

Antibodies to neutrophil cytoplasmic antigens in Wegener's granulomatosis and other conditions

D J HARRISON, R SIMPSON, R KHARBANDA, V E ABERNETHY, G NIMMO

From the University Departments of Pathology and Rheumatology and the Medical Renal Unit, Royal Infirmary, Edinburgh

ABSTRACT The use of serum antibodies to neutrophil cytoplasmic antigens (ANCA) as a diagnostic marker for Wegener's granulomatosis and other forms of vasculitis has been assessed. Although ANCA have been described by several groups the precise antigenic targets are unknown, and detection of ANCA still relies on an indirect immunofluorescence assay technique. Several different patterns of fluorescence have been produced by using sera from different groups of patients, and insufficient information is available on the frequency of positive results and of the patterns of immunofluorescence obtained when serum from patients with vasculitis as a part of a generalised connective tissue disease is used. A study was carried out on serum from 240 patients, including 23 patients with Wegener's granulomatosis, 12 with microscopic polyarteritis, and 30 with various connective tissue diseases. Three patterns of fluorescence were observed: bright coarsely granular cytoplasmic, bright non-granular cytoplasmic, and weak diffuse cytoplasmic. The bright, coarsely granular pattern was 86% specific for Wegener's granulomatosis in this series and was observed in 18 of 23 cases. Other patterns of fluorescence were found in various conditions and were not of diagnostic value. The technique is simple, inexpensive, rapid, and reproducible.

Introduction

Wegener's granulomatosis is classically defined as a necrotising granulomatous vasculitis in the upper and lower respiratory tracts associated with focal and segmental necrotising glomerulonephritis.¹ The classical features are frequently absent, however, and diagnosis is therefore delayed.

The recognition of autoantibodies directed against a neutrophil cytoplasmic antigen (ANCA) in Wegener's granulomatosis^{2,3} has led to hopes of a specific diagnostic marker for this disease. ANCA may also be useful in monitoring disease activity,^{2,3} particularly when used with measurement of C-reactive protein.⁴

It is clear that ANCA represent a group of different antibodies as different patterns of fluorescence may be obtained from different serum samples.⁵ The original description described bright, granular fluorescence of the neutrophil cytoplasm in an indirect immuno-

fluorescence assay as diagnostic of Wegener's granulomatosis.² ANCA have, however, been described in microscopic polyarteritis,^{6,7} Kawasaki disease,⁸ Churg-Strauss syndrome,⁹ and even carcinoma of the lung.¹⁰ In microscopic polyarteritis, where the pattern of fluorescence has been specified,^{6,7} a bright, diffuse cytoplasmic fluorescence has been recorded.

This study investigates the pattern of fluorescence produced by ANCA in serum samples from patients with a wide range of diseases, and assesses the clinical value of detecting ANCA by an indirect immunofluorescence assay.

Methods

IMMUNOFLUORESCENCE ASSAY

The assay used is a modification of that described by Van der Woude and colleagues.² In brief, heparinised venous blood from a healthy volunteer was mixed with half its volume of 5% Dextran 250 (Pharmacia, UK) in 0.9% sodium chloride solution, and incubated at 37°C for 40 minutes to sediment red blood cells. The neutrophil enriched supernatant was washed twice in

Address for reprint requests: Dr D J Harrison, Department of Pathology, University of Edinburgh, Edinburgh EH9 8AG.

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phosphate buffered saline and resuspended at a cell concentration of 5×10^5 cells/ml. Aliquots of 100 μ l were used to make cytospin preparations, which were fixed in absolute ethanol at 4°C for five minutes. In some experiments cytospin preparations were fixed in acetone at 4°C for five minutes. They were used either immediately or within five weeks, having been wrapped and stored at -20°C until use.

Cytospin preparations were incubated with the patient's serum serially diluted from 1:20 or 1:80 in phosphate buffered saline for 45 minutes. After two washes in the saline they were incubated with a 1:50 dilution of fluorescein conjugated rabbit antihuman IgG (Scottish Antibody Production Unit, Carlisle) for 30 minutes before examination with an ultraviolet microscope. A known positive and a known negative serum sample were included each time the assay was performed.

Cytospin preparations were scored according to the nature and brightness of fluorescence to give four groups: 1—no appreciable fluorescence; 2—weak, diffuse cytoplasmic fluorescence; 3—bright but not coarsely granular cytoplasmic fluorescence; 4—bright, coarsely granular cytoplasmic fluorescence identical to or brighter than the positive control. The presence of antinuclear antibodies was also recorded. The dilution of serum at which fluorescence disappeared was also recorded for positive cases.

CLINICAL DIAGNOSES

The diagnosis was recorded for each patient studied without prior knowledge of the presence or absence of ANCA. The diagnosis of Wegener's granulomatosis was supported in every case by the histological appearance of biopsy material obtained from at least one affected tissue. Diagnoses were not altered in the light of the ANCA findings.

Results

CLINICAL

Twenty three patients with Wegener's granulomatosis were studied. A brief summary of symptoms at presentation and biopsy findings is given in table 1. Serum samples from a further 217 patients were also studied, including 12 patients with microscopic polyarteritis, three with Churg-Strauss syndrome, 38 with various connective tissue diseases, 41 with renal disease, 10 with malignancy, and the remainder with various inflammatory and infectious disorders (table 2).

IMMUNOFLUORESCENCE

No normal volunteers had ANCA detectable at serum dilutions of 1:20 or greater. Results of the ANCA test are summarised in table 2. The scoring system for

Table 1 Details of patients with Wegener's granulomatosis

Age (y)	Sex	Presentation	Biopsy*	ANCA pattern
23	M	Haemoptysis	Nasal, renal	Coarse granular
40	M	Haemoptysis	Nasal	"
42	F	Haemoptysis	Nasal	"
53	F	Mouth or nose ulcers	Nasal, (renal)	"
45	M	Renal failure	Nasal, renal	"
39	M	Pyoderma, epistaxis	Nasal, (renal)	"
24	F	Skin vasculitis	(Nasal), (renal)	"
64	M	Epistaxis	(Nasal), renal	"
30	M	Sinusitis	Nasal, renal	"
31	M	Haemoptysis	Nasal, renal	"
39	M	Renal failure	Nasal, (lung), renal	"
38	M	Renal failure	Nasal, renal	"
38	M	Haemoptysis	Lung	"
63	M	Fever, lung consolidation	Renal	"
57	F	Haemoptysis, haematuria	(Nasal), (renal)	"
54	F	Haemoptysis	Transbronchial, renal	"
39	M	Haematuria	(Nasal), renal	"
70	F	Epistaxis, renal failure	(Nasal)	"
43	M	Renal failure	(Nasal), renal	Non-granular
26	F	Cavitating lung lesions	Lung	"
26	F	Epistaxis	Nasal, transbronchial	Weak, diffuse
28	F	Epistaxis	Nasal	"
43	M	Nasal ulceration	Nasal, (renal)	Nil

*Parenthesis indicates that although the biopsy was performed it yielded findings that did not support the diagnosis of Wegener's granulomatosis. In other cases biopsy findings were either consistent with or diagnostic of systemic vasculitis.

ANCA—antineutrophil cytoplasmic antibodies.

ethanol fixed cytospin preparations was found to be reliable and reproducible, and two pathologists not concerned with the project concurred with the scores we had assigned. The scoring was helped by the inclusion of a known positive sample in each assay. When acetone was used as a fixative the pattern of fluorescence was usually diffuse and only erratic granular staining was seen. Serum samples from 21 patients, of whom 18 had Wegener's granulomatosis, showed the very bright, coarsely granular cytoplasmic fluorescence characteristic of the positive control (fig 1).

There were 23 cases of Wegener's granulomatosis in total, giving a sensitivity of 78% for the test. Of the 217 patients studied who did not have Wegener's granulomatosis, only three had a positive result. This gives a specificity of 86%, though this would be influenced by the composition of the control group. Nineteen patients, of whom two had Wegener's granulomatosis, had bright but non-granular cytoplasmic fluorescence (fig 2).

As the fluorescence score decreased the range of diseases with detectable ANCA increased. The inclusion of patterns other than the very bright

Table 2 Results of immunofluorescence findings for antineutrophil cytoplasmic antibodies (ANCA) according to the nature of the cytoplasmic fluorescence and the clinical diagnosis

Cytoplasmic fluorescence	No of cases	Diagnosis (n)
Bright coarsely granular	21	Wegener's granulomatosis (18) Microscopic polyarteritis (2)* Churg-Strauss syndrome (1)†
Bright, non-granular	19	Wegener's granulomatosis (2) Microscopic polyarteritis (3) Mixed connective tissue disease (3) Paget's disease of bone (1) Churg-Strauss syndrome (1) Behçet's syndrome (1) Wegener's granulomatosis (treated) (2) Nephrosclerosis (2) Polymyalgia rheumatica
Weak	38	Wegener's granulomatosis (2) Microscopic polyarteritis (5) Mixed connective tissue disease (2) Rheumatoid arthritis (3) Churg-Strauss syndrome (1) Paget's disease of bone (3) Other diagnoses (22)‡
Insignificant	162	Wegener's granulomatosis (1) Microscopic polyarteritis (2) Other diagnoses, including pneumonia, nasal polyps, myeloma, Behçet's syndrome, sarcoidosis, Goodpasture's syndrome, polymyalgia rheumatica, Wegener's granulomatosis (treated), polyarteritis (treated)

*Focal and segmental necrotising glomerulonephritis.

†Eosinophilia, transbronchial biopsy.

‡Including Wegener's granulomatosis and polyarteritis during treatment, glomerulonephritis, sarcoidosis, systemic lupus erythematosus, Goodpasture's syndrome.

granular cytoplasmic fluorescence therefore resulted in reduced specificity of the assay.

ANCA were readily detectable in neutrophil cytosin preparations stored for up to five weeks. This allows the preparation of large batches of fixed slides, which is convenient if the test is to be performed rapidly as a diagnostic aid when required. Repeated freeze-thawing of serum samples resulted in a reduction in ANCA fluorescence, but samples stored for four years at -70°C retained fluorescence.

As serum giving a coarsely granular pattern was diluted we noted that the pattern of fluorescence changed to diffuse before the fluorescence signal actually disappeared. This change occurred at a serum dilution of 1:80 in one case, at 1:160 in most cases, and at 1:500 in only two cases. Repeat samples from patients having treatment sometimes showed a similar change in pattern of fluorescence from granular to diffuse, irrespective of the titre of antibody used.

The titre of antibody fell in parallel with other markers of disease activity, such as the white blood cell count, erythrocyte sedimentation rate, and serum concentration of human neutrophil elastase.¹¹ With recrudescence of clinical disease, such as mouth ulceration, lung cavitation, or deteriorating renal function, the titre of ANCA increased.

Discussion

The classification of vasculitis is difficult and at times

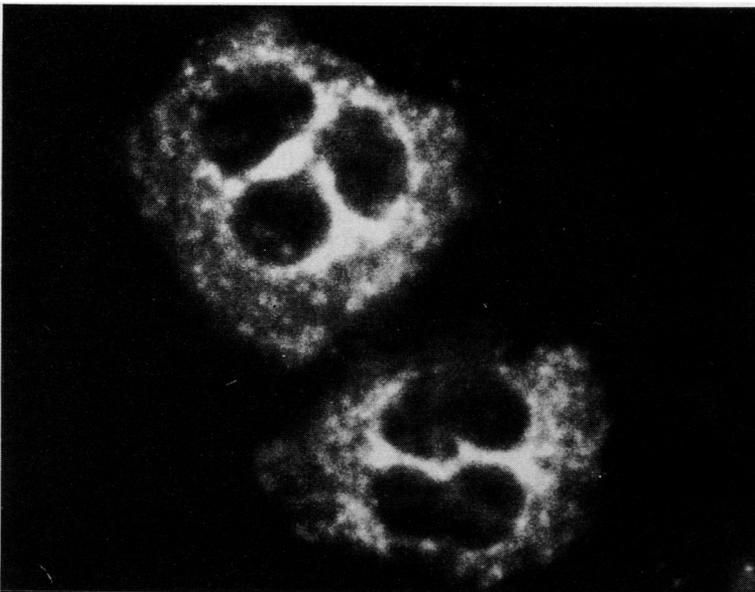


Fig 1 Photomicrograph of cytosin preparation of normal neutrophils incubated sequentially with serum from a patient with Wegener's granulomatosis and fluoresceinated antihuman IgG, showing coarse granular cytoplasmic fluorescence.

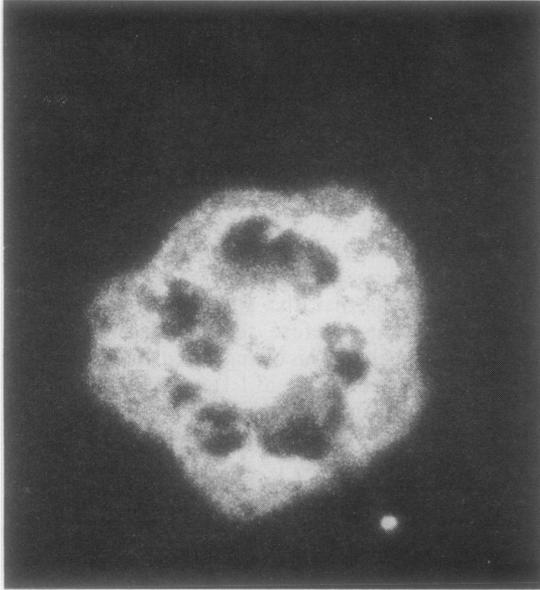


Fig 2 Photomicrograph of a neutrophil cytospin preparation with a more diffuse cytoplasmic fluorescence than in figure 1 after incubation with serum from a patient with microscopic polyarteritis.

unclear.¹² Primary vasculitis is generally accepted as having an immunological basis, probably with immune complexes damaging the vascular wall.¹ The diagnosis of Wegener's granulomatosis is frequently delayed because of the difficulty of obtaining definitive diagnostic material.^{7,13} The discovery of ANCA (a generic rather than a specific title), which are thought to be specific for Wegener's granulomatosis, was therefore hoped to be an important advance in the recognition of this disease and its differentiation from other vasculitides, in particular microscopic polyarteritis.^{2,4} The reported sensitivity of ANCA tests for active Wegener's granulomatosis has varied from 71% to 100%,^{2,4,10} which is similar to the 78% in our series. We have found ANCA in a few cases of microscopic polyarteritis and Churg-Strauss syndrome but our specificity of 86% for Wegener's granulomatosis is high, and similar to the range of 88–100% reported in other series.^{2,4,10,14} The precise figure for specificity is likely to vary a little as it will be influenced by the composition of the control group. These results are equivalent to accepted values for anti-double stranded DNA antibodies in systemic lupus erythematosus and antiacetylcholine receptor antibodies in myasthenia gravis.⁴

In addition to the very bright, granular cytoplasmic fluorescence that we regard as characteristic of Wegener's granulomatosis many samples had ANCA

activity, albeit with less intense and diffuse fluorescence, including serum from four patients with Wegener's granulomatosis, six patients with microscopic polyarteritis, and two patients with Churg-Strauss syndrome. Other groups have reported diffuse fluorescence and have included this pattern under the title of ANCA.² It seems likely that more than one antigen is recognised by ANCA¹⁵ and the pattern of fluorescence may relate to the antigen or antigens recognised by the antibodies present in the serum.¹⁶ Many cases of connective tissue diseases as well as several cases of Paget's disease of bone were included in our study group. Diffuse, weak ANCA fluorescence has been described in serum from patients with primary biliary cirrhosis,¹⁷ rheumatoid arthritis,^{14,37} bronchogenic carcinoma,¹⁰ and viral enteritis.¹⁰ The meaning of these findings is not clear but they suggest that antibodies to neutrophil cytoplasmic antigens are a heterogeneous group of antibodies directed against different antigenic determinants and not a single entity. In cases of mixed connective tissue disease there is a well described antibody to extractable nuclear antigen.¹⁸ Our present findings of cytoplasmic fluorescence in this condition may be the result of artefactual displacement of nuclear antigen during preparation of neutrophils for cytospin, in a way similar to the proposed displacement of nuclear c-myc oncoprotein during tissue fixation from the nucleus to the cytoplasm.¹⁹ Patients with Wegener's granulomatosis do not usually have other specific autoantibodies.⁴

The weak ANCA fluorescence found in cases of Paget's disease of bone are of interest. Lockwood and colleagues⁶ have proposed that the target antigen of ANCA in vasculitis is an epitope (antigenic determinant of known structure) derived from alkaline phosphatase, though this is controversial.^{17,20,21} If alkaline phosphatase is an autoantigen, then ANCA may be an epiphenomenon related to increased serum concentrations of the enzyme as a result of neutrophil degranulation. In Paget's disease the serum concentration of bone alkaline phosphatase is raised, so possibly ANCA are the result of cross reactivity between epitopes of bone and neutrophil alkaline phosphatase. A similar argument may apply to similar antineutrophil cytoplasm fluorescence detected in cases of primary biliary cirrhosis.¹⁷ In these cases the fluorescence is weak and diffuse, similar to that seen with rabbit anti-human alkaline phosphatase antiserum.²²

In conclusion, the presence of ANCA giving bright granular cytoplasmic fluorescence is of considerable value in suggesting the diagnosis of Wegener's granulomatosis and, to a lesser extent, of some other vasculitides. The indirect immunofluorescence assay is rapid, reliable, reproducible, inexpensive, and within the capabilities of most laboratories. Further identification of the antigenic epitopes concerned may

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increase both the specificity and the sensitivity of testing for ANCA in the diagnosis and follow up of Wegener's granulomatosis, and increase our understanding of the underlying disease process.

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