Antineutrophil cytoplasmic antibodies in Wegener's granulomatosis

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Abstract

The prevalence of antineutrophil cytoplasmic antibodies (ANCA) was studied in 12 children with Wegener's granulomatosis. The serum samples were taken in the active phase of disease and were screened for ANCA by indirect immunofluorescence with normal neutrophils and enzyme linked immunosorbent assay (ELISA) using crude neutrophil extract, proteinase 3, myeloperoxidase, cathepsin G, lactoferrin, and elastase as antigens. Of these 12 patients, 10 were positive for ANCA in the active phase of their illness, and they showed a predominantly cytoplasmic ANCA staining pattern on indirect immunofluorescence. There were high titres of ANCA directed against crude neutrophil extract, proteinase 3, myeloperoxidase, and cathepsin G. IgM isotypes occurred as commonly as IgG isotypes. Therefore, screening for ANCA is usually but not invariably positive in children with Wegener's granulomatosis. Specific diagnosis still relies on clinical and pathological features, and the value of ANCA in the diagnosis of paediatric Wegener's granulomatosis requires further study.

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Wegener's granulomatosis is a disorder characterised by granulomatous inflammation and necrotising vasculitis in various organs.¹ Clinically, it manifests as a febrile multisystem illness with particular involvement of the upper and lower respiratory tracts and kidneys. Since the late 1980s, there have been reports of the occurrence of antineutrophil cytoplasmic antibodies (ANCA) in these patients.²⁻⁴ In adult patients, these autoantibodies help in diagnosis,⁵ in monitoring disease activity, and in guiding treatment.⁶ They have also been shown experimentally to activate human neutrophils, and might be related to the pathogenesis of Wegener's granulomatosis and similar disorders.^{7,8} In most cases the autoantibodies are directed against proteinase 3.⁹ In children, there have been isolated reports of ANCA in Wegener's granulomatosis and other vasculitides,^{10,11} but their clinical significance remains uncertain.

Our paper reports the prevalence of ANCA in children with Wegener's granulomatosis diagnosed at Great Ormond Street Children's Hospital by indirect immunofluorescence and

enzyme linked immunosorbent assay (ELISA), and the antigenic specificities and immunoglobulin isotypes of such antibodies. The usefulness and limitations of ANCA in the diagnosis of Wegener's granulomatosis in children are discussed.

Patients and methods

The study population consisted of 12 children with Wegener's granulomatosis diagnosed at Great Ormond Street Children's Hospital, London, between January 1986 and December 1991. There were four boys and eight girls. Their age of onset ranged from 2 months to 14.2 years. They had multisystem manifestations as shown in table 1. Diagnosis of Wegener's granulomatosis was established by the presence of two or more criteria according to the American College of Rheumatology.¹² There was histological evidence of granulomatous inflammation or necrotising vasculitis in the nose, larynx, muscles, colon, or kidneys in seven patients.

All patients were in the active phase of their illness as shown by symptoms, a high erythrocyte sedimentation rate, and high concentrations of C reactive protein. Sera were obtained before immunosuppressive treatment was begun. Antinuclear factor was screened by immunofluorescence staining in rat liver cells and was positive in low titres (< 1/80) in four patients. Anti-DNA antibodies, rheumatoid factor, and other non-organ specific antibodies were also negative in all patients. Serum samples were frozen in small aliquots at -60°C until the assay. Because of the rarity of Wegener's granulomatosis, the samples had been stored from one to five years before the study.

A group of 30 healthy children (18 boys and 12 girls; mean (SD) age, 7.4 (3.8) years) admitted for elective tonsillectomy and adenoidectomy served as normal controls. They had no fever or other symptoms at the time of sampling. In addition, sera from 17 consecutive patients with other autoimmune diseases (juvenile chronic arthritis, dermatomyositis, scleroderma) were tested for ANCA by ELISA using crude neutrophil extract and they served as disease controls.

Parental consent was obtained and the study was approved by the Great Ormond Street Hospital for Children/Institute of Child Health Ethics Committee.

Serum samples were studied using: indirect immunofluorescence; ELISA using a crude neutrophil extract for IgG and IgM (crude ANCA IgG and IgM, respectively); ELISA for IgG and IgM antibodies directed against

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myeloperoxidase, lactoferrin, cathepsin G, and elastase; and assay for proteinase 3 specific IgG was also attempted using a commercial kit (BioCarb Diagnostics Ltd, Worcestershire, UK), which unfortunately used an α granule extract of neutrophils as antigen. Although this assay measured principally antibody directed to proteinase 3, there may have been some cross reactivity to myeloperoxidase and lactoferrin present.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

Patient sera were first screened for ANCA using standard indirect immunofluorescence microscopy.¹³ Neutrophils from healthy donors were separated by gravity sedimentation on methylcellulose/Hypaque. Slides prepared by cytocentrifugation were fixed in absolute ethanol at 0°C. The neutrophil preparations were incubated with patient's serum (dilution, 1/16), washed, incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-human immunoglobulin (antikappa and anti-lambda chains; Dako, High Wycombe, Bucks, UK), washed and mounted. Staining patterns by fluorescent microscopy were compared with known negative control sera. Positive controls included antiproteinase 3 and antimyeloperoxidase sera (Binding Site Ltd, Birmingham, UK). Cytoplasmic pattern (C-ANCA) was defined as granular staining of cytoplasm with accentuation between nuclear lobes. Perinuclear pattern (P-ANCA) was present when there was fluorescence around the nucleus.

ELISA PROCEDURE FOR CRUDE ANCA

Crude neutrophil extract was prepared from neutrophils of healthy donors by methylcellulose/Hypaque gradient separation, washing, and resuspension in sodium acetate (0.2 M, pH 4.2); cells were disrupted by sonication, and cell membranes and nuclear debris were removed from the supernatant antigen extract by centrifugation, as described previously.³ Microtitre plates (Nunc Life Technologies, Paisley, UK) were coated by incubation with this antigen preparation in carbonate/

bicarbonate buffer (pH 9.6) at room temperature overnight. Plates were washed thoroughly, using phosphate buffered saline (PBS) containing 2% casein and 0.1% Tween 20 between all steps. Wells were incubated serially with PBS/2% casein, patient's serum in PBS/2% casein, then peroxidase conjugated rabbit antihuman IgG or IgM immunoglobulins (Sigma Chemicals Co, Poole, Dorset, UK), and finally *o*-phenylenediamine dihydrochloride (Sigma Chemicals Co). The enzyme reaction was stopped by the addition of 2 M sulphuric acid after 20–30 minutes incubation and the absorbance was read at 492 nm. All samples were processed in duplicate, and the absorbances were averaged. Each plate included wells with PBS alone, known positive and negative sera, and control wells (without antigens but incubated with patient serum) for each sample. Results were expressed as ELISA units by reading patients' absorbance against the dilution curve of a known positive standard on a semi-logarithmic scale, and subtracting from it similar readings of the corresponding control well. The positive standard and control sera were taken from known Wegener's granulomatosis patients and they have been confirmed to have strong positive ANCA in a leading laboratory undertaking regular ANCA assays (Hammersmith Hospital, London). The intra-assay coefficient of variation was 6.2% and the interassay coefficient of variation was 12%.

ELISA PROCEDURES FOR ANTIBODIES AGAINST MYELOPEROXIDASE, LACTOFERRIN, CATHEPSIN G, AND ELASTASE

Similar procedures as above were used. The following antigen solutions were used to coat the wells at 4°C overnight: myeloperoxidase (0.2 µg/ml; Calbiochem, Nottingham, UK); lactoferrin (25 µg/ml; Calbiochem); cathepsin G (2 µg/ml; Calbiochem); elastase (10 ng/ml; Sigma Chemicals Co). Bovine serum albumin (1%) in PBS was used for blocking. Bound antibodies were detected with alkaline phosphatase conjugated goat antihuman IgG or

Table 1 Systemic manifestation of 12 patients with Wegener's granulomatosis

Case	Sex/age (years)	Presenting symptoms	Skin	MS	URT	LRT	Kidney	GI tract	CNS	Biopsy
1	F/11.7	Acute renal failure	+	+	+	–	+	+	–	Kidney, crescentic glomerulonephritis Nose, granulomatous inflammation Vasculitis
2	M/1.16	Stridor	+	–	+	+	–	–	–	Skin, granulomatous inflammation
3	F/12.7	Haemoptysis	+	+	+	+	+	+	–	Kidney, focal glomerulonephritis Nose, granulomatous inflammation Larynx, granulomatous inflammation
4	M/7.29	Polyarthrititis	+	+	+	+	+	+	–	Muscle, vasculitis Colon, vasculitis
5	F/13.94	Stridor	–	+	+	–	–	–	–	Larynx, vasculitis
6	F/7.82	Fever, epistaxis	–	+	+	+	–	+	+	Rectum, vasculitis Nose, non-specific inflammation
7	M/14.2	Haemoptysis	+	+	+	+	–	–	–	Nose, non-specific inflammation
8	M/10.5	Polyarthrititis	+	+	+	–	+	+	–	Not done
9	F/9.83	Stridor	+	+	+	–	–	–	+	Larynx, non-specific inflammation
10	F/2.1	Fever, skin nodules	+	+	–	+	–	–	–	Skin, non-specific inflammation Lung, non-specific inflammation
11	F/0.31	Stridor	+	–	+	–	–	–	–	Larynx, non-specific inflammation
12	F/0.16	Stridor, haemoptysis	+	–	+	+	–	+	–	Trachea, granulomatous inflammation Colon, vasculitis

Skin, vasculitic or purpuric rash; musculoskeletal (MS), muscle pain/tenderness, polyarthrititis; upper respiratory tract (URT), granulomatous rhinitis and sinusitis, laryngeal stenosis; lower respiratory tract (LRT), haemoptysis or shadows on chest radiography; kidney, urine abnormalities, raised serum creatinine, glomerulonephritis; GI tract, abdominal pain, bloody diarrhoea; CNS, neurological deficits.

Table 2 ANCA results in 12 patients with Wegener's granulomatosis

Case	ANCA IIF pattern	Titre (in ELISA units)		Antigenic specificities	Concomitant clinical parameters			
		Crude ANCA IgG (control < 4.1)	Crude ANCA IgM (control < 9.6)		ESR (mm/h)	Hb (g/l)	WBC ($\times 10^9/l$)	Platelets ($\times 10^9/l$)
1	P-ANCA	10.0	256.0	MPO-IgG, MPO-IgM, Cath-IgG, Elas-IgG	88	93	9.6	344
2	Negative	1.8	13.8	PR3-IgG, Lact-IgG, Cath-IgM	48	98	19.7	1098
3	C-ANCA	20.0	11.8	PR3-IgG, MPO-IgM, Cath-IgM	70	66	9.6	447
4	C-ANCA	8.0	2.6	MPO-IgG, Lact-IgG, Cath-IgG, Elas-IgG	38	123	9.4	470
5	Negative	5.5	5.2	Titre within control range	83	126	9.7	385
6	C-ANCA	4.8	11.0	MPO-IgM, Cath-IgM	90	69	14.0	375
7	C-ANCA	43.6	36.0	PR3-IgG, MPO-IgM, Cath-IgM	100	79	6.3	957
8	Negative	1.9	0.5	Titre within control range	96	78	8.6	425
9	C-ANCA	0.5	35.7	Cath-IgG, Cath-IgM	56	110	6.5	395
10	C-ANCA	10.0	0.7	PR3-IgG	40	91	14.6	375
11	Negative	4.2	13.6	MPO-IgM, Lact-IgG, Cath-IgG, Cath-IgM	50	121	23.6	607
12	Negative	3.0	8.2	Titre within control range	40	120	24.3	1033

P-ANCA, perinuclear pattern; C-ANCA, cytoplasmic pattern; MPO, antimyeloperoxidase titre; Cath, antineutrophil cytoplasmic antibody titre; Lact, antilactoferrin titre; Elas, antielastase titre; PR3, antiproteinase 3 titre; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; WBC total white blood cell count.

IgM immunoglobulins (Sigma Chemicals Co), with *p*-nitrophenyl phosphate (Sigma Chemicals Co) as substrate, and absorbance was read at 405 nm. Results were expressed in ELISA units as above, but a different positive standard was needed for different antibodies.

STATISTICAL METHODS

Because the normal control group data did not have a Gaussian distribution, and could not be normalised by transformation, an arbitrary cut off to include 90% of these readings (more than 28 samples) was chosen as the observed normal range for each antibody subtype. Non-parametric Mann-Whitney test was used to compare the difference between any two groups.

Results

Table 2 shows the results of screening for ANCA and their antigen specificity in our 12 patients with Wegener's granulomatosis. Indirect immunofluorescence demonstrated the presence of ANCA in seven patients. Six patients showed a C-ANCA pattern and one patient showed a P-ANCA pattern. Using ELISA with the crude neutrophil extract, 10 patients were found to have high titres: crude

ANCA IgG was seen in eight patients and crude ANCA IgM was seen in seven patients (five of whom were positive for both). Three of these patients showed negative indirect immunofluorescence results but low positive titres by ELISA. Figure 1 shows the crude ANCA IgG and IgM titres of Wegener's granulomatosis patients, the disease control group, and the normal control group. The titres were significantly higher in Wegener's granulomatosis patients compared with normal controls by Mann-Whitney test, while there were no significant differences between disease control and normal control groups.

Using specific antigens in the ELISA assay, we demonstrated positive titres of antibodies directed to cathepsin G in seven patients (IgG in three, IgM in six), to myeloperoxidase in six patients (IgG in two, IgM in five), to lactoferrin in three patients (IgG in all), and to elastase in two patients (IgG in all). ANCA screening using the BioCarb kit was positive in four patients. This probably indicated the presence of IgG antibodies to proteinase 3. However some cross reactivity to other impurities such as myeloperoxidase or lactoferrin might have been present, although this was unlikely

Figure 1 ANCA detected by ELISA using crude acid neutrophil extract as substrate (crude ANCA IgG and crude ANCA IgM) in patients with Wegener's granulomatosis (WG), patients with other autoimmune diseases as disease control (DC), and normal children (NC). ELISA units are on a logarithmic scale. Values are medians and 95% confidence limits. Solid circles, patients with positive IIF results; open circles, patients with negative IIF results; dotted lines indicate arbitrary upper limit of normal children.

because these four patients showed negative or low titres of IgG against other antigens.

Discussion

Wegener's granulomatosis is a rare disorder in childhood but an important differential diagnosis in ill children with non-specific multi-system symptoms. By definition, Wegener's granulomatosis is characterised by the presence of granulomatous inflammation of the respiratory tract and necrotising vasculitis affecting small to medium sized vessels.¹⁴ The diagnosis in paediatric cases is particularly difficult because it might not be possible to obtain adequate tissue from multiple sites or multiple organs to demonstrate the characteristic histological features. A negative biopsy does not exclude the diagnosis.¹⁵

In 1985, the presence ANCA with a characteristic cytoplasmic staining pattern (C-ANCA) was demonstrated in patients with Wegener's granulomatosis,² and this has provided a convenient non-invasive means of establishing the diagnosis. In adults, the demonstration of ANCA has a high sensitivity of 91% and specificity of 98% during the active phase but the sensitivity is lower (63%) during the inactive phase.¹⁶ In a meta-analysis by Bajema *et al*,¹⁵ on 349 Wegener's granulomatosis patients reported from 1979 to 1995, ANCA analysis was positive in 46 patients and negative in 11 of the 228 patients with histologically proven Wegener's granulomatosis (in the remaining 171 patients ANCA analysis was not performed). Among the 121 patients without histological proof but with typical clinical course of Wegener's granulomatosis, ANCA analysis was positive and helped in the diagnosis in 45 patients. It was not performed in 75 patients and was negative in one patient.

However, the role of ANCA in paediatric patients has not been fully established. Ellis *et al* reported five children and reviewed six previously reported children with systemic vasculitis and the presence of ANCA.¹⁰ Only three had histological proof of Wegener's granulomatosis and in two of them a C-ANCA staining pattern was seen and in one a P-ANCA staining pattern was seen. The other patients had microscopic polyangiitis or idiopathic crescentic glomerulonephritis.

All our patients satisfied two or more of the clinical criteria proposed by the American College of Rheumatology for the diagnosis of Wegener's granulomatosis.¹² Most had histological evidence of granulomas or necrotising vasculitis. Our study supported the usefulness of ANCA screening in the diagnosis of Wegener's granulomatosis. We demonstrated the presence of ANCA in 10 of the 12 patients. This was shown by means of indirect immunofluorescence in seven patients, with a C-ANCA staining pattern in six and a P-ANCA staining pattern in one. The ELISA assay using crude neutrophil extract demonstrated the presence of ANCA in all patients with positive indirect immunofluorescence results and in three patients with negative indirect immunofluorescence results. It is necessary to detect both IgG

and IgM in the ELISA assay because either might be positive, as shown in our patients. In adult patients, the combined use of indirect immunofluorescence and ELISA also leads to a diagnostic specificity of 99%.¹⁷

On the other hand, in a small minority of Wegener's granulomatosis patients, ANCA screening might be negative—two of our patients had a negative ANCA screen by indirect immunofluorescence and ELISA, one of whom had histological proof of granulomatous inflammation in the trachea. This is also supported by Bajema *et al*'s report of 11 patients with negative ANCA yet histological proof of granulomatous inflammation.¹⁵ Overall, the current diagnosis of Wegener's granulomatosis depends on one or more of the following criteria: a positive C-ANCA test, the presence of granulomas, and a clinical course typical of the disease.

In one study of adult patients, increases in serum ANCA concentrations demonstrated by indirect immunofluorescence were shown to precede clinical exacerbation by a few months or weeks,¹⁸ although this was not confirmed by a later study.¹⁹ ANCA concentrations decreased significantly or disappeared when patients were treated successfully with steroid and cyclophosphamide.²⁰ IgM ANCA have been seen in relation to haemorrhagic capillaritis in the lungs.^{21, 22} In our study, we have not attempted to study ANCA concentrations serially in our patients. In fact, all our patients were in the active phase of their disease, as shown by a raised erythrocyte sedimentation rate, and most had anaemia, leucocytosis, and thrombocytosis. In addition, because of the small number of patients, we could not demonstrate any statistical association of ANCA isotypes or ANCA titres with organ system involvement or clinical parameters.

Our studies on antigen specificities revealed interesting findings. First, antiprotease 3 and antiperoxidase (both IgG and IgM) occurred commonly in our patients. Antiprotease 3 IgG was present in only four patients. Antilactoferrin and antielastase antibodies were unusual. Second, seven patients had antibodies with multiple specificity, most to myeloperoxidase and cathepsin G. In his 1996 review,⁴ Wiik summarised the antigen specificity of ANCA in adult patients. Most Wegener's granulomatosis patients have antibodies directed to proteinase 3, giving a C-ANCA pattern on indirect immunofluorescence, although a few Wegener's granulomatosis patients and about half the patients with microscopic polyangiitis, Churg-Straus syndrome, and focal necrotising glomerulonephritis have antibodies directed against myeloperoxidase, giving a P-ANCA pattern on indirect immunofluorescence. Antibodies to elastase occurred in patients with propylthiouracil induced vasculitis, and lactoferrin is recognised as an autoantigen in a few patients with rheumatoid arthritis complicated by vasculitis, in systemic lupus erythematosus, and in ulcerative colitis. Our finding of multiple antigen specificities might be the result of non-specific binding of antibody secondary to generalised immune

activation. Its significance in the pathogenesis of paediatric Wegener's granulomatosis needs further study.

In conclusion, we found a high prevalence of ANCA in paediatric patients with Wegener's granulomatosis. These autoantibodies have variable specificities including proteinase 3, myeloperoxidase, cathepsin G, and rarely lactoferrin and elastase. The occurrence of antibodies with multiple antigenic specificities and the frequency of IgM isotypes in our patients was also noteworthy. The significance and clinical usefulness of these findings require further study.

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