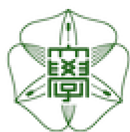

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Original article

Neutrophil fixation protocols suitable for substrates to detect anti-neutrophil cytoplasmic antibodies by indirect immunofluorescence

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A R T I C L E I N F O

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A B S T R A C T

Anti-neutrophil cytoplasmic antibodies (ANCAs) are autoantibodies that recognize neutrophil cytoplasmic antigens. The major ANCA antigens are myeloperoxidase and proteinase 3. Necrotizing small vessel vasculitis accompanied by ANCA production is called ANCA-associated vasculitis (AAV). In addition to AAV, ANCA is sometimes produced in patients with connective tissue diseases, such as systemic lupus erythematosus, and inflammatory bowel diseases. Indirect immunofluorescence (IIF) and enzyme immunoassay (EIA) have been used to detect ANCAs. Recently, the accuracy of EIA has improved and it has become the gold standard for ANCA detection. However, IIF does not lose its role in ANCA detection because EIA cannot detect ANCAs that recognize antigens other than those coated on the plate. For IIF, neutrophil substrates prepared with two different fixations, namely, ethanol fixation and formalin fixation, are used. There is a recommended protocol for ethanol fixation but not for formalin fixation. This study prepared neutrophil substrates according to the recommended protocol for ethanol fixation and protocols in the literature and original protocols for formalin fixation and then examined ANCA specificity and how storage period would influence the number of cells, antigen distribution, and antigenicity of the substrates. As a result, the number of cells and antigen distribution did not change after storage for up to 2 months regardless of fixation protocols, whereas a time-dependent decline in ANCA antigenicity and a fixation protocol-dependent difference in ANCA specificity were observed. How neutrophils are fixed on the glass slide needs to be checked upon evaluation of ANCAs by IIF.

1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCAs) are autoantibodies that recognize neutrophil cytoplasmic antigens. The major ANCA antigens are myeloperoxidase (MPO) and proteinase 3 (PR3). Necrotizing small vessel vasculitis accompanied by ANCA production is called ANCA-associated vasculitis (AAV) [1]. AAV includes microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA).

MPA develops rapidly progressive glomerulonephritis with glomerular capillary necrosis and crescent formation and, in the lungs, pulmonary hemorrhage due to capillaritis. A nationwide cohort study in Japan has demonstrated that the average age of MPA onset is in the 70s and that most patients produce MPO-ANCA [2]. GPA shows necrotizing granulomas in the upper and lower respiratory tracts and necrotizing granulomatous vasculitis in the systemic small vessels, with a predilection for patients in their 60s. There is an almost equal prevalence of MPO-ANCA and PR3-ANCA in GPA. EGPA is a systemic necrotizing granulomatous vasculitis associated with allergic symptoms, such as bronchial asthma and eosinophilia, with a peak incidence in the 50s. About half of EGPA patients are positive for MPO-ANCA, but others do not possess ANCA.

There are AAV patients in whom neither MPO-ANCA nor PR3-ANCA is detectable. In such cases, neutrophil cytoplasmic antigens other than MPO and PR3—so-called minor antigens—including bactericidal permeability increasing protein, neutrophil elastase, cathepsin G, lactoferrin, and lysosome-associated membrane protein 2, are recognized by ANCAs [3,4]. In addition to AAV, ANCA is detected in patients with connective tissue diseases, such as systemic lupus erythematosus, and inflammatory bowel diseases (IBD) [5]. In these non-AAV diseases,

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ANCAs often recognize minor antigens.

Indirect immunofluorescence (IIF) and enzyme immunoassay (EIA) have been applied for ANCA detection. IIF is an examination in which neutrophils derived from the peripheral blood of healthy subjects are fixed on glass slides, allowed to react with test sera followed by fluorescence-labeled anti-human IgG antibodies, and observed under a fluorescence microscope. Although it is a qualitative and subjective test, IIF can detect the presence of ANCAs independent of antigen specificity. In contrast, EIA is a quantitative and objective examination that measures the reactivity of solid-phase neutrophil cytoplasmic antigens with antibodies in test sera using enzyme-labeled anti-human IgG antibodies. Recently, the accuracy of EIA has improved and it has become the gold standard for ANCA detection. Today, MPO-ANCA and PR3-ANCA can be measured routinely. The limitation of EIA is that it cannot detect ANCAs that recognize antigens other than those coated on the plate. Therefore, IIF and EIA compensate for the shortcomings of each other in ANCA detection.

For IIF, neutrophil substrates prepared with two different fixations are subjected to determine the presence of ANCAs. One is ethanol fixation, and the reactivity of ANCAs can be divided into two patterns, namely, perinuclear pattern called the p-ANCA pattern and cytoplasmic pattern called the c-ANCA pattern. The major antigen of p-ANCAs is MPO, although some ANCAs that recognize minor antigens also display the p-ANCA pattern. The major antigen of c-ANCAs is PR3. The other is formalin fixation, in which both MPO-ANCA and PR3-ANCA demonstrate the c-ANCA pattern. The positively charged antigens, such as MPO, approach the negatively charged nuclear membrane during relatively slow fixation by ethanol compared to rapid and tight fixation using formalin [6].

When ethanol-fixed neutrophils are employed as substrates, it is sometimes difficult to determine p-ANCA when the test serum contains anti-nuclear antibody (ANA). However, ANCAs detected as p-ANCAs in ethanol-fixed neutrophils exhibit the c-ANCA pattern in formalin-fixed neutrophils, whereas ANA is not detectable when formalin-fixed neutrophils are used as substrates. Therefore, when p-ANCA cannot be determined in IIF with ethanol-fixed neutrophils as substrates due to the presence of ANA, it is necessary to apply formalin-fixed neutrophils as IIF substrates.

Some patients with IBD possess the anti-neutrophil antibody that recognizes an intranuclear antigen [7]. This antibody is not detected as typical p-ANCA in IIF with ethanol-fixed neutrophils as substrates. When formalin-fixed neutrophils are used as IIF substrates, a rim-like staining of the nuclear periphery is detected [8]. Therefore, when atypical ANCA is detected in the sera of IBD patients in IIF with ethanol-fixed neutrophils as substrates, it is necessary to apply formalin-fixed neutrophils as substrates, too.

The preparation protocol of ethanol-fixed neutrophils as IIF substrates has been established by Wiik [9], whereas diverse protocols, including Lock's protocol [10] and Billing's protocol [11], have been applied to prepare formalin-fixed neutrophil substrates. Although critical problems may not be present in these protocols, the standardization of methods is desirable for data comparison between institutions. To qualify ANCA-IIF substrates, it is important to maintain the number of cells, antigen distribution, and antigenicity for a while. This study prepared neutrophil substrates according to Wiik's protocol for ethanol fixation and Lock's and Billing's protocols and original protocols for formalin fixation and then examined the influence of the storage period on the cell numbers, antigen distribution, and antigenicity of the substrates.

2. Materials and methods

2.1. Serum

Sera containing MPO-ANCA ($n = 3$), PR3-ANCA ($n = 3$), and ANA ($n = 1$) and sera that do not contain ANCA ($n = 5$) were kindly provided from Medical & Biological Laboratories (MBL; Tokyo, Japan). These sera were positive and negative controls included in commercially available ANCA-IIF kits. The EIA titers of MPO-ANCA and PR3-ANCA were determined in MBL.

2.2. Peripheral blood neutrophils

Peripheral blood was obtained from healthy volunteers, and polymorphonuclear leukocytes (PMNs) were isolated by density gradient centrifugation using Polymorphprep (Axis-Shield, Dundee, Scotland). Because most cells recovered as PMNs were neutrophils, PMNs were used as neutrophils in this study.

2.3. Preparation of neutrophil substrates

Neutrophils were suspended in RPMI-1640 supplemented with 10% fetal bovine serum (1×10^6/ml), seeded onto 12-well poly-L-lysine-coated glass slides, and incubated for 30 min at 37 °C in 5% CO_2 humidified atmosphere. After removing the supernatants, glass slides that bound neutrophils were washed with phosphate-buffered saline (PBS) and air-dried immediately. Then, the glass slides were subjected to ethanol or formalin fixation. Thereafter, the glass slides were sealed with desiccant and stored at 4 °C until use.

2.4. Ethanol fixation

Ethanol fixation was carried out according to Wiik's protocol [9]. In brief, glass slides that bound neutrophils were immersed in 95% ethanol for 5 min at 4 °C and air-dried immediately (ethanol fixation protocol 1: e1).

2.5. Formalin fixation

Formalin fixation was carried out according to Lock's protocol [10], Billing's protocol [11], and the original protocols, namely, paraformaldehyde (PFA)-PBS, PFA-ethanol, and PFA-acetone protocols (Table 1).

Lock's protocol (formalin fixation protocol 1: f1): Glass slides that bound neutrophils were immersed in formalin-acetone (36 ml formalin, 180 ml acetone, and 184 ml PBS) at room temperature (RT) for 50 s, immersed in absolute ethanol at 4 °C for 15 min, and air-dried immediately.

Billing's protocol (formalin fixation protocol 2: f2): Glass slides that bound neutrophils were immersed in 1% PFA at RT for 10 min, immersed in acetone at −20 °C for 1 min, and air-dried immediately.

PFA-PBS protocol (formalin fixation protocol 3: f3): Glass slides that bound neutrophils were immersed in 4% PFA at RT for 15 min, rinsed with PBS, and air-dried immediately.

PFA-ethanol protocol (formalin fixation protocol 4: f4): Glass slides that bound neutrophils were immersed in 4% PFA at RT for 15 min, immersed in absolute ethanol at 4 °C for 1 min, and air-dried immediately.

PFA-acetone protocol (formalin fixation protocol 5: f5): Glass slides that bound neutrophils were immersed in 4% PFA at RT for 15 min, immersed in acetone at −20 °C for 1 min, and air-dried immediately.

Table 1
Protocols employed in this study.

Protocol	1st step	2nd step	Reference
e1	95% ethanol, 5 min, 4 °C	–	[9]
f1	formalin-acetone, 50 s, RT	100% ethanol, 15 min, 4 °C	[10]
f2	1% PFA, 10 min RT	Acetone, 1 min, −20 °C	[11]
f3	4% PFA, 15 min, RT	PBS	Original
f4	4% PFA, 15 min, RT	100% ethanol, 1 min, 4 °C	Original
f5	4% PFA, 15 min, RT	Acetone, 1 min, −20 °C	Original

RT, room temperature; PFA, paraformaldehyde; PBS, phosphate-buffered saline.

2.6. IIF

After the glass slides were settled at RT, a 1:20 dilution of sera containing MPO-ANCA, PR3-ANCA, and sera that do not contain ANCA was applied to each well and incubated at RT for 30 min. The ready-to-use ANA serum was applied similarly. After washing with PBS, fluorescein isothiocyanate (FITC)-conjugated anti-human IgG antibodies containing Evans blue dye were applied to each well and incubated at RT in the dark for 30 min. After washing with PBS, the glass slides were mounted with a mounting solution containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). ANCA IIF was carried out in quadruplicates, and the reproducibility of results was confirmed. Each well was observed and photographed using a fluorescence microscope. The number of cells, antigen distribution, and cellular fluorescence intensity that represented antigenicity were measured using the captured images. Image analysis was performed using ImageJ software version 1.53a (https://imagej.nih.gov/ij/; NIH, Bethesda, MD).

2.7. Statistics

The decrease in the number of cells and antigenicity on days 14, 30, and 60 compared to those on day 1 was analyzed statistically using one-way analysis of variance or Student's *t*-test. $p < 0.05$ was regarded as significant.

3. Results

3.1. Cell numbers

Neutrophils that adhered to the glass slides were fixed according to Wiik's protocol for ethanol fixation (e1), Lock's protocol for formalin fixation (f1), Billing's protocol for formalin fixation (f2), PFA-PBS protocol for formalin fixation (f3), PFA-ethanol protocol for formalin fixation (f4), and PFA-acetone protocol for formalin fixation (f5). Neutrophil substrates were stored at 4 °C until use and applied to ANCA-IIF on the day after substrate preparation (day 1), 2 weeks later (day 14), and 1 month later (day 30). The number of cells in the low-power field of view was counted, and the results were displayed as the relative values when the cell number on day 1 was set as 100 (Fig. 1). Cell numbers were maintained for up to 1 month regardless of fixation protocols. Observation under a high-power field of view confirmed the good condition of neutrophils that adhered to the glass slides.

3.2. Antigen distribution

Next, alteration in antigen distribution under each condition was evaluated. Using neutrophil substrates with ethanol fixation (e1), MPO-ANCA and PR3-ANCA demonstrated the p-ANCA and c-ANCA patterns, respectively, similar to days 1, 14, and 30 (Fig. 2A). Using neutrophil substrates with formalin fixation (f1–f5), both MPO-ANCA and PR3-ANCA exhibited the c-ANCA pattern, similar to days 1, 14, and 30 (Fig. 2B–F). Nonspecific reaction with the control sera that do not contain ANCA was minimal.

3.3. Antigenicity

Protocols for ethanol and formalin fixation employed in this study (e1 and f1–f5) did not affect the cell numbers and antigen distribution in ANCA-IIF after storage for up to 1 month. However, there was a decrease in the cellular fluorescence intensity, which represented the decline in antigenicity, over time depending on the fixation protocols (Fig. 3A). The mean fluorescence intensity (MFI) per cell on day 1 was set as 100, and the relative values on days 14 and 30 were analyzed. As a result, the MFI per cell of MPO-ANCA on day 14 in Billing's protocol (f2) and PR3-ANCA on day 14 in PFA-ethanol protocol (f4) was lower than 50% of the values on day 1 (Fig. 3B).

3.4. Cell numbers and antigen distribution after long-term storage

Concerning ANCA-IIF substrates that demonstrated less than 50% decrease in MFI per cell after storage for up to 1 month (e1, f1, f3, and f5), the storage period was extended by another month and the influence on the cell numbers and antigen distribution was examined. There was no significant alteration in the cell numbers and antigen distribution on day 60 (Fig. S1).

3.5. Antigenicity after long-term storage

Similarly, the decline in antigenicity was examined on day 60 concerning ANCA-IIF substrates that demonstrated less than 50% decrease in MFI per cell after storage for up to 1 month (e1, f1, f3, and f5). Using neutrophil substrates fixed with Wiik's protocol (e1), PFA-PBS protocol (f3), and PFA-acetone protocol (f5), the MFI per cell of MPO-ANCA and PR3-ANCA on day 60 was more than 50% of that on day 1, although the antigenicity declined significantly in e1 and f5 during the 2 months of storage. In contrast, using neutrophil substrates fixed with Lock's protocol (f1), the MFI per cell of MPO-ANCA on day 60 was less than 50% of that on day 1 (Fig. 4).

3.6. ANA in IIF with neutrophil substrates

ANA was detected when neutrophils fixed with Wiik's protocol (e1) and Billing's protocol (f2) were used as substrates but not when neutrophils fixed with Lock's protocol (f1), PFA-PBS protocol (f3), PFA-ethanol protocol (f4), and PFA-acetone protocol (f5) were used as substrates (Fig. S2).

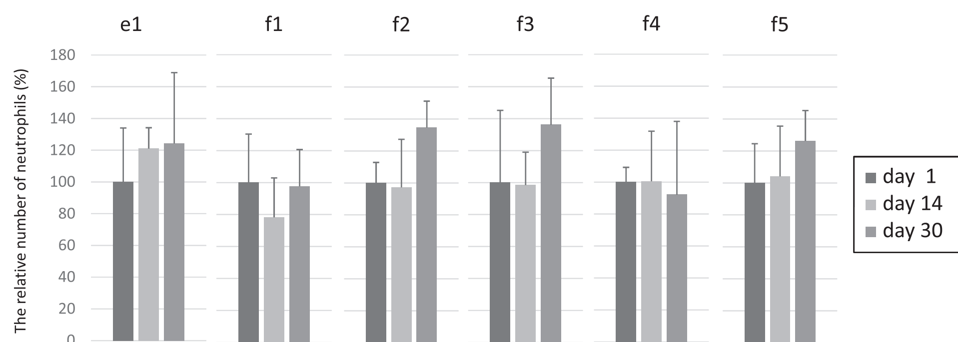


Fig. 1. Number of neutrophils that remained on the glass slides. Each well of the glass slides where neutrophils were allowed to adhere was photographed under a low-power field of view (magnification, ×100), and the number of cells was counted. The columns demonstrate the mean of four to five wells and the error bar represents the standard deviation (SD). Cells that remained in the wells after 2 weeks (day 14) and 1 month (day 30) are represented as the relative values by setting the number of cells on the day after substrate preparation (day 1) as 100. Cell numbers were maintained for up to 1 month regardless of fixation protocols. e1, Wiik's protocol; f1, Lock's protocol; f2, Billing's protocol; f3, PFA-PBS protocol; f4, PFA-ethanol protocol; f5, PFA-acetone protocol.

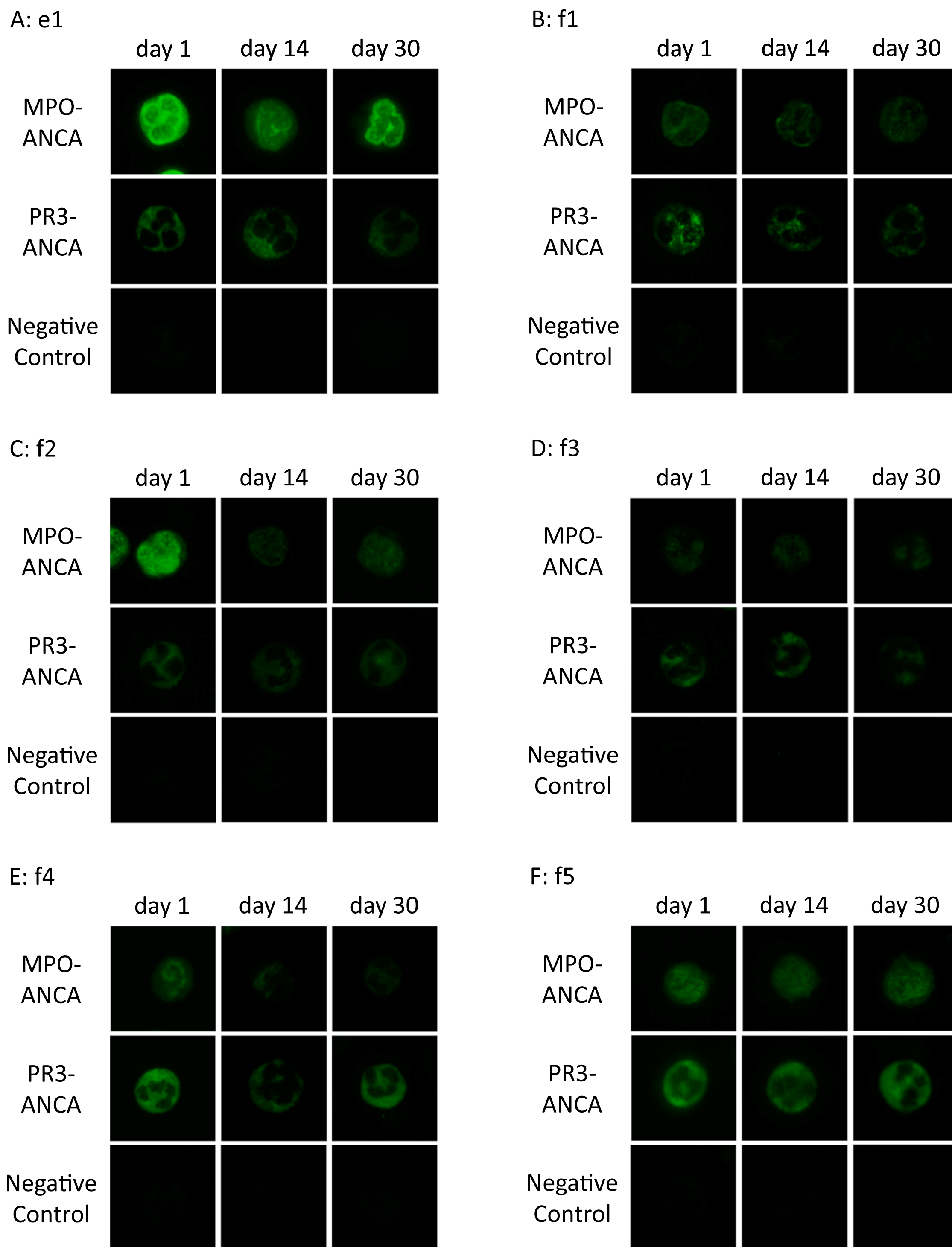
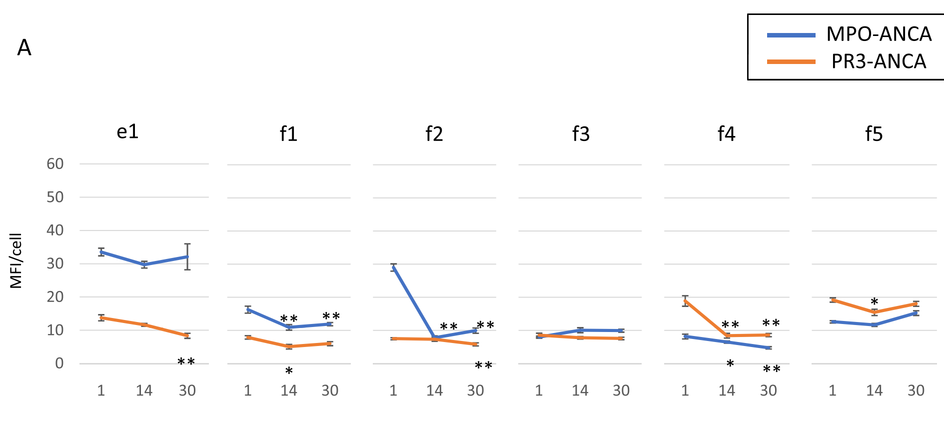


Fig. 2. Distribution of ANCA antigens. IIF was performed using the prepared neutrophil substrates. The substrates were allowed to react with a 1:20 dilution of sera containing MPO-ANCA and PR3-ANCA and sera that do not contain ANCA followed by FITC-conjugated anti-human IgG antibodies. Using neutrophil substrates prepared according to Wiik's protocol for ethanol fixation (e1), MPO-ANCA and PR3-ANCA demonstrated the p-ANCA and c-ANCA patterns, respectively, similar to days 1, 14, and 30 (A). Using neutrophil substrates prepared according to Lock's protocol (f1; B), Billing's protocol (f2; C), PFA-PBS protocol (f3; D), PFA-ethanol protocol (f4; E), and PFA-acetone protocol (f5; F) for formalin fixation, both MPO-ANCA and PR3-ANCA exhibited c-ANCA pattern, similar to days 1, 14, and 30. Representative micrographs, using MPO-ANCA and PR3-ANCA with an EIA titer of 13.0 and 135.0 U/ml, respectively, are shown.

4. Discussion

ANCA is an autoantibody discovered by Davies et al. in 1982 [12]. Among small- and medium-sized vasculitis diagnosed as periarteritis nodosa (PN), a group of diseases characterized by ANCA production was identified, and a small vessel vasculitis called AAV was isolated from PN. ANCA is not only a disease marker for AAV but also a pathogenic autoantibody that plays an important role in its pathogenesis [13].

It was recommended that ANCA should be assessed by a combination of IIF and EIA or by screening with IIF first and then measuring antibody titer with EIA (1999 consensus) [14]. EIAs commercialized in the early days varied in sensitivity and specificity depending on the suppliers and



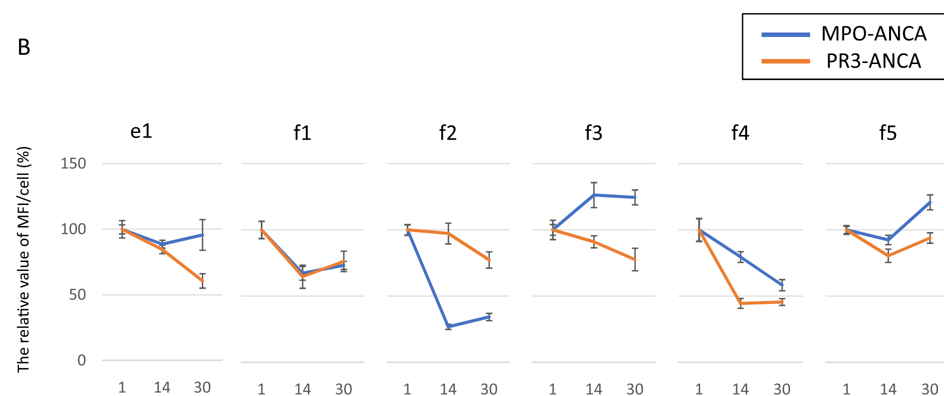


Fig. 3. Retention of antigenicity. MFI per cell was measured using images taken under a high-power field of view (magnification, ×400) in IIF (A). The dots demonstrate the MFI of cells (3–65/image), and the error bar represents the standard error (SE). The decrease in antigenicity on days 14 and 30 compared to that on day 1 was analyzed statistically. $*p < 0.05$, $**p < 0.01$. The relative values were calculated for days 14 and 30 when the MFI per cell on day 1 was set as 100 (B). The MFI per cell of MPO-ANCA on day 14 in Billing's protocol (f2) and PR3-ANCA on day 14 in PFA-ethanol protocol (f4) was lower than 50% of the values on day 1. e1, Wiik's protocol; f1, Lock's protocol; f2, Billing's protocol; f3, PFA-PBS protocol; f4, PFA-ethanol protocol; f5, PFA-acetone protocol.

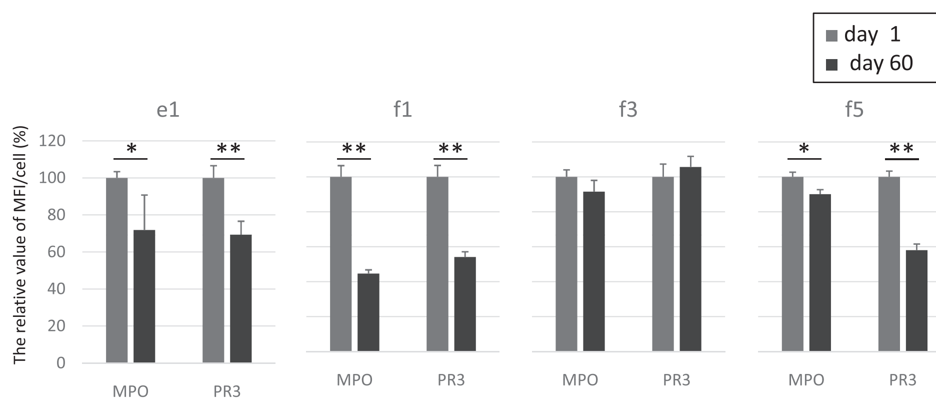


Fig. 4. Retention of antigenicity after long-term storage. MFI per cell was measured using images taken under a high-power field of view (magnification, ×400) in IIF. The relative values were calculated for day 60 when the MFI per cell on day 1 was set as 100. The columns demonstrate the MFI of cells (3–65/image) and the error bar represents the SE. e1, Wiik's protocol; f1, Lock's protocol; f3, PFA-PBS protocol; ff5, PFA-acetone protocol.

thus were used as auxiliary examinations. However, with the improvement of the antigen solid-phase technique and the development of more sensitive detection systems, the accuracy of EIAs is now as good as or better than that of IIF. Based on this current situation, the 2017 consensus recommends first screening with EIA when AAV is clinically suspected, and IIF does not need to be performed if MPO-ANCA or PR3-ANCA is found to be present [15]. If AAV is clinically suspected but EIA does not show a clear positive finding, IIF is recommended.

In this study, besides Wiik's protocol recommended for ethanol fixation (e1) and Lock's and Billing's protocols for formalin fixation (f1 and f2), ANCA-IIF substrates were prepared with three original protocols using 4% PFA, namely, PFA-PBS protocol (f3), PFA-ethanol protocol (f4), and PFA-acetone protocol (f5). The concentration of PFA was adopted as 4%, which is commonly used for cell fixation today.

IIF was performed on the day after substrate preparation (day 1), 2 weeks later (day 14), and 1 month later (day 30), and the obtained images were analyzed. As a result, no decrease in the number of cells or alteration in antigen distribution was observed regardless of fixation protocols. In contrast, the MFI of MPO-ANCA in f2 and that of PR3-ANCA in f4 dropped to less than half over time, which means a decline in antigenicity. When the storage period was extended to 2 months, more than half reduction in MPO-ANCA antigenicity was also evident in f1. Regarding the decline in antigenicity, it has not been determined what factors contributed to the difference between MPO-ANCA and PR3-ANCA. This is an important subject that should be clarified in future studies. Anyway, because ANCA-IIF determines the antibody titer semiquantitatively using serial 1:2 dilutions of serum, a decrease in fluorescence intensity of more than 50% may affect the

antibody titer determination.

In this study, e1 for ethanol fixation and f3 and f5 for formalin fixation were superior in maintaining the antigenicity. However, f3 had a disadvantage of lower MFI per cell than f5 from day 1. Microscopic observation of neutrophil substrates prepared with f3 revealed scattered precipitation of salts in the wells, and some neutrophils overlapped with the precipitated salts. It is possible that the cell membrane was damaged by PBS-derived crystals formed during the air-drying process, which caused the antigens in the cytoplasm to flow out of the cells, resulting in low MFI per cell when performing IIF.

From another point of view, it is important that ANA is not detected in IIF with formalin-fixed neutrophils as substrates to distinguish p-ANCA from ANA. Regarding this issue, formalin fixation protocols examined in this study yielded satisfactory results, except for Billing's protocol (f2). Based on collective findings, PFA-acetone protocol (f5) is the best recommendation among the formalin fixation protocols examined in this study, although the sensitivity appears to be inferior to Wiik's protocol (e1). It will be necessary to revise the protocol to enhance the sensitivity in future studies.

This study suggested that there is a recommended protocol for ethanol fixation but not for formalin fixation because of the problem of maintaining antigenicity. There are two basic procedures to fix cells and tissues: the precipitation and fixation of proteins by strong dehydration and lipid dissolution using organic solvents, such as methanol and acetone, and the cross-linking method using aldehydes, such as formaldehyde and glutaraldehyde. Formalin fixation aims to prevent protein denaturation by clothing the protein with formaldehyde by the dissociation of existing bonds in the protein, binding of formaldehyde to the end of amino acid residues, and methylene cross-linking to deform the three-dimensional structure of the protein. Therefore, a certain degree of decrease in antigenicity is unavoidable. Among the original protocols for formalin fixation, the PFA-acetone protocol (f5) demonstrated relatively good retention of antigenicity because the protein precipitation effect of acetone may have favored the retention of antigenicity.

There are two limitations to this study. First, sera that contained ANCAs that recognize antigens other than MPO and PR3 were not examined. Therefore, how the fixation protocols and the storage period would influence the detection of ANCAs that recognize minor antigens remains elusive. The other is that the effect of a storage period longer than 2 months was not examined. Further studies are needed to determine whether neutrophil substrates prepared by each fixation protocol can withstand a storage period longer than 2 months.

In the present study, neutrophil substrates for ANCA-IIF were prepared according to diverse fixation protocols, including Wiik's protocol for ethanol fixation and Lock's protocol, Billing's protocol, and three original protocols for formalin fixation, and the influence of the storage period on the number of cells, antigen distribution, and antigenicity of the substrates was investigated. The number of cells and antigen distribution did not change after storage for up to 2 months regardless of fixation protocols, whereas ANCA antigenicity decreased with time depending on the fixation protocols. Investigators should care how neutrophils are fixed on the glass slide upon evaluation of ANCAs by IIF.

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Author contributions

Y.N. and A.I. designed the research. Y.N., S.M., and Y.S. performed the research. Y.N. and A.I. wrote the paper. All authors discussed the results and contributed to the preparation of the manuscript.

Ethics approval

This study was conducted with the permission of the Ethics Committee of the Faculty of Health Sciences, Hokkaido University (Permission No. 19-86).

Data availability statement

The data underlying this article will be shared at reasonable request to the corresponding author.

Declaration of competing interest

The authors report no declarations of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2021.153661.

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