Clin. exp. Immunol. (1990) **82,** 238–243

Diverse target antigens recognized by circulating antibodies in anti-neutrophil cytoplasm antibody-associated renal vasculitides

J. A. SAVIGE, M. GALLICCHIO, T. GEORGIOU* & D. J. DAVIES *Royal Melbourne Hospital, Parkville, Victoria, and *Department of Anatomical Pathology, St Vincent's Hospital, Fitzroy, Victoria, Australia*

(Accepted for publication 4 June 1990)

SUMMARY

Antibodies that are directed against cytoplasmic constituents of neutrophils and monocytes (anti-neutrophil cytoplasm antibodies, ANCA) have been described in Wegener's granulomatosis, microscopic polyarteritis (MPA) and some cases of segmental necrotizing glomerulonephritis (SNGN). Other antibodies occasionally described in Wegener's granulomatosis and MPA include anti-nuclear antibodies (ANA) and anti-glomerular basement membrane (GBM) antibodies. We have studied the diversity of the corresponding antigens in ANCA-associated renal diseases. Sera from 46 patients with active histologically proven Wegener's granulomatosis, MPA and SNGN were tested for ANCA by indirect immunofluorescent examination of normal peripheral blood neutrophils. Thirty-four sera (74%) were positive; 16 were associated with diffuse cytoplasmic staining (cANCA) and 18 with perinuclear staining (pANCA). In addition, five demonstrated anti-neutrophil-specific nuclear staining (ANNA). On Western blotting of the neutrophil extract, five sera recognized a 29-kD molecule recently identified as neutrophil proteinase 3. Two sera with typical cANCA bound to molecules of 36, 38 and 116 kD and another to a molecule of 22 kD. The final serum associated with pANCA bound to a molecule of about 12 kD. Thirteen sera out of 46 (28%) tested in an ELISA contained anti-myeloperoxidase antibodies; 10 of these were associated with pANCA and two others with ANNA. Three sera of 17 (18%) tested contained anti-elastase antibodies; these also contained anti-myeloperoxidase antibodies and were associated with pANCA. However, eight sera with pANCA were negative for anti-myeloperoxidase antibodies and three of these were also negative for anti-elastase antibodies, suggesting further unidentified target antigen or antigens associated with the pANCA. Fifteen of the 34 sera positive for ANCA also demonstrated anti-nuclear staining on Hep-2 cells (53%) in a speckled, homogeneous, or nucleolar pattern. ANA were significantly associated with the presence of pANCA ($P < 0{\cdot}01$), and levels of ANA and ANCA fell in parallel after treatment. One serum with a pANCA was also positive for anti-GBM antibodies. Inhibition studies using ELISAs for anti-GBM antibodies indicated that there was no cross-reactivity between target molecules recognized by these antibodies. The diversity of target molecules recognized by ANCA suggests that cross-reactivity with bacterial structures is less likely as the primary aetiological event in the development of these antibodies than tissue destruction; and that cross-reactivity with vascular endothelium is also unlikely as the pathogenetic basis of vessel disease.

Keywords anti-neutrophil cytoplasm antibodies anti-nuclear antibodies anti-glomerular basement antibodies elastase myeloperoxidase

INTRODUCTION

An autoimmune basis for Wegener's granulomatosis and microscopic polyarteritis (MPA) has long been suspected on the basis of the clinical response to cyclophosphamide (Fauci & Wolff, 1971) and plasma exchange (Pusey & Lockwood, 1984) and the findings of circulating immune complexes (Howell & Epstein, 1976), rheumatoid factor, anti-nuclear antibodies (ANA) and occasionally other autoantibodies (Shillitoe *et al.*, 1974). The recent description of circulating antibodies directed against cytoplasmic constituents of human neutrophils (anti-neutrophil cytoplasm antibodies, ANCA) (Davies *et al.*, 1982; van der Woude *et al.*, 1985) has provided a highly specific immunologic marker for these conditions.

Two patterns of immunofluorescence can be distinguished when sera containing ANCA are tested on normal human

Correspondence: Dr J. Savige, Department of Haematology, Repatriation General Hospital, Private bag 1, Heidelberg West, Victoria, Australia

neutrophils (Falk & Jennette, 1988). With some, mainly from patients with Wegener's granulomatosis (Nolle *et al.*, 1989) there is uniform fine granular staining of the cytoplasm (cANCA) and the antigen in many cases is a serine proteinase with a molecular weight of 29 kD (Goldschmeding *et al.*, 1989b) that is probably human neutrophil proteinase 3 (Ludemann *et al.*, 1990). With other sera immunofluorescent staining of ethanol-fixed neutrophils results in staining in a perinuclear pattern (pANCA); this however is artefactual, and with cells fixed in formalin–acetone these same sera produce diffuse cytoplasmic staining (Falk & Jennette, 1988). Most pANCA are directed gainst myeloperoxidase (Falk & Jennette, 1988) although these antibodies may also be directed against elastase (Goldschmeding *et al.*, 1989a). pANCA are frequent in MPA, segmental necrotizing glomerulonephritis (SNGN) and in Wegener's granulomatosis confined to the kidney (Falk & Jennette, 1988) but these antibodies may also be found in occasional cases of rheumatoid arthritis (Savage *et al.*, 1987) and systemic lupus erythematosus (SLE) (Falk & Jennette, 1988).

Other auto-antibodies are common in Wegener's granulomatosis and MPA. Rheumatoid factor is recognized in 41% (Savage *et al.*, 1985), 50% (Parlevliet *et al.*, 1988) or 67% (Pinching *et al.*, 1983) of different series. The reported incidence of ANA varies from 0% (Shillitoe *et al.*, 1974; Ronco *et al.*, 1983; Pinching *et al.*, 1983) to 13% (Ludemann & Gross, 1987), 14% (Falk & Jennette, 1988), 18% (Parlevliet *et al.*, 1988) and 21% (Savage *et al.*, 1985). There have also been isolated reports of antibodies directed against glomerular basement membrane (GBM) (Wahls, Bonsib & Schuster, 1987), endothelial cells (Savage *et al.*, 1989), smooth muscle, thyroid microsomes and heart muscle (Ludemann & Gross, 1987).

The present study examines the target molecules of the antibodies found in ANCA-associated renal diseases and the associations between these antibodies.

PATIENTS AND METHODS

Patients

Sera from 46 patients with active disease and histological evidence of renal involvement by Wegener's granulomatosis (eight), MPA (20) or SNGN (18) were studied. These diagnoses were made on the basis of clinical and renal histological features. Where renal histology was insufficient to make the diagnosis of Wegener's granulomatosis or MPA, these patients were grouped under the heading SNGN.

Immunofluorescent staining of whole blood leucocytes for ANCA and anti-neutrophil-specific nuclear antibodies (ANNA)

The method is from the First International Workshop on ANCA using a concentrated cytocentrifuge preparation of whole-blood leucocytes (Wiik, 1988).

Ten millilitres of heparinized blood were centrifuged at 150 ***g*** and the platelet-rich plasma was removed. After mixing, the blood was layered on a mixture of 2% methylcellulose:32·8% sodium metrizoate (8:5) and allowed to settle for 30–40 min. The supernatant was then removed, spun and the pellet resuspended in 0·1 M phosphate-buffered saline (PBS) containing 1% human serum albumin. The leucocytes were deposited on ethanol-cleaned slides by means of a cytocentrifuge (Shandon Cytopsin) at 350 ***g*** for 5 min. The slides were fixed in 100% ethanol at 4°C, air-dried and stored at −20°C.

Slides were incubated with a 1/10 dilution of test sera for 30 min at room temperature in a moist chamber. When a positive result was observed, the titre was measured using dilutions of 1/40, 1/160, 1/320 and 1/640. After washing the slides were incubated with FITC-conjugated rabbit anti-human globulin (DaKO), diluted 1/10 in PBS for 30 min. After further washing the slides were mounted in 90% glycerol in PBS and examined by epi-illumination using incident narrow-band blue light. They were then scored for the presence and character of neutrophil and monocyte cytoplasmic immunofluorescence, and neutrophil and lymphocyte nuclear staining by two independent examiners. The titre was taken as the last dilution at which specific staining was discernible.

Each set of tests included negative and positive standards where the positive sera had been compared with the samples provided by the Danish State Serum Institute.

Western blotting of antigens recognized by ANCA

Neutrophil cytoplasm extract was used for the Western blots. The results were demonstrated to be reproducible. Neutrophils were collected by sedimentation on 6% dextran/0·9% NaCl and Ficoll gradients. They were then lysed in Triton X-100 (Sigma) in the presence of freshly prepared protease inhibitors, 1 mM PMSF, 2·5 mM EDTA and Trasylol (Bayer, 240 kallikrein inactivator U/ml). The preparation was centrifuged at 1500 ***g*** for 10 min and the optical density (OD) at 280 nm of the supernatant determined. The cytoplasmic extract was then stored at −20°C at 20 mg/ml.

The extract was diluted with an equal volume of SDS buffer (62 mM Tris HCl, pH 6·8, 0·2% SDS, 50 mM dithiothreitol, 10% glycerol) and separated on a 6–20% gradient SDS acrylamide resolving gel with a 5% stacking gel (Laemmli, 1970). A mixture of low and high molecular weight markers (BioRad) had been included in another track. Following electrophoresis the proteins were transferred to nitrocellulose sheets (BioRad) with a BioRad electroblot apparatus in transfer buffer (25 mmol/l Tris, 192 mmol glycine containing 20% methanol) for 6 h at room temperature (Towbin, Staehlin & Gordon, 1984). After transfer the remaining protein sites on the nitrocellulose were blocked at room temperature with a solution of 5% non-fat dried milk powder in PBS, pH 7·4 ('Blotto') (Johnson *et al.*, 1984). The nitrocellulose was then cut into strips about 0·5-cm wide and each strip was probed with a serum containing ANCA diluted 1/25 in Blotto for 2 h at room temperature. After washing four times with Blotto the strips were reacted with 25 μCi ^{125}I–protein A (specific activity approximately 40 μCi/μg) in 2 ml Blotto for 20 min, washed five more times, air-dried, covered with thin plastic (Gladwrap, Union-Carbide) and autoradiographed using Agfa Curix RP2 X-ray film and an intensifying screen (Cronex, Dupont).

Anti-myeloperoxidase antibodies

In preliminary checkerboard experiments, the optimal concentration of myeloperoxidase (Calbiochem) for coating plates was determined (1 μg/ml in PBS). There was no contamination of myeloperoxidase with elastase or any other major proteins on SDS–PAGE. Myeloperoxidase was coated to polyvinylchloride microtitre plates (Dynatech) for 18 h at 4°C. Each serum was assayed at a dilution of 1/8 and in triplicate and each assay

Table 1. Anti-neutrophil cytoplasm antibodies (ANCA) and anti-neutrophil-specific nuclear antibodies (ANNA) in ANCA-associated diseases

		ANCA positive							
		cANCA		pANCA		ANNA positive		ANCA positive	
Disease	*n*	*n*	%	*n*	%	*n*	%	*n*	%
WG	7	5	72	1	14	0	0	1	14
MPA	20	7	35	7	35	4	20	2	10
SNGN	19	4	21	10	53	1	5	4	21
Total	(*n*=46)	16	35	18	39	5	11	7	15

cANCA, ANCA with cytoplasmic staining on indirect immunofluorescence; pANCA, ANCA with perinuclear staining on indirect immunofluorescence; WG, Wegener's granulomatosis; MPA, microscopic polyarteritis; SNGN, segmental necrotizing glomerulonephritis.

contained a positive and negative standard. Bound antibody was detected with alkaline phosphatase-linked anti-heavy and light chain antiserum (Dako) diluted 1/500 in PBS/Tween 20 (Sigma; PBS/T20) containing 0·1% bovine serum albumin (BSA); the substrate used was *p*-nitrophenylphosphate (Sigma), 5 mg tablet in 5 ml 0·05 M carbonate buffer, pH 9·6, with 0·02% $MgCl_2$. All incubations were for 1 h and plates were washed three times with PBS/T20 between incubations. The amount of binding was determined in an ELISA plate reader at OD 405 nm (Dynatech Instruments) and results were expressed as percentages of a strongly binding reference standard. Intra-assay variation was 7% and inter-assay variation 9%. The assays for the detection of anti-myeloperoxidase and anti-elastase antibodies are to be described in detail elsewhere (manuscript submitted for publication).

Anti-elastase antibodies

Again the optimal concentration for coating elastase (Calbiochem) to microtitre plates was determined in preliminary experiments (5 μg/ml in PBS). Elastase was not contaminated with significant amounts of any other proteins on SDS–PAGE. Elastase was coated to plastic microtitre plates (Dynatech) for 18 h at 4°C. The assay was then performed as for the anti-myeloperoxidase ELISA.

Immunofluorescent staining of Hep-2 cells for ANA

Sera were tested on Hep-2 cells using a commercial kit (Quantafluor, Kallestad). Two sera were examined using rat liver as the source of nuclei. The serum dilutions and method were similar to those described above for the detection of ANCA.

Anti-GBM antibodies

The ELISA for anti-GBM antibodies is described elsewhere (Savige, Mavrova & Kincaid-Smith, 1989); briefly, the non-collagenous region of type IV collagen that contains the Goodpasture antigen was obtained by the collagenase digestion (collagenase type V, Sigma) of isolated GBM. This was coated to microtitre plates at a concentration of 30 μg/ml for 18 h at 4°C. The assay was then performed as for the anti-myeloperoxidase ELISA.

Inhibition studies were performed on the serum that contained both anti-GBM antibodies and ANCA in order to detect cross-reactive epitopes. Forty microlitres of neutrophil extract or GBM (both at 3 mg/ml) were incubated with a 1/4 dilution of serum in a total of 320 μl for 1 h at 37°C, immediately before the assay was performed. Significant inhibition was considered to have occurred if pre-incubation reduced binding by 15% (Bowman & Lockwood, 1985).

In order to study the antigens recognized by anti-GBM antibodies and ANCA, Western blots were performed as described above. GBM and neutrophil extract were electrophoresed in loading buffer without dithiothreitol, transferred to nitrocellulose, blotted with the serum that contained both anti-GBM antibodies and ANCA and exposed to X-ray film.

Statistical analysis

Results were analysed using χ^2 analysis.

RESULTS

Immunofluorescent staining of whole blood leucocytes for ANCA and ANNA

Of the 46 sera tested, 39 produced staining of whole blood neutrophils (Table 1). In 16 the staining was diffuse throughout the cytoplasm with a fine granular pattern with minimal perinuclear accentuation (cANCA); these included five of the sera from seven patients with Wegener's granulomatosis. In 18 others the staining was predominantly perinuclear (pANCA) and this pattern was found in only one patient with Wegener's granulomatosis but in about half of the patients with MPA or SNGN. In the remaining five, the staining was predominantly nuclear (ANNA) and there was no associated cytoplasmic staining.

Target molcules recognized by ANCA

Nine sera from patients were examined in Western blots (Fig. 1). By immunofluorescence, seven of these sera (no. 1, 2, 3, 5, 6, 7 and 8) contained typical cANCA but one (no. 4) contained ANNA and one (no. 9) pANCA and anti-myeloperoxidase antibodies.

Four sera with cANCA (no. 1, 2, 3 and 8) contained antibodies that recognized a 29-kD molecule. One of these also

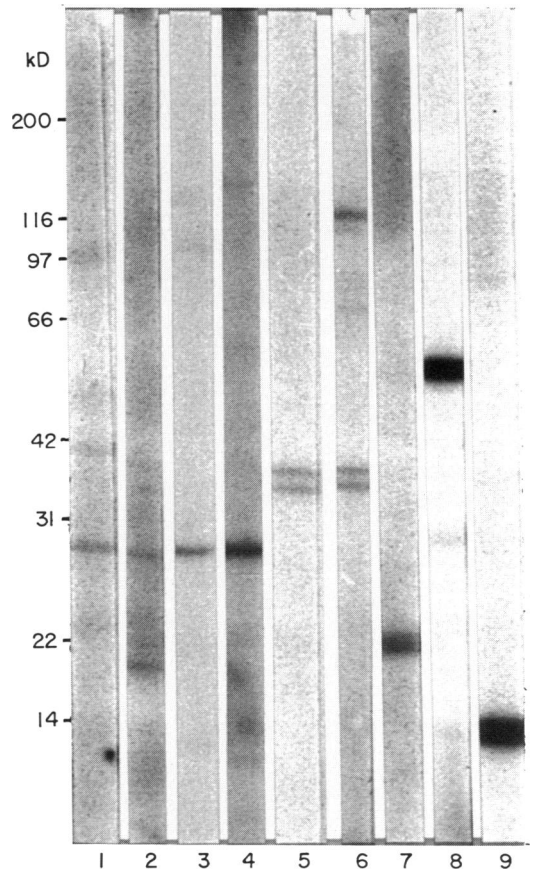

Fig. 1. Heterogeneity of antigens recognized by anti-neutrophil cytoplasm antibodies (ANCA). Western blot of ANCA-containing sera. Clinical diagnosis and pattern of staining seen with indirect immunofluorescence. Lane 1, microscopic polyarteritis (MPA), ANCA with cytoplasmic staining (cANCA); lane 2, Wegener's granulomatosis (WG), cANCA; lane 3, MPA, cANCA; lane 4, MPA, anti-neutrophil-specific nuclear antibodies (ANNA); lane 5, WG, cANCA; lane 6, WG, cANCA; lane 7, segmental necrotizing glomerulonephritis (SNGN), cANCA; lane 8, SNGN, cANCA with speckled anti-nuclear antibodies (ANA) on Hep-2 cells; lane 9, MPA, ANCA with perinuclear staining on indirect immunofluorescence (pANCA).

Table 2. Anti-nuclear antibodies (ANA) in renal diseases associated with anti-neutrophil cytoplasm antibodies (ANCA)

		Speckled		Homogeneous		Nucleolar		Negative	
Disease	*n*	*n*	%	*n*	%	*n*	%	*n*	%
WG	7	0	0	0	0	0	0	7	100
MPA	20	5	25	2	10	1	5	12	60
SNGN	19	4	21	3	16	0	0	12	63
Total	(*n*=46)	9	20	5	11	1	2	31	67

WG, Wegener's granulomatosis; MPA, microscopic polyarteritis; SNGN, segmental necrotizing glomerulonephritis.

Table 3. Inhibition studies of antibody with anti-neutrophil cytoplasm antibodies (ANCA) and anti-glomerular basement membrane (GBM) antibody activity in anti-GBM ELISA

	PBS	GBM	Neutrophil extract
Patient J	0·601	0·146 (24·3%)	0·620 (103%)

The patient's serum contains ANCA and anti-GBM antibodies. Inhibition could be readily demonstrated by pre-incubation of this serum with GBM but not by pre-incubation with the neutrophil extract.

PBS, phosphate-buffered saline.

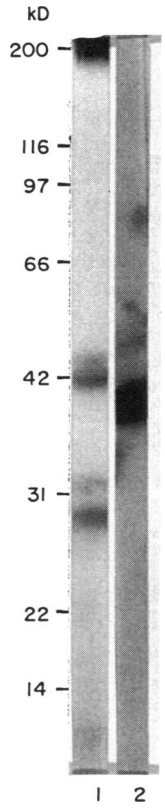

Fig. 2. Western blot of serum containing anti-glomerular basement membrane (GBM) antibodies and anti-neutrophil cytoplasm antibodies (ANCA). Lane 1, collagenase-digested GBM blotted with serum shows typical bands found with anti-GBM serum; lane 2, neutrophil extract blotted with serum shows a whole band in the 38–42-kD range that does not corespond to any of those seen on collagenase-digested GBM.

recognized a molecule of about 58 kD that would be consistent with a dimer of the 29 kD molecule. The serum with ANNA also recognized the 29-kD molecule. In contrast, two sera with cANCA recognized molecules of 36, 38 and 116 kD (in one). Finally, one serum with pANCA and anti-myeloperoxidase antibodies also recognized a molecule of about 12 kD.

Anti-myeloperoxidase antibodies

Thirteen of the 46 sera tested contained anti-myeloperoxidase antibodies (28%); 10 of these produced perinuclear staining on

indirect immunofluorescence but in two others the staining was predominantly nuclear on the normal peripheral blood neutrophils (ANNA). Low-titre anti-myeloperoxidase antibodies were found associated with a weak cANCA and speckled ANA. However, anti-myeloperoxidase antibodies were absent from eight further sera with typical perinuclear staining and from the remaining three sera with ANNA.

Anti-elastase antibodies

These were present in three of the 17 sera tested. All were associated with pANCA by immunofluorescence, and contained anti-myeloperoxidase antibodies. The remaining 14 sera that were negative for anti-elastase antibodies included six in which pANCA were demonstrated by immunofluorescence. Of the eight sera with pANCA that were negative for anti-myeloperoxidase antibodies, three were also negative for anti-elastase antibodies.

Anti-nuclear antibodies

These were detected in 15 sera. The patterns of staining in the different diagnostic groups are summarized in Table 2. In 13 sera ANA were associated with ANCA or ANNA ($P < 0{\cdot}01$).

Titres were low: 1/40 or less in nine cases and 1/160 in six. Follow-up sera were available in six patients who had both ANA and ANCA. In all cases there was a reduction in titres of both ANA and ANCA after treatment, and in one case a speckled ANA was replaced by a homogeneous pattern. In one serum initially negative for ANA, a weak homogeneous pattern developed 10 months after the initial presentation at a time when ANCA had completely disappeared.

Anti-GBM antibodies

Ten sera containing ANCA were also tested for anti-GBM antibodies. One was positive (10%). This serum contained pANCA and the presence of anti-myeloperoxidase antibodies was confirmed by ELISA.

Pre-incubation of this serum with GBM in the anti-GBM ELISA resulted in inhibition (Table 3) but there was no inhibition with an equal concentration of the neutrophil extract. This suggests that the binding of the serum in the anti-GBM ELISA is specific, and that there is no cross-reactivity between the epitopes recognized by the anti-GBM antibodies and the neutrophil extract. That is, ANCA and anti-GBM antibodies occur together in this patient incidentally.

This observation was confirmed by the results of the Western blots (Fig. 2). The serum that contained both anti-GBM antibodies and ANCA demonstrated the typical pattern of bands on GBM but a single band in the region of 38–42 kD on the neutrophil extract. The single band did not correspond to any of the bands seen on GBM.

DISCUSSION

ANCA are found commonly in Wegener's granulomatosis, MPA and in SNGN (where a vasculitic illness is suspected). However, ANCA are not specific for Wegener's granulomatosis and MPA, and have also been described in SLE (Falk & Jennette, 1988) and rheumatoid arthritis (Savage *et al.*, 1987).

Both cANCA and pANCA were identified in Wegener's granulomatosis, MPA and SNGN. pANCA were uncommon in Wegener's granulomatosis (one out of seven sera) but both cANCA and pANCA were found almost equally in MPA and SNGN. Three target molecules for cANCA and pANCA have been identified so far. The molecule corresponding to cANCA is often neutrophil proteinase 3, with a molecular weight of 29 kD, but we have demonstrated with Western blots that cANCA may be directed against other molecules, whose origin is at present unclear. Secondly, while most pANCA are directed against myeloperoxidase and elastase our data from specific ELISAs show that pANCA may be directed against other molecules and that both of these antibodies may occur together in the one serum. Thus ANCA encompasses a family of antibodies directed against neutrophil cytoplasm constituents (all of which currently identified molecules are enzymes).

In anti-GBM disease in addition to the characteristic antibodies against the non-collagenous domain of type IV collagen, antibodies directed against the triple helix of collagen type IV and other basement membrane proteins may also be detected. Likewise we have demonstrated that ANA are common in some ANCA-associated diseases, occurring in nearly half of all cases of MPA and SNGN, but rarely in Wegener's granulomatosis. ANA were of various specificities with speckled and homogeneous patterns predominating. Nucleolar antibodies were present in only one serum. ANA were found more often when pANCA were present and levels correlated with the activity of ANCA. The parallel development of these antibodies suggests a common aetiopathogenetic mechanism. Further evidence of this would come with the observation that both antibodies recurred simultaneously in disease relapse.

We noted ANNA in five sera. These antibodies have been described in a variety of disorders, including primary biliary cirrhosis, inflammatory bowel disease (Snook *et al.*, 1989), rheumatoid arthritis and the seronegative arthritides (Faber & Elling, 1966; Elling, 1967). The antigen or antigens have not been identified and may not even be nuclear in origin. The characteristic staining pattern may arise from artefactual distribution depending on the fixatives used (Briggs *et al.*, 1981) as is the case for anti-myeloperoxidase antibodies (Falk & Jennette, 1988).

pANCA were associated with anti-GBM antibodies in one case. Both antibodies were present in the initial serum and further samples were not available. There was no cross-reactivity demonstrable between ANCA and anti-GBM antibodies in this serum. It is unlikely, although possible, that the anti-GBM antibodies arose secondary to ANCA-mediated GBM damage. This mechanism has been described in membranous glomerulonephritis (Klassen *et al.*, 1974; Kurki *et al.*, 1984) and in IgA disease (Savige & Kincaid-Smith, 1989). If this were the case, anti-GBM antibodies could arise secondary to lung-localized Wegener's granulomatosis since the Goodpasture antigen against which anti-GBM antibodies are directed, is found in the alveolar basement membrane too. In the patient described here there was no histological evidence to support a diagnosis of Wegener's granulomatosis.

Because of the number of antigens identified by ANCA, ANA and other autoantibodies it is improbable that they all arise by cross-reactivity with an external stimulus such as a bacterial organism. However, infections have been implicated in both the onset and relapses of Wegener's granulomatosis and MPA. Chronic suppurative diseases of the respiratory tract such as otitis media, sinusitis, bronchiectasis and tuberculosis have been described prior to Wegener's granulomatosis (Pinching *et*

al., 1983). Infections with cytomegalovirus, hepatitis (Ronco *et al.*, 1983) and various arboviruses have been implicated in the onset of MPA and Wegener's granulomatosis. However, ANCA and ANA may develop in response to tissue breakdown, especially of neutrophils, from various causes. ANCA were first described in conditions where neutrophils lyse after multiple blood transfusions (Calabresi, Edwards & Schilling, 1959). However, ANCA are not simply markers of tissue injury since they are not commonly found in SLE, rheumatoid arthritis, primary biliary cirrhosis or inflammatory bowel disease. Their origin and significance remain to be elucidated.

ACKNOWLEDGMENTS

This work was supported in part by the National Health and Medical Research Council of Australia. We would like to thank the many physicians who provided us with sera.

REFERENCES

BOWMAN, C. & LOCKWOOD, C.M. (1985) Clinical application of a radioimmunoassay for auto-antibodies to glomerular basement membrane antibodies. *J. clin. Lab. Immunol.* **17,** 197.

BRIGGS, R.C., GLASS, W.F., MONTIEL, M.M. & HNILICA, L.S. (1981) Lactoferrin: nuclear localisation in the human neutrophilic granulocyte? *J. Histochem. Cytochem.* **29,** 1128.

CALABRESI, P., EDWARDS, E.A. & SCHILLING, R.F. (1959) Fluorescent antiglobulin studies in leukopenic and related disorders. *J. clin. Invest.* **38,** 2091.

DAVIES, D.J., MORAN, J.E., NIALL, J.F. & RYAN, G.B. (1982) Segmental necrotising glomerulonephritis with anti-neutrophil antibody: possible arbovirus etiology? *Br. med. J.* **285,** 606.

ELLING, P. (1967) On the incidence of anti-nuclear factors in rheumatoid arthritis. *Acta rheumatol. Scand.* **13,** 101.

FABER, V. & ELLING, P. (1966) Leucocyte-specific anti-nuclear factors in patients with Felty's syndrome, rheumatoid arthritis, systemic lupus erythematosus and other diseases. *Acta medica Scand.* **29,** 1128.

FALK, R.J. & JENNETTE, J.C. (1988) Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotising and crescentic glomerulonephritis. *N. Engl. J. Med.* **318,** 1651.

FAUCI, A.S. & WOLFF, S.M. (1971) Wegener's granulomatosis: studies in eighteen patients and a review of the literature. *Medicine*, **52,** 535.

GOLDSCHMEDING, R., COHEN-TERVAERT, J.W., VAN DER SCHOOT, C.E., VAN DER VEEN, C, KALLENBERG, C.G.M. & VON DEM BORNE, A.E.G.K. (1989a) ANCA, anti-myeloperoxidase and anti-elastase: 3 members of a novel class of autoantibodies against myeloid lysosomal enzymes. *APMIS*, **97,** 48.

GOLDSCHMEDING, R., TEN BOKKEL HUININK, D., FABER, N., TETTEROO, P.A.T., VROOM, T.M., HACK, C.E. & VON DEM BORNE, A.E.G.K. (1989b) Identification of the 'ANCA antigen' as a novel myeloid lysosomal serine protease. *APMIS*, **97,** 46.

HOWELL, S.B. & EPSTEIN, W.V. (1976) Circulating immune complexes in Wegener's granulomatosis. *Am. J. Med.* **60,** 259.

JOHNSON, D.A., GAUTSCH, J.W., SPOTTSMAN, J.R. & ELDER, J.H. (1984) Improved technique utilising non-fat dry milk for analysis of protein and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* **1,** 3.

KLASSEN, J., ELWOOD, C., GROSSBERG, A., MILGROM, F., MONTES, M., SEPULVEDA, M. & ANDRES, G.A. (1974) Evolution of membranous nephropathy into anti-glomerular basement membrane glomerulonephritis. *N. Engl. J. Med.* **290,** 1340.

KURKI, P., HELVE, T., VON BONSDORFF, M., TORNROTH, T., PETTERSSON, E., RISKA, H. & MIETTINEN, A. (1984) Transformation of membranous glomerulonephritis into crescentic glomerulonephritis with anti-glomerular basement membrane antibodies. *Nephron*, **38,** 134.

LAEMMLI, U.K. (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680.

LUDEMANN, G. & GROSS, W.L. (1987) Autoantibodies against cytoplasmic structures of neutrophil granulocytes in Wegener's granulomatosis. *Clin. exp. Immunol.* **69,** 350.

LUDEMANN, J., CSERNOK, E., ULMER, M., LEMKE, H., UTECHT, B., RAUTMANN, A. & GROSS, W.L. (1990) Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis: immunodiagnostic value, monoclonal antibodies and characterisation of the target antigen. *Neth. J. Med.* **36,** 157.

NOLLE, B., SPECKS, U., LUDEMANN, J., ROHRBACH, M.S., DEREMEE, R.A. & GROSS, W.L. (1989) Anti-cytoplasmic autoantibodies: their immunodiagnostic value in Wegener's granulomatosis. *Ann. intern. Med.* **111,** 28.

PARLEVLIET, K.J., HENZEN-LOGMANS, S.C., OE, P.L., BRONSVELD, W., BALM, A.J.M. & DONKER, A.J.M. (1988) Antibodies to components of neutrophil cytoplasm: a new diagnostic tool in patients with Wegener's granulomatosis and systemic vasculitis. *Q. J. Med.* **249,** 55.

PINCHING, A.J., LOCKWOOD, C.M., PUSSELL, B.A., REES, A.J., SWENY, P., EVANS, D.J., BOWLEY, N. & PETERS, D.K. (1983) Wegener's granulomatosis: observations on 18 patients with severe renal disease. *Q. J. Med.* **208,** 435.

PUSEY, C.D. & LOCKWOOD, C.M. (1984) Plasma exchange for glomerular disease. In *Nephrology* (ed. by R.R. Robinson), p. 1474. Springer-Verlag, New York.

RONCO, P., VERROUST, P., MIGNON, F., KOURILSKY, O., VANHILLE, P., MEYRIER, A., MERY, J.P. & MOREL-MAROGER, L. (1983) Immunopathological studies of polyarteritis nodosa and Wegener's granulomatosis: a report of 43 patients with 51 renal biopsies. *Q. J. Med.* **206,** 212.

SAVAGE, C.O.S., POTTINGER, C., LOCKWOOD, C.M. & PEARSON, J.D. (1989) Anti-endothelial antibodies (AECA) and anti-neutrophil cytoplasm antibodies (ANCA) in Wegener's granulomatosis and microscopic polyarteritis. In *Proceedings of the Seventh International Congress of Immunology, Berlin* p. 508. Fischer, Stuttgart.

SAVAGE, C.O.S., WINEARLS, C.G., EVANS, D.J., REES, A.J. & LOCKWOOD, C.M. (1985) Microscopic polyarteritis: presentation, pathology and prognosis. *Q. J. Med.* **220,** 467.

SAVAGE, C.O.S., WINEARLS, C.G., JONES, S., MARSHALL, P.D. & LOCKWOOD, C.M. (1987) Prospective study of radio-immunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. *Lancet*, **i,** 1389.

SAVIGE, J.A. & KINCAID-SMITH, P.S. (1990) Anti-glomerular basement membrane antibody disease associated with mesangial proliferative glomerulonephritis. *Am. J. Kidney Dis.* (In press).

SAVIGE, J.A., MAVROVA, L. & KINCAID-SMITH, P.S. (1990) Inhibitable anti-glomerular basement membrane antibody in Alport's syndrome. *Transplantation*, (In press).

SHILLITOE, E.J., LEHNER, T., LESSOF, M.H. & HARRISON, D.F.N. (1974) Immunological features of Wegener's granulomatosis. *Lancet*, **ii,** 281.

SNOOK, J.A., CHAPMAN, R.W., FLEMING, K. & JEWELL, D.P. (1989) Anti-neutrophil nuclear antibody in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis. *Clin. exp. Immunol.* **76,** 30.

TOWBIN, H., STAEHLIN, T. & GORDON, J. (1979) Electrophoretic transfer of proteins for polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natl. Acad. Sci. USA*, **76,** 4350.

VAN DER WOUDE, F.J., RASMUSSEN, N., LOBATTO, S., WIIK, A., PERMIN, H., VAN ES, L.A., VAN DER GIESSEN, M., VAN DER HEM, G.K. & THE, T.H. (1985) Auto-antibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet*, **i,** 425.

WAHLS, T.L., BONSIB, S.M. & SCHUSTER, V.L. (1987) Co-existent Wegener's granulomatosis and anti-glomerular basement membrane disease. *Hum. Pathol.* **18,** 202.

WIIK, A. (1988) Delineation of a standard procedure for indirect immunofluorescence of ANCA. *APMIS*, **97,** 12.