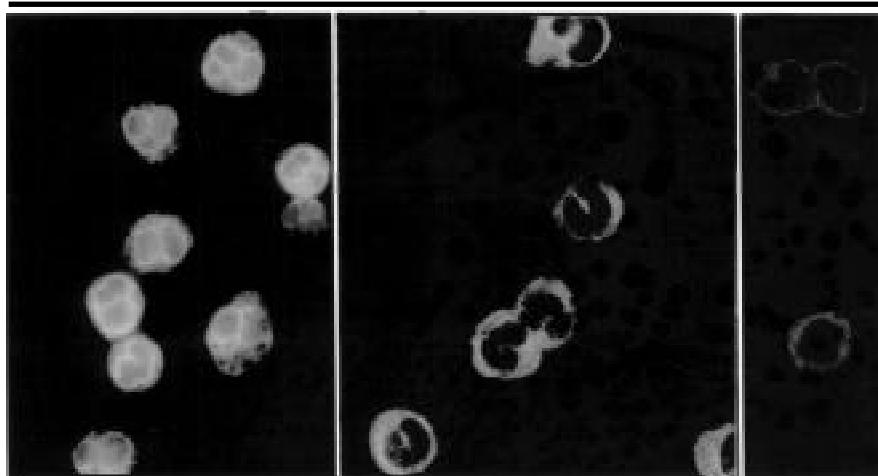
trophils 30/30 (100%) of the UC P-ANCA+ sera were positive with the same pattern versus only 6/30 (20%) of the vasculitic P ANCA+/MPO-ANCA+

sera (Fig. 1, Fig. 2). Interestingly enough, one of these 6 sera was ANA positive and 2 were anti-lactoferrin positive (Table II).

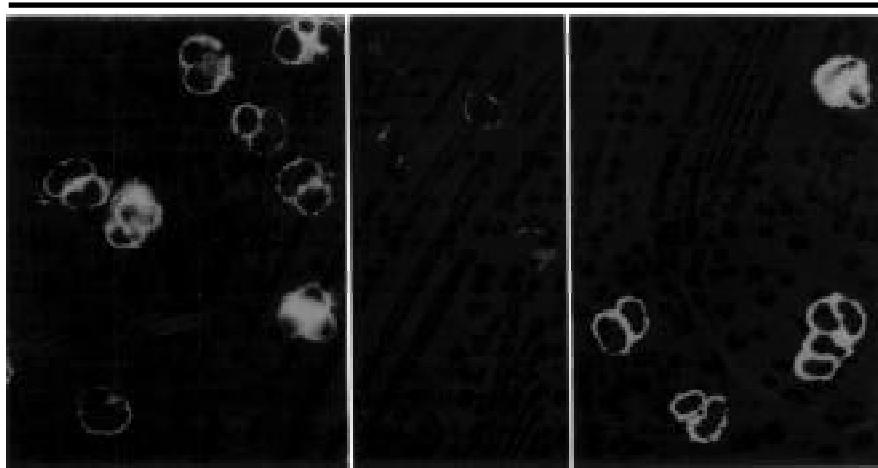
**Table II.** Features of the only six vasculitis patients who were positive by indirect immunofluorescence also on methanol-fixed granulocytes.

Patient	Indirect immunofluorescence			MPO-ANCA		Notes
	Ethanol	Methanol	Formalin	ELISA (NV	15 a.u.)	
1	P	P	C	1140		ANA+
2	P	P	C	102		LF+
3	P	P	C	200		
4	P	P	neg.	80		No reactivity on formalin-fixed cells
5	P	P	C	80		LF+
6	P	P	C	44		Nuclear staining*

\* In addition to perinuclear staining there was intense nuclear fluorescence on granulocytes.



**Fig. 1.** Vasculitic P-ANCA+/MPO-ANCA+ serum: IIF on ethanol, formalin and methanol-fixed substrates (from left to right).



**Fig. 2.** UC P-ANCA positive serum: IIF on ethanol (left), formalin (middle) and methanol-fixed (right) slides.

Methanol fixation had no effect on the PR3-ANCA and ANA positive sera. DNase treatment of methanol-fixed granulocytes before IIF completely abolished the reactivity of the ANA positive sera and of P-ANCA from UC patients, but had no effect on the PR3 positive sera. Control buffer treatment did not significantly influence the staining.

#### ELISAs

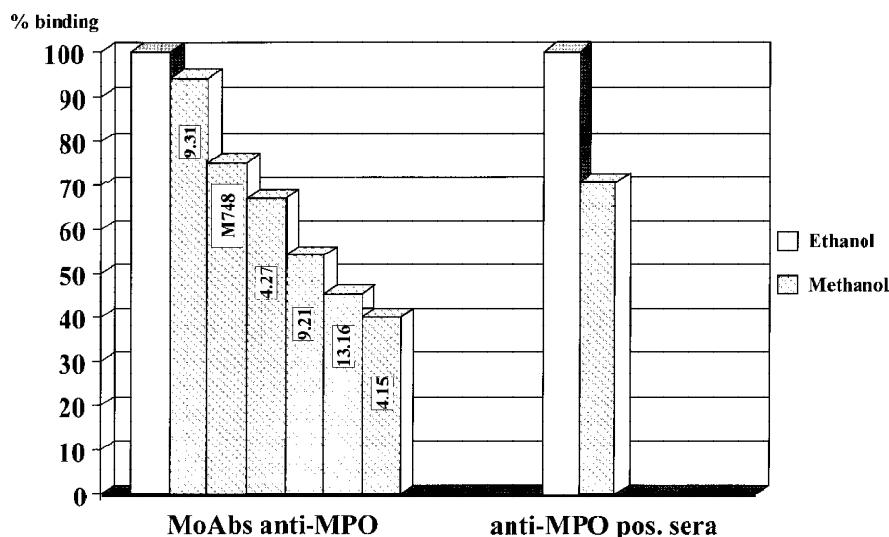
The solid phase assays performed using as antigens PR3, MPO, LF and BPI confirmed previous findings: only a minority of the UC-ANCA+ sera were positive on Ag-specific ELISAs. All 30 UC sera were negative for MPO-ANCA, 1 and 3 were positive at borderline levels for PR3-ANCA and LF-ANCA, respectively, and 5 of them contained low levels of autoantibodies to BPI (data not shown).

When methanol was used as the coating diluent for the MPO-ANCA ELISA, the binding of MPO positive sera (4) and of some of the MoAbs to MPO was significantly ( $p < 0.05$ ) reduced in comparison to ethanol (Fig. 3). Similar results were obtained with PR3 whereas methanol had no effect on the results of the LF-ANCA ELISA (Fig. 4).

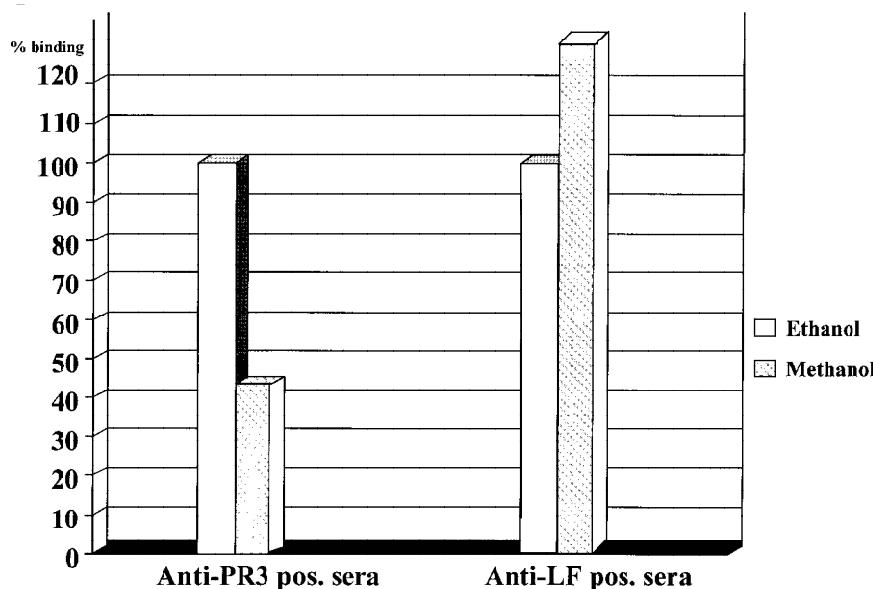
#### Discussion

Indirect immunofluorescence, utilising ethanol-fixed human neutrophils as substrate, is still the method of choice for detecting ANCA. Recently, the addition of PR3 and MPO Ag-specific ELISAs to the IIF ANCA assay has been found to significantly improve the diagnostic specificity for vasculitis of the ANCA results (5, 36-38) and should be used routinely. Unfortunately, for a number of ANCA, mainly P-ANCA positive sera, the antigenic targets have not been identified yet. This is particularly true for patients with rheumatic and inflammatory bowel diseases, and therefore we have to rely on the IIF results alone in these categories of patients.

In our study we found that by utilising different fixatives we were able to distinguish among ANCA with different Ag specificities and disease associations, and between "true" ANCA and anti-nuclear antibodies. Indeed, the vast majority of P-ANCA/MPO-ANCA positive



**Fig. 3.** Binding reduction of MPO positive sera (mean of 4) or MoAbs to different epitopes of MPO (9.31, M748, 4.27, 9.21, 13.16, 4.15) to the ethanol vs methanol-coated ELISA microplates.



**Fig. 4.** Differences in binding of PR3 positive and LF positive sera (mean of 4 for each specificity) to the ethanol versus methanol-coated plates in ELISA assays.

sera changed, as expected, their pattern of reactivity from P to C when the cells were fixed with formalin, and became negative when methanol was used as fixative. On the contrary, P-ANCA positive sera from UC patients remained positive with methanol and turned out to be negative with formalin. There were a few exceptions, which could be explained by other findings such as the concomitant presence of ANA and of anti-lactoferrin antibodies.

The different behavior of P-ANCA vasculitic sera from that of P-ANCA UC sera could be due to the nature of the

antigen involved or, even more likely, to the localization of the Ag itself (26, 28). P-ANCA UC sera had almost the same behaviour as the ANA positive sera, raising questions about the true localization of their target Ags. In fact, they were negative in our study using formalin-fixed cells and ANA sera. Formalin fixation has, however, yielded different and/or inconsistent results in other laboratories, probably due to technical differences such as formalin concentration, the presence/absence of an association with acetone, incubation time etc. (8). Recently, evidence has accumulated regard-

ing the possible nuclear localization of UC-associated P-ANCA Ag on the inner side of the nuclear periphery in association with nuclear DNA (heterochromatin). This finding would appear to indicate that DNase pre-treatment of cell-slides abolished the reactivity of P-ANCA from UC patients (26, 28, 29). Why MPO positive sera become negative when methanol was used as fixative is not clear. We found that the use of methanol instead of ethanol as the coating diluent significantly decreased the binding to MPO-coated plates and that this was probably due to a denaturing effect of this alcohol. The possibility that methanol might have had some negative effects on the efficiency of MPO coating is ruled out by the fact that the different MoAbs had a broad range of specific reactivity (from 40% to 93%). However, a similar denaturing effect of methanol was also found for other ANCA Ags such as PR3, which behaved differently than MPO when ANCA-IIF was performed using methanol as fixative. However, one can hypothesize that since PR3 is not redistributed around the nucleus, its antigenicity might be better preserved than that of MPO when performing IIF. Unfortunately, nothing can be said about the behaviour of other ANCA Ags, such as elastase and cathepsin G, since no sufficiently positive sera were available for these Ags. However, our *in vitro* results suggest that these Ags could have the same behaviour as MPO, since they are also redistributed around the nucleus with alcohol fixation.

In conclusion, our results suggest that performing the ANCA-IIF test, with the use of different fixatives and well-standardized methodology, can help to differentiate among the different types of ANCA and between ANCA and ANA. In particular, P-ANCA from patients with UC has a different behaviour than MPO-ANCA. Moreover, their sensitivity to the DNase treatment of cells, along with their behaviour using formalin and methanol fixation, suggest a possible nuclear (or nuclear-associated) origin of their antigenic target.

Whether the different methods of fixation are of clinical use for the distinction between ANCA in vasculitis and ANCA in UC remains to be ascertained.

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