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Anti-neutrophil cytoplasm antibodies in rheumatoid arthritis

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SUMMARY

Anti-neutrophil cytoplasm antibodies (ANCA) occur occasionally in rheumatoid arthritis (RA), but their incidence and clinical significance have been unclear. In this study we have investigated 58 patients with RA. In 22 patients the disease was inactive and the remaining 36 with active disease were further subdivided into those without clinical evidence of vasculitis (26), those with cutaneous vasculitis (8) and those with systemic vasculitis (2). ANCA were demonstrated by indirect immunofluorescence in 10 of the 58 patients (17%). While both perinuclear (pANCA) and cytoplasmic (cANCA) staining were detected, pANCA were more common (70%). Neutrophil-specific anti-nuclear antibodies (ANNA) were demonstrated in a further eight sera (14%) and ANA were detected on Hep-2 cells in 30 of the 58 sera (52%). ELISAs for the detection of anti-myeloperoxidase and anti-elastase antibodies were then established. Five sera with pANCA and five that contained ANNA were negative for both anti-myeloperoxidase and anti-elastase antibodies, suggesting other as yet unidentified cytoplasmic antigens as the target molecules. However, anti-myeloperoxidase or anti-elastase antibodies were found in four sera that had homogeneous or speckled ANA on both Hep-2 cells and neutrophils. One serum contained both antibodies. The presence of ANCA detected by indirect immunofluorescence or of anti-myeloperoxidase or anti-elastase antibodies in these patients with RA was not associated with disease activity nor with the demonstration of cutaneous vasculitis or renal disease (*P* NS). A possible association with systemic vasculitis remains to be confirmed. There is an incomplete correlation between indirect immunofluorescence patterns and antibody specificity in ELISA systems.

Keywords arthritis vasculitis anti-neutrophil cytoplasm antibodies anti-nuclear antibodies

INTRODUCTION

Anti-neutrophil cytoplasm antibodies (ANCA) are directed against extra-nuclear components of neutrophils and monocytes and are common in certain small vessel vasculitides (Davies *et al.*, 1982; van der Woude *et al.*, 1985; Savage *et al.*, 1987), such as Wegener's granulomatosis and microscopic polyarteritis. Two major groups of antibodies can be distinguished on indirect immunofluorescence; these produce patterns that are either cytoplasmic (cANCA) or perinuclear (pANCA) in distribution (Falk & Jennette, 1988). cANCA are typically found in Wegener's granulomatosis (Nolle *et al.*, 1989) and are usually directed against a 29-kD molecule (Goldschmeding *et al.*, 1989b) recently identified as neutrophil proteinase 3 (Ludemann, Utecht & Gross, 1989) but other specificities also occur. pANCA are found more commonly in microscopic polyarteritis and segmental necrotizing glomerulonephritis (Falk & Jennette, 1988) and the perinuclear immunofluorescence is found only when sera are tested on alcohol-fixed neutrophils, presumably after the leakage of granular components with alcohol fixation (Falk & Jennette, 1988). When blood smears are fixed with acetone the pattern of staining with pANCA is diffusely cytoplasmic. In many cases of the renal vasculitides, sera that are positive for pANCA can be shown to have antibodies directed against myeloperoxidase (Falk & Jennette, 1988), elastase (Goldschmeding *et al.*, 1989a) and lactoferrin (Falk *et al.*, 1990). The significance of these antibodies is unclear but their levels appear to parallel disease activity in Wegener's granulomatosis and microscopic polyarteritis (Savage *et al.*, 1987) and they may be useful diagnostically when clinical features are also taken into account. ANCA have been recorded in rheumatoid arthritis (RA) (Savage *et al.*, 1987) although the frequency and association with vasculitis have not been investigated. Here we have studied the incidence of ANCA in RA, the corresponding antigen specificities and possible clinical correlations.

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Table 1. Clinical characteristics of RA patients studied

Clinical category	*n*	M:F	Disease duration (years) median (range)
(i) Inactive	22	10:12	18 (3–31)
(ii) Active, no vasculitis	26	7:19	10 (1–27)
(iii) Active, cutaneous vasculitis	8	2:6	12 (5–23)
(iv) Active, systemic vasculitis	2	1:1	0, 37

PATIENTS AND METHODS

Patients

Fifty-eight patients with definite RA by American Rheumatism Association (ARA) criteria were studied; these included 38 females and 20 males with a median age of 61 years (range 25–78) and a median disease duration of 14 years (range 1–31) (Table 1). Thirty-nine of the 50 patients whose sera were tested for rheumatoid factor by Rose-Waaler assay were positive (titres greater than 1/32).

Patients were divided by T.J.C. and A.S. into the following groups:

(i) Inactive disease (four out of six ARA criteria of disease remission, Arnett *et al.*, 1988) ($n=22$).
(ii) Active disease without vasculitis ($n=26$).
(iii) Active disease with cutaneous vasculitis (nail fold infarcts, necrotic nodules, vasculitis ulcers or digital infarcts, $n=8$).
(iv) Active disease with systemic vasculitis (visceral, neural or extensive tissue involvement, $n=2$).

The following investigations were performed on sera, collected before the initiation of specific treatment for vasculitic lesions.

Indirect immunofluorescence for ANCA

ANCA were detected by the standard method of the First International Workshop on ANCA (Wiik, 1988). From 10 ml of heparinized blood collected by venepuncture, platelets were removed by centrifugation at 300 *g* and the supernatant plasma removed. After mixing the blood thoroughly this was layered upon the erythrocyte sedimentation agents, 2% methylcellulose (eight parts) and 32·8% sodium metrizoate (five parts). After 30–40 min the supernatant was washed and the leucocytes were resuspended in 0·1 M phosphate-buffered saline (PBS) containing 1% human serum albumin. These were then deposited on cleaned slides by means of a cytocentrifuge (Shandon Cytospin, Shandon, London, UK) at 300 *g* for 5 min. All sera were tested using neutrophil preparations that had been fixed in 100% ethanol at 4°C, air-dried and stored at −20°C until required for use.

Smears were then incubated with 1/10 dilutions of sera for 30 min at room temperature. These were then washed, incubated with fluorescein-conjugated rabbit anti-human IgG (Dako), washed again and mounted in glycerol. Slides were examined by epi-illumination using narrow-band blue light. Each set of tests included negative and positive control sera that had been compared with the international standards for ANCA supplied by the Danish Serum Institute. When a positive result was observed, the pattern of staining and titre was noted.

In addition, sera from 15 patients were tested using leucocytes that had been fixed in a formaldehyde/acetone mixture (55 ml of 0·45% formalin in 2·2 M PBS:45 ml acetone). Sera were only tested at a dilution of 1/10 but the procedure was otherwise as for the detection of ANCA.

Indirect immunofluorescence for anti-nuclear antibodies

A commercial Hep-2 cell kit system ('Quantafluor', Kallestad) was used. The method of examination was similar to that described above for the detection of ANCA but sera were initially examined at a dilution of 1/40. Sera were also examined for anti-nuclear antibodies (ANA) on peripheral blood neutrophils.

Antigens recognized by ANCA in Western blots

Neutrophil cytoplasm extract was prepared as follows. 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 units/ml). The preparation was centrifuged at 1500 *g* for 10 min and the OD_{280} of the supernatant determined. The cytoplasmic extract was stored at −20°C.

The extract was diluted with an equal volume of sodium dodecyl sulphate ($NaDodSO_4$) buffer (62 mM Tris HCl, pH 6·8, 0·2% $NaDodSO_4$, 50 mM dithiothreitol, 10% glycerol), about 50 μg of protein loaded per track and separated on a 6–20% gradient $NaDodSO_4$ acrylamide resolving gel with a 5% stacking gel (Laemmli, 1979). 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, Richmond, CA) 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, 1979). 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'). The nitrocellulose was then probed with serum corresponding to cANCA, or anti-myeloperoxidase or anti-elastase antibodies, diluted 1/25 in 'blotto' for 2 h at room temperature. After washing four times with 'blotto' for 2 h 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 a further five times, air-dried covered with thin plastic (Gladwrap, Union-Carbide Australia) and autoradiographed using Agfa Curix RP2 X-ray film and an intensifying screen (Cronex Dupont, USA).

ELISA for anti-myeloperoxidase antibodies

Myeloperoxidase (Calbiochem Behring Corp., La Jolla, CA) (1 μg/ml in PBS) was coated to polyvinylchloride microtitre plates (Dynatech, Plochingen, Germany) for 18 h at 4°C. Each serum was assayed at a 1/8 dilution, in triplicate, and each assay 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

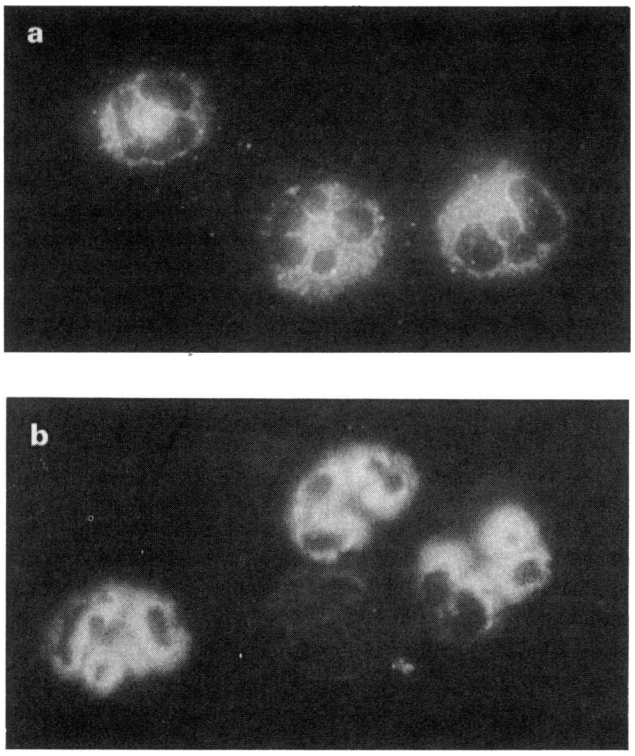

Fig. 1. (a) cANCA demonstrated by indirect immunofluorescence using 1/10 dilution of serum from a patient with RA examined on ethanol-fixed normal peripheral blood neutrophils (×495). (b) pANCA demonstrated by indirect immunofluorescence using 1/10 dilution of serum from patient with RA examined on ethanol-fixed normal peripheral blood neutrophils (×416).

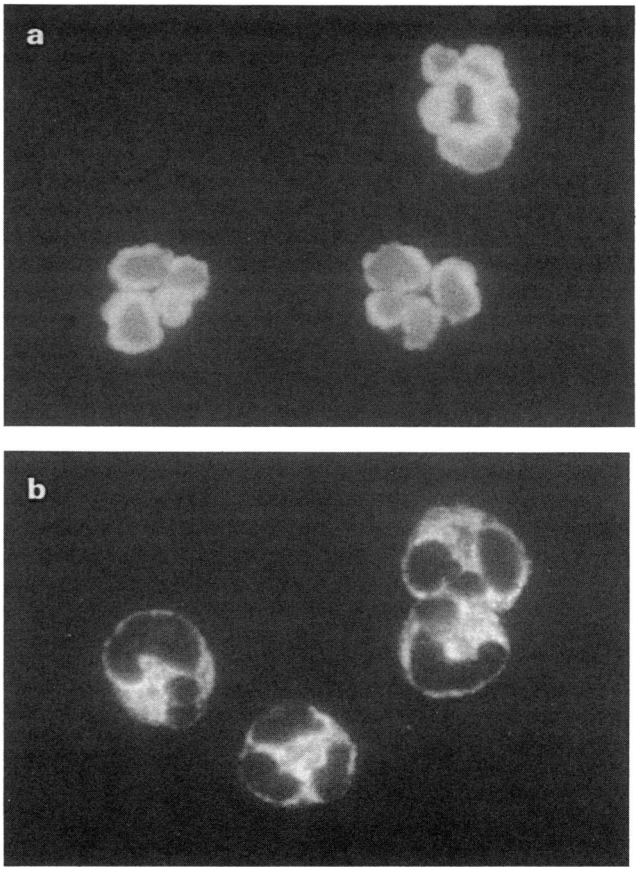

Fig. 2. (a) Neutrophil-specific ANA (ANNA) with perinuclear accentuation demonstrated by indirect immunofluorescence using 1/10 dilution of serum from a patient with RA examined on ethanol-fixed normal peripheral blood neutrophils (×378). (b) The same serum used to demonstrate cytoplasmic staining in (a) is examined using formalin-acetone fixed normal peripheral blood neutrophils and demonstrates diffuse cytoplasmic staining (×378).

three times with PBS/T20 between incubations. The amount of binding was determined in an ELISA plate reader at OD 405 nm (Dynatech) and results were expressed as percentages of a strongly binding reference standard. Positive sera bound at levels greater than the mean of 25 normal laboratory workers, +4 s.d.

ELISA for anti-elastase antibodies

Elastase (Calbiochem) (5 μg/ml in PBS) was coated to plastic microtitre plates (Dynatech) for 18 h at 4°C. The assay was then performed as for the anti-myeloperoxidase ELISA. A serum was considered positive if binding was greater than mean of 25 normal laboratory workers, +4 s.d.

Renal abnormalities

In all patients urine was tested by dipstick for the presence of blood and protein (Haema-combistix, Ames). Where positive for blood, a mid-stream urine specimen was examined for excess glomerular erythrocytes (>8000/ml) (Fairley & Birch, 1982); proteinuria was confirmed on 24 h urine collections. Renal biopsies were performed for clinical indications.

Statistical analysis

Results are expressed as the median plus range. The Wilcoxon rank sum test for unpaired samples was used for testing statistical significance.

RESULTS

Patients

Clinical details are described in Table 1. One patient with systemic vasculitis had mononeuritis multiplex and the other had a cutaneous vasculitis and extensive lung involvement.

Indirect immunofluorescence for ANCA (Figs 1a, b, 2a, b, Table 2)

ANCA were detected by indirect immunofluorescence in 10 of the 58 patients with RA (17%).

Both pANCA and cANCA were present in the sera tested and were found in all clinical groups except those with cutaneous vasculitis alone. However, one of the patients with systemic involvement also had a cutaneous vasculitis and cANCA. pANCA was more common, with seven of the 10 positive sera having this pattern of immunofluorescence (70%). When four of these sera were tested using formaldehyde/acetone fixed leucocytes, the perinuclear pattern was replaced by diffuse cytoplasmic staining of weak to moderate intensity. Two of the sera with pANCA also contained weak ANA (homogeneous and speckled) on Hep-2 cells. Three sera produced cANCA,

Table 2. ANCA, ANNA, ANA and rheumatoid factor (RF) in patients with RA

Clinical category (*n*)	ANCA		ANNA	ANA-Hep-2 cells	RF present
	pANCA	cANCA			
(i) Inactive (22)	2	1	1	9 (h8, s1)	15/18
(ii) Active, no vasculitis (26)	4	1	6	15 (h10, s5)	15/23
(iii) Active, cutaneous vasculitis (8)	0	0	1	5 (h5)	7/8
(iv) Active, systemic vasculitis (2)	1	1	0	1 (s)	2/2
Total	7	3	8	30 (h23, s7)	39/50

h, homogeneous; s, speckled.

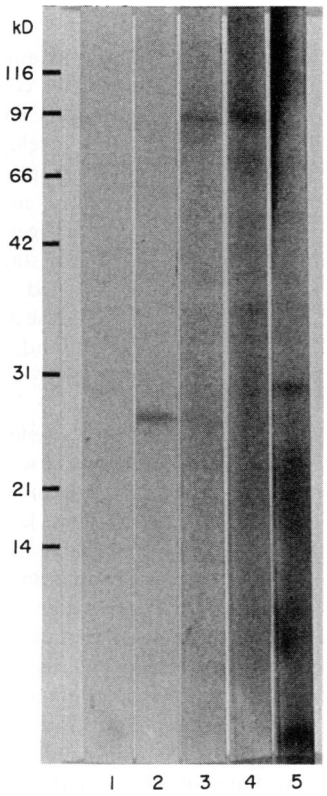

Fig. 3. Western blot of sera from a normal control (track 1) and patients (tracks 2–5). This demonstates binding to neutrophil proteinase 3 (associated with cANCA, from active systemic disease, track 2), myeloperoxidase (associated with homogeneous ANA, active disease, no vasculitis, tracks 3 and 4) and elastase (associated with speckled ANA, active disease, no vasculitis, track 5).

which was finely granular in two and homogeneous in the third. The two sera associated with the finely granular cANCA were associated with homogeneous and speckled staining of Hep-2 cells and the third serum with low intensity homogeneous nuclear staining. Two of the three sera with cANCA had very high titres of rheumatoid factor.

In addition, eight sera contained neutrophil-specific ANA (ANNA) where there was no staining of lymphocyte or Hep-2 cell nuclei. When two of these sera were tested using formaldehyde/acetone-fixed cells the nuclear staining was replaced with moderately intense diffuse staining of neutrophil cytoplasm. This was most pronounced in a serum in which anti-myeloperoxidase antibodies were detected by ELISA.

Indirect immunofluorescence for ANA (Table 2)

Thirty sera were positive for ANA on Hep-2 cells. Twenty-three of these demonstrated a homogeneous pattern of staining and seven were speckled. Fourteen of the sera positive for ANA on Hep-2 cells were also positive on neutrophil nuclei. ANCA were not found more often when ANA or neutrophil-specific ANA were present (*P* NS).

Antigens recognized by ANCA in Western blots (Fig. 3)

Molecules corresponding in size to neutrophil proteinase 3, myeloperoxidase and elastase were identified in sera with cANCA (neutrophil proteinase 3) or pANCA (myeloperoxidase and elastase).

ELISA for anti-myeloperoxidase antibodies

Thirty-eight sera were tested for anti-myeloperoxidase antibodies, and two of these were positive. Both contained homogeneous ANA on Hep-2 cells. None of the six sera tested that contained pANCA or neutrophil-specific ANA was positive for anti-myeloperoxidase antibodies.

ELISA for anti-elastase antibodies

Thirty-five sera were tested for anti-elastase antibodies, and four were positive. Three had homogeneous (2) or speckled (1) ANA on Hep-2 cells. One of these sera contained both anti-myeloperoxidase and anti-elastase antibodies. None of the five sera with pANCA that were tested for anti-elastase antibodies was positive. One serum with neutrophil-specific ANA but no ANCA contained anti-elastase antibodies.

Disease activity, presence of vasculitis and renal disease

ANCA were not detected more commonly in active disease nor in association with cutaneous vasculitis (*P* NS; *P* NS) (Table 2). Antibodies were present in three of the 22 patients who were clinically inactive (14%); in five of the 26 who had active disease without vasculitis (19%), in none of those with cutaneous vasculitis alone but in the two patients with a systemic vasculitis.

Renal function was tested in 55 patients. Of the 15 patients with ANCA by indirect immunofluorescence or anti-myeloperoxidase or anti-elastase antibodies, only one had urinary or renal abnormalities (Table 3). This was a mesangial proliferative glomerulonephritis and was associated with pANCA. Of the seven with neutrophil-specific ANA, two had renal abnormali-

Table 3. Renal abnormalities in RA patients with or without ANCA

Clinical category (*n*)	Renal abnormalities		
	ANCA positive	ANNA positive	ANCA negative
(i) Inactive (22)	0/3	0/1	1/17 (proteinuria)
(ii) Active, vasculitis (26)	0/5	1/5 (membranous gn)	2/14 (proteinuria, mesangial proliferative gn)
(iii) Active, with cutaneous vasculitis (8)	0/0	1/1 (haematuria)	2/6 (papillary necrosis haematuria)
(iv) Active, with systemic vasculitis (1)	1/2 (mesangial proliferative gn, pANCA) (pANCA)		

ANNA, neutrophil-specific anti-nuclear antibodies; gn, glomerulonephritis.

ties including membranous glomerulonephritis and haematuria. Of those without ANCA, two had proteinuria, one had a mesangial proliferative glomerulonephritis and one had renal papillary necrosis.

DISCUSSION

ANCA as demonstrated by indirect immunofluorescence were not uncommon in RA. This incidence needs to be confirmed since some sera were perhaps referred to us because of our interest in ANCA. Both pANCA and cANCA were demonstrated in our series. The presence of cANCA in RA without renal disease implies that these antibodies are not specific for Wegener's granulomatosis nor for a glomerular vasculitis. cANCA are also common in microscopic polyarteritis and we have also recognized them in a patient with atrial myxoma (Savige *et al.*, 1988) and they are common in HIV infections (Koderisch *et al.*, 1990). The pANCA were probably directed against some antigens distinct from those found in the glomerular vasculitis seen in microscopic polyarteritis, since anti-myeloperoxidase and anti-elastase antibodies were not detected. The target antigens for these antibodies remain to be determined. The presence of ANCA in RA was not associated with disease activity, nor with the presence of cutaneous vasculitis or glomerulonephritis.

Neutrophil-specific ANA were demonstrated in a number of these patients. Neutrophil-specific ANA have been commonly described in RA (Elling, 1967) and also in primary sclerosing cholangitis and ulcerative colitis (Snook *et al.*, 1989) and an antibody that stains the perinuclear region of human buccal mucosal cells has recently been described in RA (Westgeest *et al.*, 1989). Whether the targets are nuclear or cytoplasmic has been unclear. We have demonstrated that antibodies that bind specifically to neutrophil nuclei on ethanol-fixed preparations bind to the cytoplasm of neutrophils when fixed with formaldehyde/acetone and that the nuclear immunofluorescent staining may be artefactual. It has been recognized elsewhere that the pANCA pattern is virtually identical to that pattern produced by 'granulocyte-specific antinuclear antibodies' (Falk *et al.*, 1990). Whether all ANNA are forms of pANCA or whether sera with ANNA also contain pANCA activity is not clear. If the sera with ANNA also contained pANCA activity then destruction of nuclear targets with the formalin–acetone mixture would explain the lack of nuclear binding with that technique.

In addition, anti-myeloperoxidase and anti-elastase antibodies were found commonly in sera that contained homogeneous ANA on Hep-2 cells and neutrophils, but no ANCA on indirect immunofluorescence. Since both myeloperoxidase and elastase are stored in the cytoplasm of neutrophils, this suggests either that elastase and myeloperoxidase are artefactually distributed within the nuclei or that the homogeneous pattern of nuclear staining masks possible perinuclear staining. Thus the incidence of pANCA may be underestimated where ANA are also present. The ELISA for myeloperoxidase and elastase was more sensitive than immunofluorescence and the presence of these antibodies could not be excluded solely on the absence of cytoplasmic staining on neutrophils.

ANCA are typically associated with a renal vasculitis and sometimes with a systemic or cutaneous vasculitis. The cutaneous vasculitis in RA results in nail fold and digital infarcts, necrotic nodules and vasculitic ulcers. These lesions may result from a necrotizing arteritis indistinguishable from microscopic polyarteritis but they may also arise from intimal proliferation, thrombosis and vessel wall infiltration of digital arteries or from fibrinoid necrosis of venules associated with a neutrophil infiltrate and nuclear debris (leucocytoclasis) (Scott, Bacon & Tribe, 1981). Immunofluorescent examination of the skin lesions of rheumatoid vasculitis demonstrates immunoglobulin and complement in the walls of small blood vessels, suggesting an auto-antibody-mediated mechanism (Conn, Schroeder & McDuffin, 1976). However, we were unable to demonstrate any significant association between the presence of ANCA and a cutaneous vasculitis in RA. The heterogeneity of pathogenesis of cutaneous vasculitides may explain this if indeed an association is demonstrated in the future.

The systemic vasculitis of RA differs from the cutaneous vasculitis by the lack of a close HLA-B_8 DR_3 association (Cunningham *et al.*, 1982), absence of hypocomplementemia (Scott *et al.*, 1981; Scott & Bacon, 1984) and a milder clinical course (Gordon, Stein & Broder, 1973) in cutaneous vasculitides. The pathogenesis probably also differs. We have demonstrated ANCA by indirect immunofluorescence in two cases with systemic vasculitis and have examined sera from a further four patients in an ELISA (unpublished observations). ANCA was detected in only one of these four and this patient had a membranous glomerulonephritis. However, it appears that the neutrophil extract used as the basis of the ANCA ELISA does not always contain myeloperoxidase and thus the assay does not necessarily detect anti-myeloperoxidase antibodies. More

patients need to be studied before an association between ANCA and the systemic vasculitis in RA is established or dismissed.

A number of autoantibodies other than ANCA are recognized in RA and these include ANA, anti-collagen antibodies and rheumatoid factor. The origin of these antibodies is unclear but may be related to tissue breakdown. The large number of antibodies implies that cross-reactivity with bacterial or viral proteins is unlikely as the major aetio-pathogenetic event. The origin of ANCA is also obscure but has been postulated to relate to tissue breakdown, sometimes related to an infection: ANCA were first described in two multiply-transfused individuals (Calabresi, Edwards & Schilling, 1959); 33% of a series of patients with Wegener's granulomatosis had had a previous episode of suppurative or tuberculous respiratory infection (Pinching *et al.*, 1983); and relapses in patients with these antibodies are often associated with intercurrent infection (Pinching *et al.*, 1980). A role for neutrophil degradation in the aetiology of ANCA has yet to be demonstrated in RA.

There are two mechanisms by which ANCA might initiate the vasculitic process. The first is if there is cross-reactivity between the epitopes recognized in neutrophil cytoplasm and with vascular endothelium and thus ANCA bind to vascular endothelium. In one series of experiments, shared antigens were demonstrated between granulocytes, monocytes, tissue macrophages and vascular endothelial cells (Hogg *et al.*, 1984; Knowles *et al.*, 1984); furthermore, an antibody directed against one fraction of the neutrophil extract bound to glomerular endothelium and epithelium (Jones *et al.*, 1987), as well as to vascular endothelium (Abbott *et al.*, 1987). This work has not however been reproduced elsewhere. An alternative hypothesis suggests that antibody-facilitated intravascular lysis of leucocytes may be an early event in the process of vessel wall injury (Donald, Edwards & McEvoy, 1976; Falk *et al.*, 1990). It appears that priming of neutrophils by cytokines such as tumour necrosis factor results in the transfer of intracellular target epitopes to the surface of neutrophils (Falk *et al.*, 1990). Myeloperoxidase-ANCA and neutrophil proteinase 3-ANCA complexes can induce live neutrophils to release reactive oxygen species and lytic enzymes.

In conclusion, while ANCA are common in RA they are not associated with disease activity, the presence of cutaneous vasculitis nor with glomerular disease, and thus ANCA cannot represent the sole mechanism for these manifestations.

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