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Use of Immunofluorescence and Confocal Laser Scanning Microscopy in Identifying Rare Cases of Anti-Neutrophil Cytoplasmic Antibodies (ANCA) Showing Dual Specificities to Myeloperoxidase and Proteinase3

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Anti-neutrophil cytoplasmic antibodies (ANCA) are the immunodiagnostic markers for idiopathic necrotizing crescentic glomerulonephritis affecting mainly medium to small sized blood vessels. The diagnosis of ANCA associated vasculitis (AAV) is mainly based on clinical and histopathological characteristics along with the serological evidence. Immunofluorescence microscopy (IIF) is considered as the "gold standard" for ANCA detection, and ANCA showing two major patterns ie, cytoplasmic (c-ANCA) and perinuclear (p-ANCA) react with different antigenic targets of neutrophils like Proteinase3 (PR3) and Myeloperoxidase (MPO). A third unusual and rare immunofluorescence pattern called as "X-ANCA" or atypical ANCA is also sometimes seen. The difficulty in identification of ANCA immunofluorescence patterns is mainly seen due to the rare dual patterns seen in the same sera and also the additional nuclear immunofluorescence seen due to presence of anti-nuclear antibodies. ANCA testing by immunofluorescence and Confocal Laser scanning microscopy, as well as by specific ELISAs for detection of anti-PR3 and anti-MPO antibodies have helped in improving the diagnosis. Patients having dual specificities to MPO and PR3 in a patient is a rare finding. Among 425 clinically and histopathologically proven cases of AAV, eight patients (1.9%) had dual specificities, of which five patients showed mixed immunofluorescence pattern and 3 patients showed X-ANCA pattern which was confirmed by both immunofluorescence and Confocal Laser scanning microscopy and the dual specificities to MPO and PR3 were detected by individual ELISAs.

KEY WORDS: Anti-neutrophil cytoplasmic antibodies (ANCA); indirect Immunofluorescence test (IIF); enzyme linked immunosorbent assay (ELISA); anti-myeloperoxidase (anti-MPO); anti-Proteinase3 (anti-PR3).

INTRODUCTION

Vasculitis is an inflammation of blood vessel walls and is part of a group of inflammatory disorders with diverse clinical manifestations. The clinical and pathological features of vasculitis are variable and mainly depend on the size and type of blood vessel affected and the final

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List of abbreviations: 'α granules': Alpha granules; AAV: ANCA associated vasculitis; ANA: Anti-nuclear antibodies; ANCA: Anti-neutrophil cytoplasmic antibodies; Anti-dsDNA: Anti-double stranded DNA; Anti-GBM: Anti-glomerular basement membrane; Anti-MPO: Anti-Myeloperoxidase; Anti-PR3: Anti-Proteinase 3; BVAS: Birmingham Vasculitis Activity Score; c-ANCA: Cytoplasmic ANCA; CWG: Classical Wegener's granulomatosis; ELISA: Enzyme linked immunosorbent assay; FITC: Fluorescein isothiocyanate; ICGN: Idiopathic crescentic glomerulonephritis; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IIF: Indirect immunofluorescence test; LN: Lupus nephritis; LSM: Laser scanning microscope;

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diagnosis is mainly based on clinical and histopathological characteristics along with serological evidence [1,2]. Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies which are reactive with neutrophil cytoplasmic granules and have valuable immunodiagnostic and disease monitoring potential in patients with Wegener's granulomatosis, renal limited vasculitis, and some other forms of necrotizing and/or idiopathic crescentic glomerulonephritis (ICGN) [3,4].

ANCA show two major immunofluorescence patterns by immunofluorescence and Confocal Laser scanning microscopy, the classical cytoplasmic (c-ANCA) immunofluorescence pattern having the serine protease, Proteinase 3 (PR3) as the major antigenic target. Anti-PR3 antibodies are found in nearly 90% of patients with active Wegener's granulomatosis (WG) who show upper and lower respiratory tract involvement along with kidney involvement in few cases [5]. Also the perinuclear or "p-ANCA" immunofluorescence pattern on ethanol fixed polymorpho nuclear cells (PMN) is also seen which is mainly directed against Myeloperoxidase (MPO), but sometimes to other cytoplasmic antigens as well. Antibodies to MPO (anti-MPO) are mainly associated with Microscopic polyangiitis (MPA), ICGN and other forms of vasculitis. A third rare immunofluorescence pattern called as "X-ANCA" or the atypical pattern is also sometimes seen on ethanol fixed neutrophils and causes some difficulty in interpretation of ANCA patterns due to the dual patterns shown by the same sera and also sometimes due to also an additional nuclear fluorescence present in some sera [6].

The immunofluorescence and Confocal Laser scanning microscopy are basically screening assays for ANCA detection while specificity is identified by ELISAs for either MPO or PR3, and to other specificities like Lactoferrin (LF), Cathepsin G (CG), Elastase etc. However, mixed patterns of immunofluorescence having dual specificities to MPO and PR3 in the same serum are occasionally seen [7]. ANCA testing by both IIF and ELISA has greatly helped in diagnosing and management of AAV patients. In the present study patients showing the dual specificities of ANCA were identified during ANCA screening on suspected "pauci-immune" vasculitis cases with renal and systemic involvements, and these samples were taken up separately for an in depth study. The strength of individual antibodies, immunoglobulin profile, and their correlation with disease expression was studied. The Birmingham Vasculitis Activity Score (BVAS) is a clinical index of disease severity and is useful to evaluate several forms of necrotizing vasculitis. This score is based on signs and symptoms in nine separate organ systems, and clinical features are only scored if they are attributable to active vasculitis. This clinical activity index allows assessment of organ involvement in vasculitis, where the cumulative scores provide useful standards by which to assess serological markers, and the need for further therapy [8].

MEM: Minimal essential medium; MPA: Microscopic polyangiitis; MPO: Myeloperoxidase; NCCS: National Centre for Cell Sciences; OD: Optical density; p-ANCA: Perinuclear ANCA; PMN: Polymorpho nuclear cells; PR3: Proteinase3; SD: Standard deviation; U/mL: Units/mL; WG: Wegener's granulomatosis; X-ANCA: Atypical ANCA.

MATERIAL AND METHODS

Clinically suspected cases of "pauci-immune" small vessel vasculitis were referred by clinicians from the Nephrology and Medicine units of some major public hospitals in Mumbai. This specific study was carried out after obtaining the requisite Ethics Committee permission. Five ml of clotted blood was aseptically collected and the separated serum was stored at -20°C. Relevant clinical, histopathological and laboratory findings were recorded in a proforma. Classification criteria for systemic vasculitis was as per those published by the American College of Rheumatologists [9]. Confirmation of the diagnosis of renal vasculitis necessitated renal biopsies examined by light microscopy and was done by staining slides with Hematoxalin, Eosin and Periodic Acid Schiff (PAS) and also by immunofluorescent microscopy using anti-IgG, anti-IgM, anti-IgA, anti-Complement3, anti-Complement4 and anti-fibrinogen FITC conjugates on renal tissues in 10% buffered formalin and preparing 3 μm thick sections from paraffin embedded blocks.

The identification of ANCA was done by IIF screening test which is considered as the "Gold standard" for ANCA screening [10] using PMN as well as a human promyelocytic leukemic cell line (HL-60) obtained from NCCS (Pune) and maintained in Minimal Essential Medium (MEM) as a continuous culture and harvested at log phase of growth. The cells were used to prepare substrate slides using Hettich Universal 16A cytocentrifuge, and were fixed with 96% ethanol, also, some slides were fixed with formalin, before being treated with patient's sera, and were probed using FITC tagged polyvalent anti-human globulin serum using a fluorescent microscope, Nikon, Optiphot II, Japan. Microphotography was done using an automated photography system, Nikon AFX II A, also Confocal Laser scanning microscopy (LSM, 510, Carl Zeiss, Germany) was used [11], to reexamine the slides under higher clarity and magnification, and image rotation on X, Y, or Z axis, which gave a very clear picture showing the immunofluorescence staining patterns. Ten control sera having specificities to MPO and others to

PR3 were a gift from scientists from Germany, Denmark and Hongkong were used as required.

Anti-neutrophil cytoplasmic antibodies (ANCA) were also detected by a rapid ELISA using ultrasonicated purified neutrophil cytoplasm extract called as "α granules" as per method described by Rasmussen *et al*. [12]. Briefly the "α granule" ELISA is described as follows. The ultrasonicated PMN extract after purification was estimated for protein concentration at 260 nm and 280 nm and was adjusted to 50 μg/mL. Round bottomed microtiter plates (Nunc, Denmark) were coated with 100 μL of "α granules" and after incubation and blocking, test sera were reacted and the reaction was read using alkaline phosphatase conjugated anti-human globulin at 405 nm after adding 2 mg/mL p-nitro phenyl phosphate (Sigma, USA). A cutoff using normal human serum + 2SD was considered as positive.

Specificity of the antibodies were identified by antigen binding ELISAs for anti-Myeloperoxidase (MPO) and anti-Proteinase3 (PR3) using kits from Genesis (UK). A value <3.0 u/mL was negative, 3–5 u/mL was equivocal and >5 u/mL were considered as positive. Fluid phase soluble inhibition experiments were done on sera showing dual specificities. These sera were diluted 1:50 and were individually preincubated with MPO (Sigma, USA, 10 μg/mL) and PR3 (Pharmacia, Germany, 0.7 μg/mL) for one hour at 37°C. and were tested by IIF microscopy and on individual ELISAs using the same protocols. These antigen concentrations were determined by dose response, where concentration of antigen that gives more than 80% inhibition of the positive standard serum at 1:50 dilution. Inhibition was considered to have occurred when the difference in units/mL was greater than 10% between the inhibited and uninhibited test sample [8]. These sera were also tested for non specific binding on plates coated with human albumin instead of antigen. The percentage inhibition was calculated using the following formula [13].

$$\% \text{ Inhibition} = ((\text{OD}_{\text{without inhibitor}} - \text{OD}_{\text{with inhibitor}})/(\text{OD}_{\text{without inhibitor}})) \times 100.$$

Anti-nuclear antibodies (ANA) were qualitatively and quantitatively tested by IIF test using HEp-2 cells obtained from Enterovirus Research Center, ICMR, Mumbai. Cells were maintained in a continuous culture and harvested at log phase of growth. The results were interpreted in terms of titers i.e. sera showing immunofluorescence at highest dilution. A cutoff for positivity was 1:10 dilution of test serum for ANCA and ANA testing [14,15]. Sera giving a positive immunofluorescence were tested thrice for confirmation of patterns, and were also confirmed using a Confocal Laser scanning microscope which gives better clarity of images as they can be enlarged and rotated on the screen for better viewing. Anti-double stranded DNA antibodies (anti-dsDNA) were tested by ELISA [16].

RESULTS

Totally, eight sera from patients with crescentic glomerulonephritis were studied, comprising of 5 cases showing mixed immunofluorescence pattern and 3 cases showing atypical immunofluorescence patterns. These cases were identified during screening of a large number of samples for ANCA associated "pauci-immune" vasculitis, where 425 clinically and histopathologically proven cases of AAV were investigated, and an incidence of 1.9% of dual specificities was noted. These eight patients had initially shown a very confusing immunofluorescence pattern which was neither the typical cytoplasmic immunofluorescence nor the perinuclear immunofluorescence. However, Confocal Laser scanning microscopy and the X, Y and Z axis rotation of the captured image gave a clear picture of dual fluorescence, suggesting the presence of two distinct patterns of immunofluorescence and also the ELISA showed presence of anti-MPO and anti-PR3 antibodies.

Of the five cases (Cases 1–5) (Table I), of which first three cases were having only renal manifestations and the 4th and 5th cases, were the "Classical" Wegener's granulomatosis (CWG) type having a triad of renal, upper respiratory tract and lower respiratory tract involvement showed a "mixed pattern" by IIF microscopy using ethanol fixed neutrophils, when further tested on formalin fixed neutrophils, showed a cytoplasmic c-ANCA immunofluorescence pattern. The mixed immunofluorescence patterns showed a strength of 1:80–1:320 titers and by ELISA, showed higher values of 20–40 units/mL for anti-MPO and lower values of 5–18 units/mL for anti-PR3. The same phenomenon was seen with the other 3 cases showing atypical pattern of immunofluorescence, which were different from the mixed dual immunofluorescence pattern seen in the first 5 cases which was due to the presence of ANA as an additional antibody.

The "α granules" broad spectrum screening ELISA also showed positive results with all eight cases which by specific ELISAs showed presence of both anti-MPO and anti-PR3 antibodies. The dual antibody specificities to MPO and PR3 was also confirmed by significant soluble inhibitions or neutralization of activity using soluble MPO and PR3 purified antigens as inhibitors. The immunoglobulin subclass of ANCA was mainly IgG, but in one CWG case (4), it was IgG + IgM. The BVAS was lower in patients with only renal manifestations while it was higher in patients with renal along with other clinical manifestations. BVAS and ANCA titers were higher in

Table I. ANCA in Patients with Dual Specificities

Case No. name (age/sex)	Diagnosis (BVAS)	ANCA by IIF			ANCA by ELISA			% Soluble inhibition		ANA/ anti-dsDNA
		IIF pattern	Ethanol (mixed)	Formalin (cytoplasmic)	Anti-MPO	Anti-PR3	IgG class	By MPO	By PR3	
RS (18/M)	MPA (18)	Mixed	1 : 160	1 : 80	40	18	IgG	20	31	Neg/Neg
KK (21/M)	MPA (18)	Mixed	1 : 160	1 : 160	40	15	IgG	73	50	Neg/Neg
GG (50/M)	ICGN (18)	Mixed	1 : 80	1 : 80	10	8	IgG	46	75	Neg/Neg
AW (30/M)	CWG (31)	Mixed	1 : 320	1 : 320	20	5	IgG + M	55	35	Neg/Neg
SB (35/F)	CWG (28)	Mixed	1 : 320	1 : 320	25	5	IgG	60	25	Neg/Neg
RR (45/F)	LN (24)	Atypical	1 : 80	1 : 80	18	12	IgG	22	30	1:160/77
VS (62/F)	LN (20)	Atypical	1 : 160	1 : 80	40	15	IgG	72	28	1:160/98
SS (65/F)	MPA (20)	Atypical	1 : 160	1 : 160	15	5	IgG	27	78	1:80/Neg

CWG patients with upper and lower respiratory tract involvement.

The second group comprised of three patients (Cases 6–8) where cases 6 and 7 were clinically and histopathologically proven Class IV lupus nephritis (LN) having focal proliferative glomerulonephritis (FPGN) with crescents and the 8th case was a case of anti-GBM disease. The two patients of LN had ANA and anti-dsDNA antibodies as well and on clinical examination showed that they had renal, skin, URT and joint involvement and had the atypical pattern of ANCA along with the nuclear fluorescence. On formalin fixed PMNs, these cases showed a cytoplasmic pattern as well as a bright nuclear fluorescence. Existence of the dual specificity to MPO and PR3 antigens in them was later identified by ELISA. Antibodies to Lactoferrin (LF) and Cathepsin G (CG) were absent in these patients, where as anti-glomerular basement membrane (anti-GBM) antibodies were seen in two cases (Case 4 and case 8).

DISCUSSION

Normally ANCA's show two distinct and separate immunofluorescence patterns on neutrophils during screening by immunofluorescence microscopy, the diffuse granular immunofluorescence staining of the cytoplasm showing the perinuclear pattern (p-ANCA) and the classical cytoplasmic staining called as c-ANCA pattern. The mixed pattern of immunofluorescence which is a combination of p-ANCA and c-ANCA and also the "atypical" or X-ANCA immunofluorescence pattern are rarely seen and are difficult to interpret on immunofluorescence microscopy, though the Confocal Laser scanning microscopy shows good clarity of the mixed immunofluorescnce patterns [17]. The perinuclear immunofluorescence pattern is observed when neutrophils are fixed with ethanol, as the MPO granules in the cytoplasm get deposited around the nucleus due to solubilization of cytoplasm with ethanol and cause an artifactually distorted distribution of MPO thus showing the perinuclear immunofluorescence pattern. However after formalin treatment they get immobilized and fixed in the cytoplasm itself and express as a cytoplasmic pattern [18,19].

Detection of ANCA by indirect immunofluorescence is considered as the "Gold Standard" screening assay, but identification of various specificities of ANCA is possible with use of solid phase binding assays using various purified antigens or by using commercial kits. Some earlier reports on dual specificities have also mentioned that the existence both anti-MPO and anti-PR3 antibodies, in the same sera, is a rare finding and can be detected only by individual ELISAs [20,21].

The European ANCA assay standardization project [22], had provided useful information from their evaluation of IIF and ELISA methods for detection of ANCA where they concluded that combining the immunofluorescence test for c-ANCA and p-ANCA along with ELISA testing for anti-PR3 and anti-MPO antibodies respectively is more accurate than either IIF or ELISA alone, We too observed that it is a more useful combination for diagnosing AAV patients where a confirmed positive ANCA result becomes highly diagnositc for "pauci immune" crescentic glomerulonephritis.

Applying BVAS to these 8 cases has helped in differentiating the more severe cases having renal involvement along with upper respiratory tract (URT), lower respiratory tract (LRT), joint or skin involvement. The identification of dual specificity cases is interesting, and shows that, probably the dual specificity of ANCA may have developed due to clone specific challenges and in some cases may be also due to polyclonal activation, however many factors could have played a role. Inhibition studies using purified MPO and PR3 have shown antigen specific varying antibody inhibition separately and singularly in these cases.

Our study has identified 1.9% of ANCA positive sera showing dual specificities among 425 cases investigated for ANCA and 3.5% (8/227) among all ANCA positives. Hauer *et al*, observed that both anti-MPO and anti-PR3 antibodies existing in the same serum, though rare is seen in cases of WG and MPA [23]. Similar findings were noted in our study, where dual specificities were noted in cases of WG, MPA, class IV LN and anti-GBM patients. However Teeratkul *et al*. observed a higher incidence of 23% for dual specificities in ANCA positive patients [24]. It has been also been reported that polyreactivity exists between ANCA specificities, and other nuclear autoantibodies, where results obtained only by IIF may be further strengthened to identify the presence of ANA showing fluorescence as this is often confused with p-ANCA pattern [25]. In such type of cases the interpretation often becomes difficult and one may have to resort to Confocal Laser scanning microscopy which may help to clear the doubts.

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