Anti-Neutrophil Cytoplasmic Auto Antibodies (ANCA) in Pakistani Patients with Systemic Vasculitides

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Tahir A. Ahmed, Iftikhar A. Malik (Armed Forces Institute of Pathology, Rawalpindi.)
Ashfaq A. Khan, Syed T. A. Shah, Ahsan Hameed, Sahibzada A. Haleem (Military Hospital, Rawalpindi.)
Abid Farooqui, Safia J. Amin (Pakistan Institute of Medical Sciences, Islamabad.)

Abstract

Anti-neutrophil cytoplasmic auto antibodies are directed against antigens in the neutmphil granules. Their detection by the indirect immunofluorescence clearly divides them into two distinct types, namely c-ANCA (Classical antineutrophil cytoplasmic antibody) and the p-ANCA (Perinuclear antineutrophil cytoplasmic antibody). These antibodies have been found to be useful as non-invasive markers to help establish the diagnosis in patients with systemic vasculitides. The antibodies also help in monitoring disease activity in some patients with systemic necrotising vasculitides. This study was aimed at demonstrating the utility of these auto-antibodies in the management of our patients presenting with systemic vasculitides. Fifty-six patients presenting with features of systemic vasculitides were examined over a sixmonth period out of whom eight were found positive for these antibodies. The detection of these antibodies helped in early diagnosis and the institution of specific treatment. Six months follow-up in one patient with Wegener\'s granulomatosis, the disease activity related closely with the ANCA levels JPMA 44:272,1994).

Introduction

Anti-neutrophil cytoplasmic auto antibodies (ANCA) were first reported by Davies et al[1] in eight patients suffering from haematuria and arthralgias or myalgias. Later, van der Would et al[2] reported the detection of these antibodies in 25 of the 27 serum samples from patients suffering from active Wegener\'s granulornatosis. In a prospective study in 19 patients with Wegener’s granulomatosis they were able to show that these antibodies also related to the underlying disease activity. Subsequent studies demonstrated that these antibodies were associated specifically not only with Wegener\'s granulomatosis but they were also demonstrable in the sera of the patients suffering from idiopathic crescentic glomerulonephritis, micropolyartentis, polyarteritiis nodosa and other systemic vasculitides[3-5]. The method of detection remained indirect immunofluorescence though ELISA and later RIA were developed[6,7]. The target antigens were identified as the myeloperoxidase enzyme for the p-ANCA and proteinase 3 for the c-ANCA. The nomenclature was standardised as c (classical) and p (perinuclear) patterns of staining[3,8]. Later it was demonstrated that these antibodies were also targeted against other neutrophil constituents in a small subgroup of patients suffering from systemic vasculitides. A number of studies have shown that c-ANCA is specifically related to Wegener’s granulomatosis with a good direct relationship with the disease activity. The specificity of this marker for active Wegener\'s granulomatosis is shown to be more than 95% and the sensitivity in the range of 60-70%[9,10]. The direct and the specific relationship with the disease activity was utilised in one study in the form of the institution of specific immune suppression based on ANCA titres. The results of this study indicated that this approach may result in ultimately decreased requirements for iminunosuppression in patients with Wegener’s granulomatosis[11]. The p-ANCA was found to be useful in the management of patients with idiopathic necrotising crescentic glomerulonephritis where the immune deposits are minimal and it is difficult to establish the diagnosis. Though the disease activity is not related to the p6-ANCA

levels[12,13], the underlying immuno pathogenic mechanisms remain to be fully elucidated. The p-ANCA auto antibodies have been shown to induce neutrophil degranulation and oxidative burst activity in in-vitro studies[14].

Material and Methods

Patients

The patients were selected by treating physicians from the departments of dermatology, rheumatology and nephrology out patients with features of systemic necrotising vasculitis. Serum specimens were collected and sent to AFIP, Rawalpindi, which were tested as soon as possible to provide the required information to the concerned physicians.

ANCA Test

ANCA was measured in the patients serum using the standardised indirect immune fluorescence technique as recommended in the First International Workshop on ANCA which was held in Copenhagen (Denmark), in 1988. The human neutrophils were used as substrate[15].

Cell Substrate Preparation

Blood was collected in heparinised containers ensuring 10-15 units of sodium heparin per ml of blood. It was then gently mixed with dextran 70 and incubated at room tempera ture for 45 minutes. The leucocyte rich plasma was siphoned off and the count adjusted to 1000 neutrophils per micro litre of the medium RPMI 1640 (Sigma)+5% Foetal calf serum (FCS flow) afterX2 washes in the same medium. The cells were then smeared on to the glass slides using cytospin II (Shandon) at 900 RPM for 5 minutes. They were then immediately fixed in absolute ethanol at 4°C for 5 minutes. These cell smears were prepared in batches and stored at -20°C for later use, for upto 2 weeks.

Indirect Intmunofluorescence

The neutrophil smears were taken out of storage at -20°C, brought to the room temperature and the patients serum was layered on to the smears at 1:10 dilution in phosphate buffered saline (PBS Flow). The unbound fraction was washed off after 20 minute incubation in PBS. Goat anti-human IgG conjugated with Fluorescein isothiocyanate (FITC Binding Site) was then used to detect patients antibodies sticking on to the neutrophils. Two smears were made on each slide and a normal human serum pool was used as a negative control on each slide, along-side each patient's serum. Positive control was provided by Prof Dumonde and Mr. Swana from St. Thomas' Hospital, London. In addition, this test was included in the UK National Quality Assurance Scheme (NEQAS) for autoimmune serology for external quality control. All specimens which were found to be positive on screening were tested again for titration. The formalin fixed neutrophils and He p 2 cell were used to differentiate between c- ANCA and the p-ANCA patterns of staining and to exclude all the results due to the anti-nuclear antibodies. The results were read immediately upon completion of the test procedure with the help of Nikon microscope with a 50 watt mercury lamp as UV source.

Table I. The patterns of staining for c-ANCA and p-ANCA as observed after the indirect immunofluorescence staining.

Pattern of staining	Alcohol fixed neut.	Formalin fixed neut.	Hep 2 cells
c-ANCA	Cytoplasmic	Cytoplasmic	Negative
p-ANCA	Perinuclear	Cytoplasmic	Negative
Anti-nuclear	Nuclear/perinuclear	Nuclear/perinuclear	Nuclear

c-ANCA= Classical anti-neutrophil cytoplasmic antibody
p-ANCA= Perinuclear antineutrophil cytoplasmic antibody
Neut.= Neutrophils

The scheme outlined in Table I was used to differentiate between different patterns of staining. The c-ANCA pattern of staining is characteristically confined to the cytoplasm with somewhat central accentuation (Figure 1).

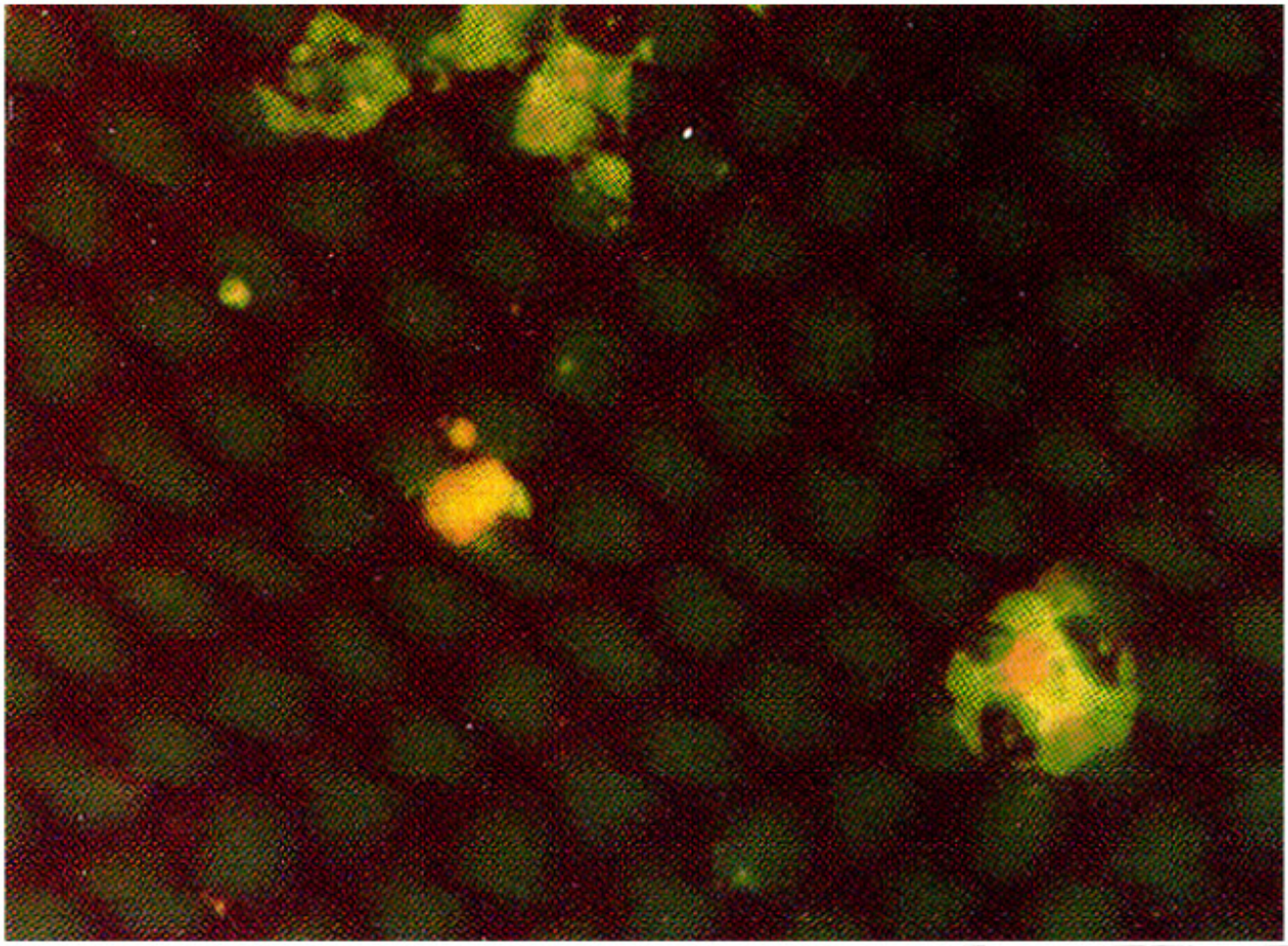

Figure 1. c-ANCA pattern of staining as seen on human neutrophils (magnification 40X).

The p-ANCA pattern of staining is confined around nucleus. The nucleus itself and the cytoplasm remain devoid of any staining. It is absolutely essential that a combination of alcohol fixed neutrophils, formalin fixed neutrophils and Hep-2 cells are used to differentiate it from the common anti-nuclear antibody and the c-ANCA (Figure 2).

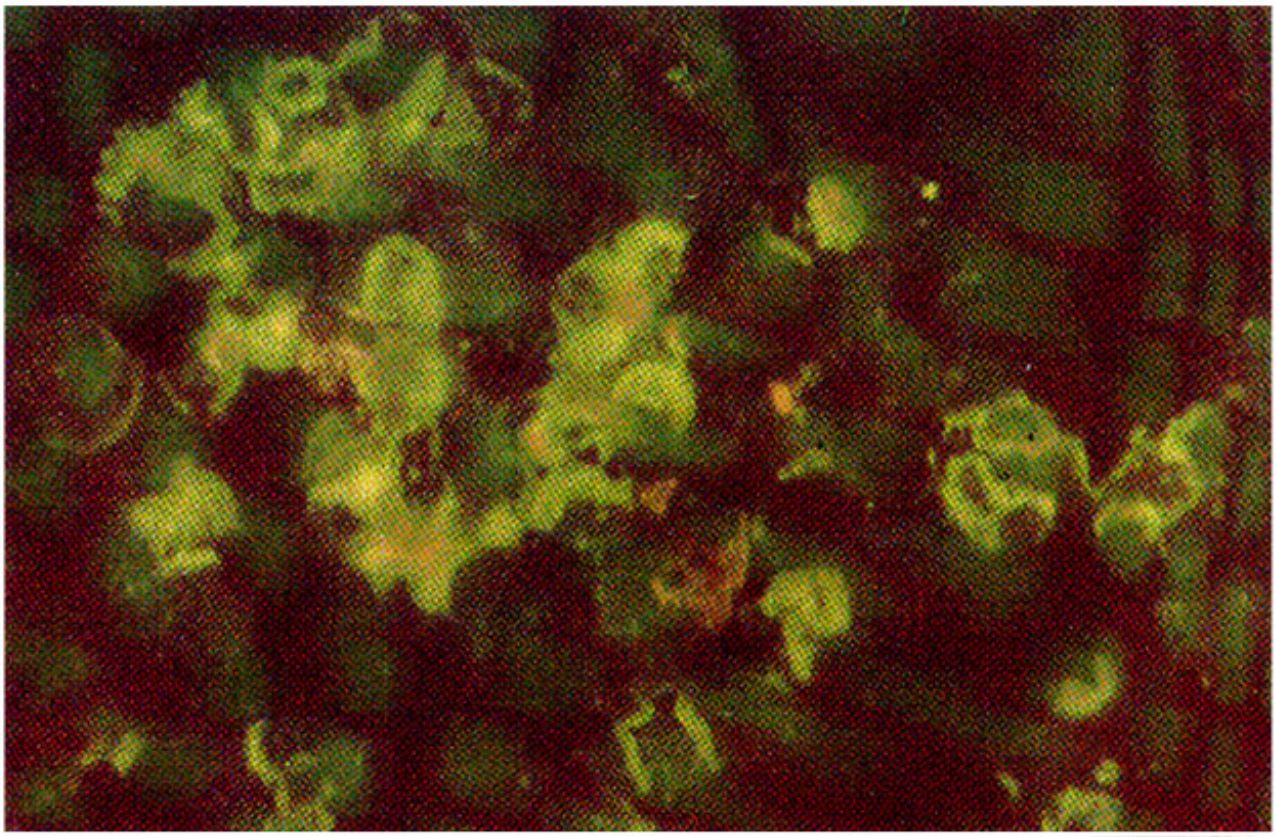

Figure 2. p-ANCA pattern of staining as observed on human neutrophils (magnification 40X).

Results

A total of 56 patients were screened for the presence of ANCA out of whom 8 were found positive. Four of these eight patients had c-ANCA; all of whom were later diagnosed to be suffering from Wegener's granulomatosis. Rest of the four ANCA positive patients had p-ANCA antibody and three of these patients presented with primarily renal manifestations. The details of the diagnoses are outlined in Table II.

Table II. The distribution of the c-ANCA and the p-ANCA in patients suffering from the systemic vasculitides.

Disease	c-ANCA	p-ANCA
Wegener's granulomatosis	4 (M)	0
Polyarteritis nodosa	0	1 (F)
Henoch-schonlein purpura	0	1 (M)
SCLE	0	1 (F)
Ac Renal Failure	0	1 (M)

SCLE= Subacute cutaneous lupus erythematosis

M= Male

F= Female

Six of our 8 ANCA positive patients were males. These patients were managed with primarily inimuno suppression. Outcome of the treatment is given in Table III.

Table III. Response to the immunosuppressive therapy.

Diagnosis	Remission	Partial remission	Death	Lost to follow up
Wegener's	1	2	0	1
Polyarteritis	0	0	0	1
Henoch-schonlein	1	0	0	0
SCLE	1	0	0	0
Ac Renal Failure	0	0	1	0

SCLE= Subacute cutaneous lupus erythematosis.

One patient with Wegener's granulomatosis is being followed closely for the past six months and the ANCA titres have related directly and specifically with the disease activity. This patient reported with arthralgia, conjunctivitis, erythematous rash over the dorsum of his hands, haematuria and one episode of epistaxis. His serum urea and C reactive protein (CRP) levels were markedly elevated and the ANCA litre was >640 (normal <1 0) at the time of presentation. With the institution of the immunosuppressive therapy his CRP, serum urea and the ANCA levels came down to normal levels in about 6 weeks. This patient then developed cough with haemoptysis while still admitted in the hospital. At this juncture it was important to decide whether it was an exacerbation of the Wegener's granulomatosis activity or was it due to some other disease. A review of his investigations revealed that his ANCA level was within normal limits and his serum urea and creatinine were not affected though CRP level was raised. At about the same time acid fast bacilli were demonstrated in his sputum. His symptoms settled down with anti- tuberculosis therapy and CRP levels also dropped down to normal levels. Later, in the course of his illness the patient developed productive cough, his CRP went up but the ANCA levels and the serum urea and creatinine were unaffected (Figure 3).

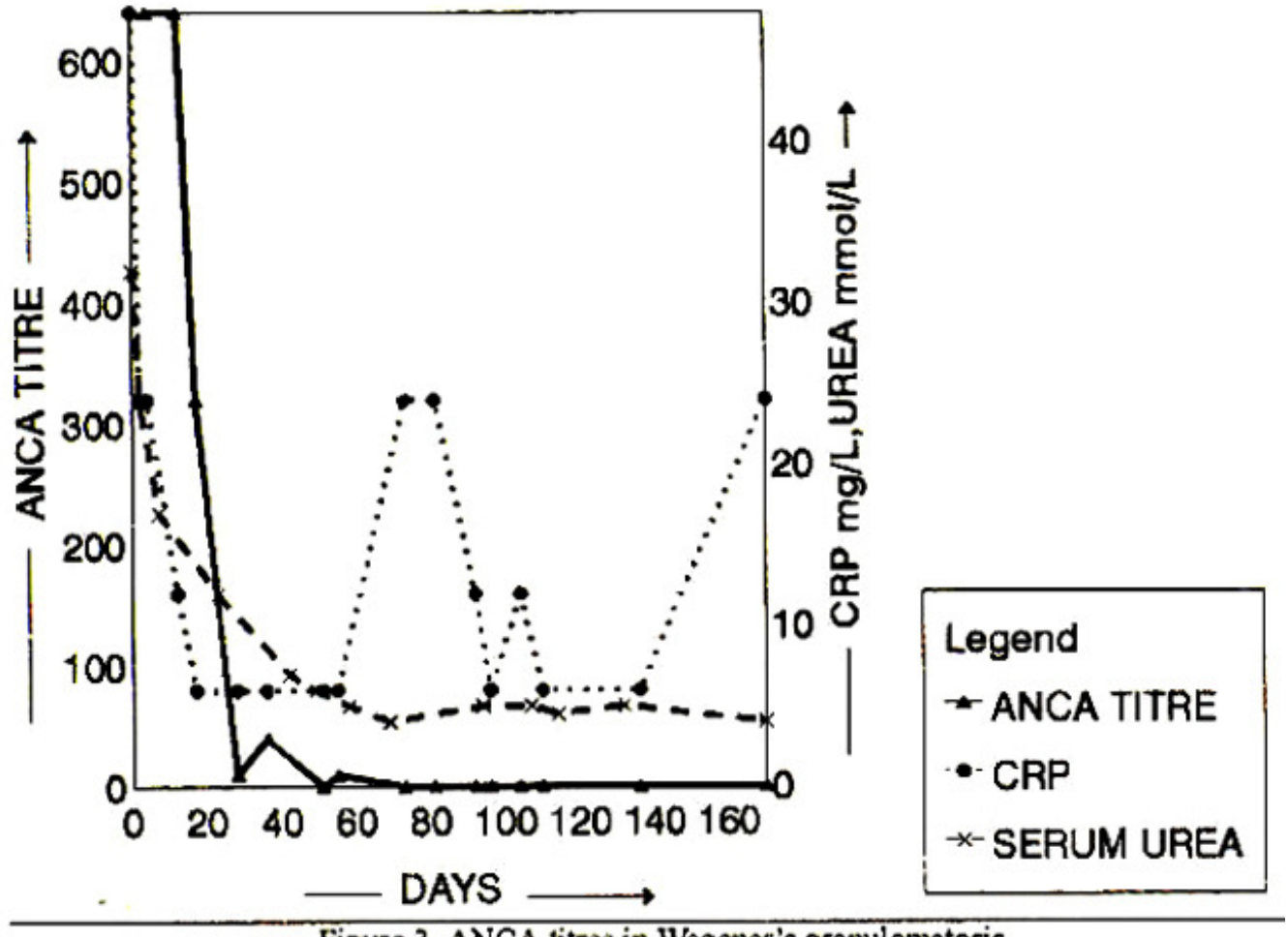

Figure 3. ANCA titres in Wegener's granulomatosis.

Discussion

ANCA has proved to be useful non-invasive marker for the management of the patients suffering from systemic vasculitides[13]. It has been suggested that the incidence of this particular group of diseases seem to be increasing, a fact which can be largely attributed to the recognition of the milder forms of these diseases. The increased physician awareness in recent years and the use of ANCA as a diagnostic test, has played a key role in establishing these diagnoses[16]. The pathogenesis of the systemic vasculitides remains largely unknown and based on some in-vitro experimental data it has been suggested that ANCA may be able to activate neutrophils in-vivo and thus may have a role in pathogenesis of this group of diseases[14]. However, it is well known that the disease exacerbations in Wegener's granulomatosis are often preceded by upper respiratory tract infections. This results in pulmonary neutrophil response in which neutrophils are activated and the contents of their granule are released. This may lead to an autoimmune response, which may be perpetuated by the auto-antibodies directed against neutrophil constituents[17]. There is little clinical or experimental evidence to support this hypothesis at present. However, the specific association of ANCA with Wegener's granulomatosis and other forms of the systemic vasculitides has been established in a number of studies[12,13,18]. But it should be kept in mind that there is a significant number of the patients who may be negative for ANCA inspite of suffering from systemic vasculitides including the Wegener's granulomatosis, specially in the inactive stages of the disease. We were able to establish a nearly and specific diagnosis

in patients with systemic vaseulitides with the use of ANCA as a diagnostic test. This resulted in early institution of specific therapy. The disease activity related closely and specifically with the ANCA levels in one of our patients suffering from Wegener's granulomatosis. We would recommend that ANCA testing should be considered as a useful diagnostic tool in investigations of all patients who present with clinical features of systemic vasculitides without a well charaeterised underlying disease process.

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