Clinical Implications and Utility of Antineutrophil Cytoplasmic Antibodies in Rheumatoid Arthritis, Spondylarthropathy and Ulcerative Colitis

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of
Finn-Medi, Lenkkeilijänkatu 6, Tampere,
on June 9th, 2000, at 12 o'clock.

University of Tampere
Tampere 2000

”Ei riitä, että tähtää – on osuttava.”

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals:

I Mustila A, Korpela M, Mustonen J, Helin H, Huhtala H, Soppi E, Pasternack A and Miettinen A (1997): Perinuclear antineutrophil cytoplasmic antibody in rheumatoid arthritis. A marker of severe disease with associated nephropathy. Arthritis Rheum 40:710-717.

II Mustila A, Paimela L, Leirisalo-Repo M, Huhtala H and Miettinen A: Antineutrophil cytoplasmic antibodies in patients with early rheumatoid arthritis. An early marker of progressive erosive disease. Arthritis Rheum. In press.

III Mustila A, Leirisalo-Repo M, Turunen U, Stenman S and Miettinen A (1999): Antineutrophil cytoplasmic antibodies in patients with spondylarthropathies. A predictor of chronic and progressive inflammatory joint disease. J Rheumatol 26:1421-1422.

IV Aitola P, Miettinen A, Mattila A, Matikainen M and Soppi E (1995): Effect of proctocolectomy on serum antineutrophil cytoplasmic antibodies in patients with chronic ulcerative colitis. J Clin Pathol 48:645-647.

V Mustila A, Turunen U, Färkkilä M and Miettinen A: Antineutrophil cytoplasmic antibodies in ulcerative colitis patients treated with long-term ciprofloxacin. Increased risk of treatment failure. Submitted.

ABBREVIATIONS

α1AT	alpha 1-antitrypsin
AKA	antikeratin antibodies
ANA	antinuclear antibodies
ANCA	antineutrophil cytoplasmic antibodies
APF	antiperinuclear factor
AS	ankylosing spondylitis
BPI	bactericidal/permeability-increasing protein
CD	Crohn's disease
CG	cathepsin G
DMARD	disease-modifying antirheumatic drug
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
GN	glomerulonephritis
GS-ANA	granulocyte-specific antinuclear antibodies
HAQ	health assessment questionnaire
HLE	human leukocyte elastase
IBD	inflammatory bowel disease
IIF	indirect immunofluorescence
LZ	lysozyme
MPO	myeloperoxidase
PMN	polymorphonuclear leukocytes
PR3	proteinase 3
PSC	primary sclerosing cholangitis
RA	rheumatoid arthritis
RF	rheumatoid factor
SLE	systemic lupus erythematosus
SpA	spondylarthropathy
TNFα	tumor necrosis factor α
UC	ulcerative colitis
VAS	visual analogue scale
WG	Wegener´s granulomatosis

INTRODUCTION

Antineutrophil cytoplasmic antibodies (ANCA) are directed against components of neutrophil granules and monocyte lysosomes. They were first reported to occur in patients with segmental necrotizing glomerulonephritis (GN) whose sera were found by indirect immunofluorescence techniques (IIF) to produce diffuse cytoplasmic staining of neutrophils by (Davies et al. 1982). The same antibody reaction was subsequently reported in patients with Wegener´s granulomatosis (WG) (van der Woude et al. 1985), a disease characterized by necrotizing granulomatous inflammation of the upper and lower airways in conjunction with systemic vasculitis and necrotizing crescentic GN.

Two different ANCA staining patterns on IIF were reported by Falk and Jennette (1988). In addition to cytoplasmic (c-) ANCA, they detected perinuclear (p-) ANCA in several forms of systemic vasculitis and glomerulonephritis. The first antigen recognized by p-ANCA was identified as myeloperoxidase (MPO), a constituent of the azurophilic granules of the neutrophil (Falk and Jennette 1988). The antigen recognized by c-ANCA proved to be proteinase 3 (PR3), another constituent of the azurophilic granules of the neutrophil (Niles et al. 1989).

New target antigens and disease associations have since been recognized. ANCA (p-ANCA and atypical ANCA) have been documented to occur in many inflammatory disorders, e.g. inflammatory bowel disease, primary sclerosing cholangitis (Snook et al. 1989, Saxon et al. 1990), and rheumatic diseases (Nässberger et al. 1989, Mulder et al. 1993, Hauschild et al. 1993, Locht et al. 1995, Mulder et al. 1997). The specific ANCA antigens and the clinical correlations of a positive ANCA test result in these disorders are still for the most part obscure. The aim of the present study was to investigate the prevalence of, the target antigens for, the clinical associations of and the possible prognostic role of ANCA in patients with various inflammatory disorders other than vasculitis.

REVIEW OF THE LITERATURE

1. Methods and terminology

The standard method of ANCA detection is the IIF technique on ethanol-fixed neutrophils. According to the guidelines established at the 1st workshop on ANCA in 1988 (Wiik 1989), neutrophils isolated from heparinized blood are cytocentrifuged, fixed in absolute ethanol on glass slides, and then incubated with dilutions of the patient´s serum. After staining with fluorescent-labeled anti-human immunoglobulin the fluorescence pattern is read under a fluorescence microcope.

On IIF, four distinct staining patterns can be distinguished (Savige et al. 1999). Cytoplasmic ANCA (c-ANCA) refers to a diffuse granular cytoplasmic staining pattern with central accentuation. Atypical cytoplasmic ANCA (c-ANCA atypical) consists in a diffuse homogeneous staining pattern without central accentuation. Perinuclear ANCA (p-ANCA) involves a perinuclear or nuclear staining on ethanol-fixed granulocytes, and atypical ANCA includes all other neutrophil-specific or monocyte-specific IIF reactivity, most commonly a combination of cytoplasmic and perinuclear fluorescence. The p-ANCA staining pattern is regarded as an artifact of ethanol fixation due to rearrangement of positively charged granule constituents around the negatively charged nuclear membrane (Falk and Jennette 1988). Crosslinking fixatives such as formalin during the preparation of the neutrophil substrates prevent this perinuclear rearrangement and thereby allow the distinction of true p-ANCA from antinuclear antibody (ANA), with or without granulocyte specificity. Formerly, granulocyte-specific antinuclear antibodies (GS-ANA) were regarded as a distinct staining pattern first reported by Wiik et al. (1974). GS-ANA were distinguished from ANA reacting with tissue nuclei by titrating. A GS-ANA titer more than two dilution steps higher than the ANA titer was taken to indicate true specificity for granulocytes (Wiik 1980). On formalin-fixed neutrophils, true p-ANCA will show predominantly diffuse cytoplasmic staining, whereas a serum containing ANA will show nuclear staining. According to a recent international consensus statement on detection of ANCA, p-ANCA and GS-ANA cannot be distinguished on ethanol-fixed neutrophils by IIF, and all nuclear and

perinuclear staining are therefore regarded as p-ANCA. ANA should be tested in all p-ANCA-positive sera. However, p-ANCA and ANA can occur simultaneously and therefore specific ANCA-ELISAs are recommended (Savige et al. 1999).

The enzyme-linked immunosorbent assay (ELISA) is used for target antigen-specific determination of ANCA. Two types of such solid-phase assays are in use. The target antigen can be coated directly onto a plastic microtiter well (standard ELISA) or it can be linked to the microtiter well via target antigen-specific mouse monoclonal or rabbit polyclonal antibodies (capture or sandwich ELISA). PR3, a serine protease present in the azurophilic granules of neutrophils, is the major target antigen associated with the c-ANCA fluorescence pattern on IIF (Goldschmeding et al. 1989, Niles et al. 1989). Antibodies to multiple antigens in the cytoplasm of neutrophils may be responsible for the p-ANCA fluorescence pattern. The principal p-ANCA antigen is MPO, an enzyme present in the azurophilic granules of neutrophils (Falk and Jennette 1988). Other antigens with p-ANCA reactivity on IIF include human leukocyte elastase (HLE), cathepsin G (CG), azurocidin (AZ), lactoferrin (LF), lysozyme (LZ), and bactericidal/permeability-increasing protein (BPI) (Hoffman and Specks 1998). In many cases, the target antigens of sera with p-ANCA or atypical ANCA reactivity have not been characterized (Hauschild et al. 1993, Mulder et al. 1993, Hertervig et al. 1995, Schnabel et al. 1995, Braun et al. 1996). Antibodies to MPO, LZ or BPI can on occasion cause atypical cytoplasmic staining on IIF, and vice versa, antibodies to PR3 can cause perinuclear staining on IIF (Hoffman and Specks 1998).

2. Disease associations of ANCA

2.1. ANCA in systemic vasculitides

The strong association between c-ANCA on IIF or PR3-ANCA on ELISA and Wegener´s granulomatosis (WG) is well documented. WG is a disease with granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels (i.e. capillaries, venules, arterioles and arteries). Necrotizing GN is common (Jennette et al. 1994). Most WG patients (80-95 %) are positive for c-ANCA on IIF (Cohen Tervaert et al. 1990, Venning et al. 1990, Savige et al. 1990, Gaskin et al. 1991, Hauschild et al. 1993, Jayne et al. 1995). The sensitivity of

the assays depends on the extent, severity and activity of the disease. In a recent meta-analysis (Rao et al. 1995), the pooled sensitivity of the IIF assay ranged from 63 % (inactive disease) to 91 % (active disease). The overall sensitivity of the assay has been estimated to be 65 %, specificity 95-99 % (Rao et al. 1995, Hagen et al. 1998). Combined utilization of IIF and PR3-ANCA testing has enhanced the specificity to approximately 99 %, but the sensitivity has remained at a level of approximately 70 % (Hagen et al. 1998). Serial ANCA determinations have been used to monitor disease activity in WG (Cohen Tervaert et al. 1990), although some studies have challenged this approach on the grounds that it may lead to overtreatment with immunosuppressive drugs (Kerr et al. 1993).

In microscopic polyangiitis (MPA), a systemic idiopathic vasculitis of small vessels (i.e. capillaries, venules or arterioles), p-ANCA/MPO-ANCA have been reported in 40-80 % of patients. About 10-25 % of ANCA-positive patients have PR3-antibodies (Falk et al. 1990, Gaskin et al. 1991, Bosch et al. 1992, Hauschild et al. 1993, Davenport et al. 1994, Hagen et al. 1998). However, the sensitivity and specificity of ANCA in MPA are difficult to assess due to the controversy regarding the distinction between MPA and classic polyarteritis nodosa (PAN), the latter being regarded as a systemic idiopathic vasculitis of medium-sized or small arteries (Cohen Tervaert et al. 1993, Guillevin et al. 1993, Hauschild et al. 1994, Jennette et al. 1994, Guillevin et al. 1995, Hoffman 1998). Generally, positivity for ANCA is taken to be associated with MPA, in which patients also frequently have GN and/or pulmonary capillaritis, but not with classic PAN (Jennette et al. 1994).

Patients with idiopathic rapidly progressing crescentic GN, a disease characterized by the absence or paucity of immune complex deposits as visualized by IF microscopy, are frequently (45-65 %) positive for ANCA (Falk and Jennette 1988, Cohen Tervaert et al. 1990). In most patients, the staining pattern of ANCA on IIF is perinuclear and the target antigen is MPO (Falk and Jennette 1988, Cohen Tervaert et al. 1990, Hagen et al. 1998). The disease is commonly associated with WG and MPA and it is thus not known whether idiopathic crescentic GN represents a limited form of these systemic diseases. In addition, antiglomerular basement membrane disease and ANCA-associated renal disease may coexist in up to 20 % of patients with rapidly progressive GN (Jayne et al. 1990, Short et al. 1995).

The Churg-Strauss syndrome (CSS) is an eosinophil-rich and granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels associated with asthma and eosinophilia (Jennette et al. 1994). Approximately 40-60 % of patients with CSS are positive for ANCA and the predominant staining pattern on IIF is perinuclear (Venning et al. 1990, Gaskin et al. 1991, Cohen Tervaert et al. 1993, Guillevin et al. 1993 Hauschild et al. 1993). Both MPO and PR3 have been described as target antigens, although the former is more common.

Henoch-Schönlein purpura, Kawasaki disease, giant cell arteritis, Takayasu arteritis and Behcet´s disease are regarded as not ANCA-associated although in some studies, ANCA of IgA isotype have been documented in patients with Henoch-Schönlein purpura (Hoffman et al. 1998).

2.2. ANCA in rheumatic diseases

In patients with rheumatoid arthritis (RA), ANCA [p-ANCA, granulocyte-specific antinuclear antibodies (GS-ANA) and atypical (c-)ANCA] have been detected in the sera of 0-70 % of cases, as can be seen in Table 1. ANCA have likewise been detected in the synovial fluid of patients with RA and the prevalence of ANCA in synovial fluid has been the same as that in serum (Wiik et al. 1974, Afeltra et al. 1996). ANCA have been documented as occurring especially in those patients with RA who have a longstanding (Mulder et al. 1993, Röther et al. 1994) and severe disease, and who are positive for rheumatoid factor (RF) and ANA (Röther et al. 1994, De Bandt et al. 1996, Braun et al. 1996). An association of ANCA with vasculitic and pulmonary involvement has also been proposed (Braun et al. 1996). In some studies no association has been found between disease activity and ANCA (Savige et al. 1991, Mulder et al. 1993).

Table 1. ANCA findings in RA patients in previous studies.

Study	No. of RA patients	ANCA+ (%)	Staining pattern on IIF		
			p-ANCA	c-ANCA	atypical
Savige et al. 1991	58	10 (17)	7	3	-
Gross et al. 1991	241	6 (2)	6	-	-
Lassoued et al. 1992	75	13 (17)	13	-	-
Juby et al. 1992	62 without FS	0	-	-	-
	32 with FS	11 (34)	4	-	7
Coremans et al 1992	49 with RV	21 (43)	1	1	19
	50 without RV	18 (36)	4	0	14
Braun et al. 1993	268	41 (15)	41	-	-
Mulder et al. 1993	94	66 (70)	66	-	-
Röther et al. 1994	94	46 (49)	41	-	5
Bosch et al. 1995	47	23 (49)	23	-	-
Afeltra et al. 1996	28	10 (36)	3	2	5
		11 (39) SF	4	2	5
De Bandt et al. 1996	84	28 (33)	16	1	11
Braun et al. 1996	385	61 (16)	61	-	-

FS = Felty´s syndrome, RV = rheumatoid vasculitis, SF = synovial fluid

Sera from ANCA-positive patients with RA recognize several different antigens in ELISAs, e.g. LF, HLE, CG and MPO, antibody levels are mostly low, and more than one antigen specificity is often found in the same serum (Brimnes et al. 1997). Coremans et al. (1992) report anti-LF antibodies in association with rheumatoid vasculitis. Cambridge et al. (1993) report positivity for anti-MPO antibodies to be more common in RA patients with nodular disease and lung involvement. This group also documented anti-MPO antibodies as being of IgG1 and IgG3 subclass in contrast to the IgG4 subclass predominance in patients with ANCA-positive vasculitis. However, in many ANCA-positive patients with RA, no specific target antigen can be detected (Afeltra et al. 1996, Brimnes et al. 1997).

In systemic lupus erythematosus (SLE), ANCA are present in about 15-30 % of patients (Schnabel et al. 1995, Merkel et al. 1997, Galeazzi et al. 1998). The predominant staining pattern is perinuclear, but atypical ANCA are also commonly found. Reactivity against LF, HLE, LZ, MPO and even PR3 in specific ELISAs has been reported, but the main target antigen remains unknown. Conclusions as to the significance of a positive ANCA test result in patients with SLE have been controversial. Some studies have shown no associations between positive ANCA and defined clinical manifestations of SLE such as vasculitis (Schnabel et al. 1995, Merkel et al. 1997), whereas Galeazzi et al. (1998) documented positive correlations between IIF ANCA and serositis, livedo reticularis, venous thrombosis and arthritis. Moreover, they also reported anti-LF antibodies in SLE to be associated with serositis and livedo reticularis. Nevertheless, their multivariate analyses showed other autoantibodies such as anticardiolipin antibodies and anti-SSA/Ro antibodies to be more strongly associated with the aforementioned clinical features. However, c-ANCA with anti-PR3 antibodies do not usually occur in patients with SLE and can therefore be used as a tool to distinguish vasculitis associated with SLE from idiopathic systemic vasculitides (Schnabel et al. 1995, Merkel et al. 1997, Galeazzi et al. 1998).

Spondylarthropathies (SpA) constitute a group of inflammatory joint diseases in which the patient suffers from inflammatory spinal pain or asymmetric synovitis usually predominantly in the lower limbs together with a positive family history, psoriasis, inflammatory bowel disease, urethritis, acute diarrhea, alternating buttock pain, enthesopathy or sacroiliitis (Dougados et al. 1991). In these disorders ANCA have been reported in 0-55 % of patients, p-ANCA as the predominant staining pattern (Gross et al. 1991, Helsloot et al. 1995, Koh et al. 1995, Weinerth et al. 1996, Locht et al. 1995, Stoffel et al. 1996). Atypical ANCA are also reported, but c-ANCA with PR3-specificity is a rare finding. Results on the occurrence and associations of ANCA in patients with SpA are controversial. Locht et al. (1995) reported positive ANCA as determined by ELISA, i.e. anti-LF and anti-MPO antibodies to be a common (55 %) finding in patients with reactive arthritis. They also observed ANCA positivity significantly more frequently in patients with chronic than in those with acute disease. In contrast, Stoffel et al. (1996) found no reactivity for ANCA in their study on patients with reactive arthritis. Koh et al. (1995) reported three patients with long-standing chronic ankylosing spondylitis (AS) to evince atypical ANCA on IIF. Two of them were

also suffering from peripheral arthritis and intestinal disorders. In contrast, Weinerth et al. (1996) found no ANCA on IIF in their patients with AS.

2.3. ANCA in inflammatory bowel disease

In the case of ulcerative colitis (UC), ANCA (mostly p-ANCA) have been detected in the sera of about 50-90 % of patients in contrast to a prevalence of approximately 10-20 % in patients with Crohn´s disease (CD), as shown in Table 2.

Table 2. The prevalence of ANCA in patients with UC and CD in previous studies.

Study	No. of UC patients	ANCA + (%)	No. of CD patients	ANCA + (%)
Saxon et al. 1990	25	21 (84)	25	5 (25)
Rump et al. 1990	34	20 (59)	30	3 (10)
Duerr et al. 1991a	19	15 (79)	-	-
Duerr et al. 1991b	40	24 (60)	18-	1 (6)
Cambridge et al. 1992	50	27 (54)	50	5 (10)
Oudkerk Pool et al. 1993	120	95 (79)	105	14 (13)
Hardarson et al. 1993	21	16 (76)	25	2 (8)
Hauschild et al. 1993	72	17 (24)	84	5 (6)
Kossa et al. 1995	49	20 (49)	33	3 (10)
Castellino et al. 1995	108	43 (40)	92	11 (12)
Hertervig et al. 1995	155	78 (50)	128 44 with colitis only	31 (24) 16 (36)

UC = ulcerative colitis, CD = Crohn´s disease

ANCA were thus first regarded as serologic markers distinguishing between UC and CD (Saxon et al. 1990, Rump et al. 1990). Further studies have rendered this conception of a distinctive role of ANCA between UC and CD in inflammatory bowel disease (IBD) controversial due to substantial number (up to 40 %) of ANCA-positive patients in CD, especially in cases with UC-resembling left-sided colonic inflammation (Hertervig et al. 1995, Vasiliauskas et al. 1996). Moreover, antibodies to the same ANCA antigens, namely CG, LF, LZ, HLE, BPI, are detected in both diseases

(Halbwachs-Mecarelli et al. 1992, Peen et al. 1993, Kossa et al. 1995, Stoffel et al. 1996). More recently it has been suggested that the difference in the prevalence of p-ANCA in UC and CD is very likely due to the different cytokine profile in these diseases and therefore to different types of inflammation (Fiocchi 1998). Moreover, the heterogeneity in immunoregulation within these disease entities is very considerable (Dalekos et al. 1993, Fiocchi 1998, Facklis et al. 1999). Some investigators have regarded p-ANCA-positivity as a marker of genetic susceptibility for UC, since healthy first-degree relatives of p-ANCA-positive UC patients are significantly more frequently p-ANCA-positive than environmental controls (Shanahan et al. 1992). However, positivity for ANCA is a useful marker to distinguish IBD from other colitides or diarrheal diseases (Duerr et al. 1991, Bansi et al. 1996).

In most studies, no correlation has been shown between presence or titer of ANCA and clinical disease activity, localization of the disease or outcome in IBD (Cambridge et al. 1992 Oudkerk Pool et al. 1993, Kossa et al. 1995, Castellino et al. 1995, Bansi et al. 1996). Positivity for ANCA also persists after colectomy (Oudkerk Pool et al. 1993, Reumaux et al. 1993). However, in some studies anti-CG antibodies have been shown to be associated with a refractory type of ulcerative colitis (Sobajima et al. 1996).

Recently, nuclear antigens have also been documented as target antigens for ANCA in IBD. They were first detected by laser confocal microscopy and localized by electron microscopy primarily over chromatin concentrated toward the nuclear periphery. However, these antigens were not DNA (Billing et al. 1995). Further studies have revealed histone H1 and nuclear lamina proteins, lamins A, C, B1, and lamin B receptor to colocalize with the antigens recognized by p-ANCA (Eggena et al. 1997, Terjung et al. 1998). Also non-histone chromosomal proteins belonging to high mobility groups (HMG) 1 and 2 have been demonstrated to be target antigens for p-ANCA in UC (Sobajima et al. 1997). These antigens are distributed in the nuclei and cytoplasm of eukaryotic cells and act as transcription factors. It has moreover been shown that anti-HMG1/HMG2 antibodies are significantly related to disease activity in UC (Sobajima et al. 1997).

2.4. ANCA in hepatobiliary diseases

Primary sclerosing cholangitis (PSC), a chronic cholestatic liver disease characterized by inflammation and fibrosis of the bile ducts, is frequently associated with UC (Greenstein et al. 1976, Monsén et al. 1990). A high percentage (65-85 %) of patients with PSC (with and without UC) are positive for ANCA, predominantly presenting with a perinuclear fluorescence pattern on IIF (Duerr et al. 1991a, Seibold et al. 1992, Oudkerk Pool et al. 1993, Mulder et al. 1993, Bansi et al. 1996). The same autoantigens for ANCA have been reported to occur in patients with both IBD and PSC (Halbwachs-Mecarelli et al. 1992, Mulder et al. 1993, Peen et al. 1993, Stoffel et al. 1996). Thus, a common underlying immunological disturbance in IBD and PSC has been suggested. Although ANCA detection by IIF has not been generally regarded as an ideal means of discriminating between specific subsets of the disease, antibodies to BPI and CG have been reported to be associated with the presence of cirrhosis. Antibodies to LF have been more frequently detected in patients with coexisting UC than in those without (Roozendaal et al. 1998). More recently, catalase and alpha-enolase have been characterized by Western blotting and partial amino acid sequencing as specific autoantigens for ANCA in PSC not occurring in patients with UC. Anti-catalase antibodies in PSC have also been reported to predict a more severe course of disease compared with PSC patients without these antibodies (Orth et al. 1998). The possible pathogenic role of ANCA in PSC is unclear. Their prevalence before and after liver transplantation is almost the same, although some decrease in titers may be observed. Nevertheless, no recurrence of PSC in the transplanted liver can be seen (Haagsma et al. 1993). Some studies have however suggested a possible pathogenic role for ANCA in PSC due to their association with anti-endothelial antibodies and the effect of anti-catalase antibodies on the production of oxygen-derived free radicals (Gur et al. 1995, Orth et al. 1998).

Sera from patients with autoimmune hepatitis, but not with other forms of chronic liver disease, are also frequently (65-95 %) positive for (perinuclear or atypical cytoplasmic) ANCA (Hardarson et al. 1993, Pokorny et al. 1994). More recently, Targan et al. (1995) showed positivity for p-ANCA to be a selective and independent marker especially for type I autoimmune hepatitis (AIH), a chronic liver inflammation with periportal hepatitis and autoantibodies against smooth muscle and/or ANA. In contrast, patients with type II autoimmune hepatitis (with autoantibodies against liver/kidney microsome

6type-1) were negative for ANCA. Further studies revealed the specific autoantigen in type I autoimmune hepatitis to be actin (Orth et al. 1997, Zauli et al. 1997). p-ANCA occurring in some patients with primary biliary cirrhosis (PBC) have been proposed to indicate a PBC/AIH overlap syndrome, but this observation has yet to be confirmed (Orth et al. 1997).

2.5. ANCA in infections

ANCA are also found in some infections. Such observations are of particular value in that differential diagnosis between systemic vasculitis and some infections in which ANCA have been detected, for example endocarditis (Wagner et al. 1991, Soto et al. 1994) or respiratory tract infections (Davenport et al. 1994), is sometimes difficult. The association between infection and breakthrough of an autoimmune disease is still obscure, but must be considered as one possible mechanism for the first flare-up of an autoimmune inflammatory disease. Reports on ANCA in different infections have mostly been case reports and further studies are thus needed to confirm the underlying mechanisms. Nevertheless, ANCA have been detected in patients with HIV (Savige et al. 1994), invasive amebiasis (c-ANCA with positive PR3-antibodies) (Pudifin et al. 1994), chromomycosis (Galperin et al. 1996), and malaria (Wenisch et al. 1996). These observations are of special significance in that false-positive ANCA results may occur in these conditions.

3. Pathogenetic role of ANCA

Marked progress has been achieved during the past few years in the understanding of the underlying pathogenetic mechanisms in ANCA-associated vasculitis, i.e. Wegener´s granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome. The main issues are briefly reviewed here.

Normally in inflammatory conditions, leukocytes migrate across the endothelial vessel wall, this involving a complex process, to arrive at the site of inflammation without damage to the endothelium of the vessels. In ANCA-associated vasculitides, autoantibodies may interact with neutrophils and monocytes in the circulation, resulting

in activation and microvascular adherence of leukocytes and subsequent vascular inflammation and necrosis.

3.1. Etiology of vasculitis

The etiology for vasculitic disease and aggravated host response is not known. Several environmental agents have been thought to be involved. Exposure to silicon-containing compounds has been documented as associated with chronic renal failure or vasculitis (Cohen Tervaert et al. 1998). Many reports have described treatment with hydralazine or antithyroid drugs such as propylthiouracil and methimazole as an inducing agent in ANCA-associated vasculitides. Also penicillamine, minocycline and allopurinol have been singled out in a number of case reports (Nässberger et al. 1990, Jones et al. 1992, Dolman et al. 1993, Kawachi et al. 1995, Elkayam et al. 1996).

Genetic factors may also be implicated in ANCA-associated vasculitis. An association has been revealed between ANCA activity and the deficiency alleles of alpha 1-antitrypsin (α1AT) (Esnault et al. 1993, Griffith et al. 1996). α1AT is the main inhibitor of neutral serine proteases like PR3 and HLE, and it is inactivated by MPO. Thus, lack of PR3 inhibitor exposes increased amounts of active enzyme to the immune system, and the diminished neutralization may cause increased damage at the site of inflammation. Moreover, ANCA are capable of inhibiting the interaction of PR3 with α1AT (Dolman et al. 1993). Genetic polymorphism in tumor necrosis factor α (TNFα) production has been proposed to influence genetic vulnerability to WG, as the clinical activity can be correlated to the amount of TNFα production (Kekow et al. 1992). TNFα performs as a significant primer of polymorphonuclear leukocytes (PMN) in ANCA-associated vasculitides. Phenotype 2/2 has been shown to produce higher amounts of TNFα compared with phenotypes 1/1 or 1/2. The prevalence of the TNFα 2/2 phenotype has been shown to be slightly elevated in patients with WG, although the difference did not reach statistical significance (Mascher et al. 1998). Moreover, PR3 may be expressed on the surface membrane of normal PMNs in healthy individuals, increasing the risk for WG (Jenne et al. 1996). Polymorphism is also seen in Fcγ receptors on PMNs. An association between the FcγRIIIb-NA1 allele (FcγRIIIb has two serologically defined allelic forms NA1 and NA2) with severe renal disease in WG has recently been documented (Wainstein et al. 1996).

Generally, infections are regarded as the most significant inductors of ANCA-associated vasculitides. Inflammatory mediators and especially proinflammatory cytokines released by leukocytes during an infection, for example TNFα, interleukin-1 (IL-1) or IL-8, are capable of priming neutrophils and monocytes *in vitro*. Upon priming, intracellularly stored ANCA antigens are released and bind to the cell membrane by charge interactions or via membrane-bound molecules such as serpin-enzyme complex receptor and β2-integrins. Intracellular antigens thus become available for ANCA. Furthermore, reactive oxygen metabolites and cytokines are produced (Falk et al. 1990, Perlmutter et al. 1990, Charles et al. 1991, Csernok et al. 1994, Ralston et al. 1997, Johansson et al. 1997, Zimmermann et al. 1998). However, it has been proposed that granular antigens may also be reached by ANCA on apoptotic PMNs (Gilligan et al. 1996).

The cytokine dependency of ANCA-mediated neutrophil activation *in vitro* corresponds to clinical observations. Circulating neutrophils in WG patients express PR3 and MPO on their cell surface during active disease and relapses (Csernok et al. 1994, Muller Kobold et al. 1996). However, in clinically quiescent patients high titers of ANCA can be observed, suggesting that ANCA alone are not sufficient to induce disease activity. Instead, exacerbations of disease activity are frequently preceded by infections and an increased incidence of ANCA-associated vasculitides is noted during winter months (Pinching et al. 1980, Falk et al. 1990). Furthermore, nasal carriage of *Staphylococcus aureus* is an important risk factor for relapses of WG (Stegeman et al. 1994). Nasal carriage of *Staphylococcus aureus* has also been shown to be associated with the activity of WG in the upper respiratory tract (Gadola et al. 1997).

3.2. Neutrophil activation by ANCA

Upon binding of ANCA to their antigens at the cell membrane, simultaneous cross-linking of surface molecules acting as signal transducers on the surface of PMNs is needed, since PR3 and MPO do not contain a transmembrane domain and cytoplasmic tail. β2-integrins, serpin-enzyme complex receptor and Fcγ receptors have been proposed to be possible candidates for signal transduction (Porges et al. 1994). Three classes of Fcγ receptors have been described in man: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Neutrophils mostly express FcγRIIa and FcγRIIIb while monocytes express FcγRI, FcγRIIa and FcγRIIIa. The Fcγ receptors differ in their ability to bind

IgG and interact differently with the various subclasses of IgG (van de Winkel et al. 1993). Fcγ receptors can initiate a proinflammatory tissue injury program through the oxidative burst and degranulation. With translocation of the ANCA target to the cell surface, the critical elements for the triggering of phagocytes for endothelial injury are available in a patient positive for ANCA: a phagocyte-displaying ANCA target, an antibody, and Fcγ receptors available to bind the Fc portion of the antibody and trigger a tissue injury program (Edberg et al. 1997).

IgG consists of four subclasses which differ in their ability to activate complement or the affinity for certain Fc receptors (van de Winkel et al. 1993). FcγRIIa has a particular affinity for the subclass IgG3 (van de Winkel et al. 1993). A correlation between the neutrophil-activating capacity of serum IgG fractions from remission to relapse and increases in levels of IgG3 subclass ANCA in patients with WG has been documented *in vivo* (Mulder et al. 1995). In addition, Brouwer et al. (1991) showed the IgG3 subclass of ANCA to be associated with renal involvement in patients with WG. Signal transduction then leads to degranulation and enhanced release of reactive oxygen radicals, and production of inflammatory mediators such as TNFα, IL-1, IL-8 and leukotriene B4, amplifying the inflammatory process (Casselman et al. 1995, Grimminger et al. 1996, Brooks et al. 1996, Ralston et al. 1997).

The clinical manifestations are known to differ in necrotizing crescentic GN patients with anti-PR3 and anti-MPO antibodies; thus the antigen-specificity of the ANCA involved has been held to have an effect on neutrophil activity (Franssen et al. 1995, Cohen Tervaert et al. 1990). Recently, Franssen et al. (1999) demonstrated *in vitro* that anti-PR3 antibodies had a greater capacity to activate neutrophils than anti-MPO antibodies.

3.3. Interaction between vascular endothelial cells and ANCA

ANCA-induced neutrophil activation only occurs when the primed cells are bound to a surface, i.e. vascular endothelial cells. ANCA-induced activation could not be demonstrated when adherence of the cells was prevented by continuous stirring of the suspension or by addition of blocking antibodies directed against FcγRIIa or β2-integrins, thus confirming that β2-integrin-mediated outside-in signalling is instrumental (Lub et al. 1995). PMNs activated by ANCA express increased levels of adhesion

molecules, including β2-integrins, facilitating their binding and transmigration through the endothelial monolayer (Johnson et al. 1997). Endothelial cells are then damaged by toxic products released from activated leukocytes. During the vasculitic inflammatory process, PR3 and MPO are also released by degranulation and can serve as planted antigens on the endothelial cell surface and thus as new targets for ANCA. Thus, in situ ANCA binding is also a possible mechanism eventually causing vessel wall destruction (Savage et al. 1993, Ballieux et al. 1994).

Some investigators have suggested a capability of ANCA to directly activate endothelial cells, since incubation of endothelial cells with IgG ANCA has resulted in increased expression of adhesion molecules (E-selectin, VCAM-1 and ICAM-1) or tissue factor (Mayet et al. 1993, Mayet et al. 1996, Johnson et al. 1997, De Bandt et al. 1997, Sibelius et al. 1998). Kain et al. (1995) documented a membrane protein of PMNs, (human lysosomal-associated membrane protein h-lamp-2) and a membrane protein of renal microvascular cells (a 130 kD glycoprotein gp 130) as being autoantigenic targets for ANCA in patients with active NCGN. In some reports it has been proposed that PR3 can be expressed by endothelial cells, or that PR3 expression on endothelial cells may be induced by proinflammatory cytokines similarly to neutrophils (Mayet et al. 1993). Addition of anti-PR3 antibodies in the presence of primed neutrophils then results in lysis of primed endothelial cells (Mayet et al. 1993). However, some series have been unable to demonstrate PR3 expression on endothelial cells (King et al. 1995). Furthermore, immune deposits in ANCA-associated vasculitides are absent or scanty (Horn et al. 1974, Ronco et al. 1983). Controversy thus prevails on the hypothesis of direct binding of ANCA to endothelial cells as a pathophysiological mechanism.

3.4. Monocyte activation by ANCA

PR3 and MPO are constituents of monocyte granules, which would render these cells equally feasible targets for ANCA. ANCA have been reported to activate monocytes to produce toxic oxygen radicals, although the effect was not dependent on priming. In contrast to neutrophils, endothelial cell lysis is not observed in the presence of ANCA-activated monocytes (Ewert et al. 1991). However, ANCA stimulate monocyte chemoattractant protein-1 production in monocytes (Casselman et al. 1995), and this might play an important role in the formation of granulomas by amplification of local monocyte recruitment *in vivo*.

3.5. T-cell reactivity in ANCA-associated vasculitis

In the acute phase of vasculitis, ANCA-associated vascular injury is characterized by a mural and perivascular influx of neutrophils (Churg and Churg 1989). Upon the progression of inflammatory lesions, a chronic mononuclear infiltrate develops, sometimes presenting as granulomas containing mononuclear phagocytes and CD4+ T-cells (Gephardt et al. 1983, ten Berge et al. 1985, Rastaldi et al. 1996). Since immune deposits are absent at the sites of vasculitic inflammation, it has been suggested that T-cells also contribute to the pathophysiology of ANCA-associated vascular injury (Mathieson et al. 1995). PR3- and MPO antigen-specific T-cells have been identified and demonstrated to persist during disease remission in the peripheral blood of patients with ANCA-associated vasculitis. Thus, T cells may contribute to the propensity of patients with vasculitis to relapse (Griffith et al. 1996, King et al. 1998). Whether the recruited T cells play a regulatory or cytotoxic role after entering the tissue is not known. Generally it is believed that initial lesions are neutrophil-dependent and subsequent T-cell recruitment and infiltration is a secondary event. However, interactions through CD40 expressed on activated endothelial cells and CD40 ligand expressed on activated CD4+ T cells may allow cross-talk between endothelial cells and activated T cells promoting inflammation (Karmann et al. 1995, Karmann et al. 1996, Dechanet et al. 1997). More recently, Csernok et al. (1999) have observed T-cells in patients with WG to have a Th1 cytokine profile producing predominantly interferon-gamma, which activates local macrophages and granulocytes to produce proinflammatory cytokines and toxic metabolites. They thus suggest that T cells may also have a triggering role in the pathogenesis of WG.

3.6. Animal models

To date no fully satisfactory animal model for ANCA-associated vasculitis has been developed. Exposure to mercuric chloride of Th2-responder-type rat strains such as the Brown Norway has been shown to induce an autoimmune syndrome resulting in tissue injury in multiple organs. Antibodies to MPO are developed in addition to a multitude of IgG autoantibodies (Esnault et al. 1992). MPO-ANCA have also been demonstrated in 22 % of female MRL-lpr mice, a species which spontaneously develop lymphoproliferation, glomerulonephritis, arteritis and arthritis. These MPO-ANCA-positive mice develop a clinical syndrome of vasculitis and GN distinct from immune complex disease (Harper et al. 1998). It has also been shown in several studies reviewed

by Heeringa et al. (1998) that injection of products of activated neutrophils would lead to GN and vasculitis in MPO-immunized rats. However, the presence of ANCA in itself is not sufficient to cause tissue injury; proinflammatory factors are needed to prime neutrophils and activate the endothelium (Heeringa et al. 1998).

In the idiopathic dysregulation theory it is assumed that during bacterial or viral infections antibodies are generated directed against bacterial or viral antigens containing autoimmune idiotypes (Shoenfeld 1995). In the models based on this theory, mice are immunized with a pathogenic autoantibody derived from patients with the respective autoimmune disease. Blank et al. (1995) injected purified human PR3-ANCA into Balb/c mice. This caused production of mouse antibodies against human PR3-ANCA (Ab1), followed by production of anti-Ab1 antibodies (Ab2) which recognized human PR3. The mice developed renal and pulmonary vasculitis similar to WG. However, more recently, Jenne et al. (1997) identified and characterized murine PR3, which was not recognized by PR3-ANCA from WG patients despite the strong similarities between human and murine PR3. It is therefore unlikely that the disease observed in mice after immunization with PR3-ANCA was caused by pathogenic antibodies against mouse PR3 (Jenne et al. 1997). Nevertheless, *in vivo* animal models strongly suggest albeit do not definitely prove, the pathogenetic role of ANCA in vasculitides.

3.6. Pathogenetic role of ANCA in diseases other than vasculitis

As outlined above, ANCA are also detected in a high percentage of patients with rheumatic diseases, inflammatory bowel disease, autoimmune liver diseases and drug-induced illnesses, and also in some infections. However, anti-PR3 and anti-MPO antibodies occur only in a minority of ANCA-positive cases. A variety of cytoplasmic or nuclear antigens are detected, and in many cases the specific target antigens remain unknown. In most of these diseases, no relationship between disease activity and ANCA titer has been documented. Nevertheless, p-ANCA-producing B-cell clones have been demonstrated in UC mucosa (Targan et al. 1995), and p-ANCA are detected in the synovial fluid of patients with RA (Afeltra et al. 1996). Thus the possibility remains that ANCA might be implicated in the pathogenesis of these diseases. However, further studies are needed before any conclusions can be drawn as to the exact role of ANCA in these diseases.

4. Clinical utility of ANCA detection

4.1. ANCA in vasculitis

As with any diagnostic test, the predictive value of ANCA depends on the pretest probability of the disease. Since ANCA-associated vasculitides are rare diseases and have a wide variety of manifestations, no predictive values can be calculated for ANCA in these cases or can be calculated or only for well-defined patient subgroups. Thus, determining ANCA in a normal population on a "scattergun" basis produces confusing results void of clinical relevance. Early studies on ANCA showed these autoantibodies to be highly sensitive and specific for classifying patients who already had a definite diagnosis, i.e. WG (van der Woude et al. 1985). Serial ANCA determinations were reported to mirror disease activity in patients with WG and MPA, with titers rising about six weeks before a clinical relapse and then decreasing upon effective treatment (Cohen Tervaert et al. 1990). However, decisions on treatment should not be made solely on the basis of ANCA test results, since up to one-third of patients with clinically quiescent disease may have a persistent high titer of ANCA, whereas some patients with active disease may be ANCA-negative (Kerr et al. 1993, Pettersson et al. 1992, De'Oliviera et al. 1995). A recent consensus statement on the appropriate use of ANCA determination recommends that positive ANCA on IIF should always be tested for specific ANCA antigens by ELISA (Savige et al. 1999). By this means the diagnostic value of the IIF test for ANCA detection in ANCA-associated vasculitides can be greatly increased, i.e. c-ANCA/anti-PR3 and p-ANCA/anti-MPO antibodies (Hagen et al. 1998). Moreover, ANCA test results should be interpreted in context with other clinical and laboratory manifestations. Nevertheless, patients who experience a significant rise in ANCA titer should be advised about the possibility of a relapse and should be closely monitored (Hoffman et al. 1998).

4.2. ANCA in other diseases

As noted above, ANCA are a common finding in many diseases other than vasculitis, i.e. rheumatic diseases, inflammatory bowel disease, hepatobiliary diseases and some infections. However, a c-ANCA pattern by IIF and anti-PR3 or anti-MPO antibodies by ELISA are seldom found in these diseases, as opposed to vasculitis. Staining patterns by IIF in these diseases are mainly p-ANCA or atypical ANCA, and antibodies to a wide variety of cytoplasmic and nuclear antigens of leukocytes are commonly (and even

simultaneously) found. Additionally, in many studies the target antigens for ANCA presenting in these disorders have remained obscure.

To date, positive ANCA can be used to differentiate IBD from other colitides and diarrheal diseases (Duerr et al. 1991b, Bansi et al. 1996). Similarly, autoimmune hepatititis type 1 and primary sclerosing cholangitis can be differentiated from other chronic liver diseases by positive ANCA (Duerr et al. 1991a, Seibold et al. 1992, Oudkerk Pool et al. 1993, Mulder et al. 1993, Targan et al. 1995, Bansi et al. 1996). So far, ANCA titers would not appear to constitute a useful marker indicating disease activity in these diseases (Savige et al. 1991, Cambridge et al. 1992, Mulder et al. 1993, Oudkerk Pool et al. 1993, Castellino et al. 1995, Kossa et al. 1995, Schnabel et al. 1995, Bansi et al. 1996, Merkel et al. 1997). No long-term prospective studies on the clinical outcome of ANCA-positive and –negative patients in these clinical disorders have been made. Thus, ANCA determination in diseases other than vasculitis is not in every-day diagnostic use.

AIMS OF THE PRESENT STUDY

The aims of the present study were to establish the prevalence, clinical correlates and possible predictive role of antineutrophil cytoplasmic antibodies in patients with inflammatory diseases other than vasculitis. More specifically, the objectives were:

1. to evaluate the prevalence and clinical correlations of ANCA in a cross-sectional population of patients with RA of long duration. Special interest was focused on the possible association between these autoantibodies and the occurrence of RA-associated nephropathy (I).

2. to evaluate the prevalence and clinical correlations of ANCA in patients with early RA. Special interest was focused on the possible predictive role of ANCA determined in early RA for the outcome of patients during a seven-year follow-up period (II).

3. to evaluate the prevalence, clinical associations, and possible predicitive role of ANCA in patients with spondylarthropathies. Special interest was focused on the possible role of ANCA in differentiating between patients with and without symptoms and signs of bowel inflammation (III).

4. to evaluate the effect of proctocolectomy on serum ANCA in patients with UC (IV).

5. to evaluate the outcome of UC patients positive and negative for ANCA treated with long-term ciprofloxacin in a prospective, double-blind, and placebo-controlled study (V).

SUBJECTS AND METHODS

1. Subjects and samples

1.1 *Study I*

The material for this study was divided into three groups. Group A consisted of 99 patients with RA (68 female, 31 male) with clinical renal disease probably related to RA itself and/or to antirheumatic drugs, for whom renal biopsy findings were available (Helin et al. 1995). The indications for renal biopsy had been isolated proteinuria in 34 patients, isolated hematuria in 29, proteinuria combined with hematuria in 19, nephrotic syndrome in 16, and acute renal failure in 1. Renal morphologic studies disclosed mesangial GN in 18, focal proliferative GN in 4, minimal-change nephropathy in 2, arteriosclerosis in 2, diabetic glomerulosclerosis in 2, acute interstitial nephritis in 1, and normal morphologic findings in 6. Three patients had concomitant mesangial GN and amyloidosis, and 1 had concomitant membranous GN and amyloidosis. Fifty-two (53 %) of the patients in group A received immunosuppressive drug therapy (oral corticosteroids in 45 patients, and azathioprine, methotrexate, or cyclophosphamide with or without corticosteroids in 7). The mean age of the patients in this group was 52 years (range, 11-76 years), mean duration of RA 11 years (range, 1-52 years).

The original group B for this study comprised 102 patients who yielded clinical and laboratory findings of renal and urinary tract disease in a prospective, population-based study of 604 patients with RA (Korpela et al. 1993, Korpela et al. 1995). Twenty-four patients had urologic causes for hematuria and were excluded. Thus the final group B consisted of 78 RA patients (56 female, 22 male). Renal biopsy was performed in 28 cases, where histologic data were included with those of group A. Fifty not biopsied had clinical and laboratory findings indicative of renal disease (isolated proteinuria in 16, isolated hematuria in 15, hematuria combined proteinuria in 3, and reduced renal function without hematuria or proteinuria (serum creatinine $\geq$ 100 μmoles/l in females and $\geq$ 115 μmoles/l in males) in 16. Twenty-nine (37 %) of the patients in this group were on oral corticosteroids, combined with azathioprine, methotrexate or

cyclophosphamide in 10 patients. The mean age of the patients was 62 years (range, 31-85 years), mean duration of RA 16 years (range, 1-53 years).

Group C (controls) comprised 97 patients with RA (72 female, 25 male). These patients were matched for age, sex and duration of RA with patients in the original group B from the population-based study of 604 patients. No clinical or laboratory findings suggestive of renal disease, i.e. abnormal urinalysis results, urine albumin excretion, or renal function, were observed. Eighteen (19 %) of the group C patients received oral corticosteroids, combined with methotrexate in 1 patient. The mean age of the patients was 63 years (range, 32-90 years), mean duration of RA 13 years (range, 2-45 years).

Altogether, the study population consisted of 246 patients with RA of long duration (176 female, 70 male), 149 of whom had clinical or laboratory findings suggestive of nephropathy (group A and group B), and 93 histologically verified nephropathy (group A). All patients had definite or classic RA according to the criteria of the American College of Rheumatology (formerly the American Rheumatism Association) (Ropes et al. 1958). In this study, serum samples from all these 246 patients were analysed. Specimens were stored at -20˚C until detection of ANCA.

1.2. Study II

The study population in this case consisted of 82 consecutive patients (67 female, 15 male) with early RA, i.e. with a duration of symptoms ≤ 12 months at diagnosis. The mean age of the patients was 44.4 years (range, 18-65 years). All fulfilled the American College of Rheumatology (ACR) revised criteria for RA (Arnett et al. 1988). They entered a prospective seven-year follow-up study of early RA, in which clinical, radiologic and laboratory data were collected at study entry, and thereafter at 12 months, 36 months, 60 months and 84 months. Before entry, none of the patients had received any disease-modifying antirheumatic drugs (DMARD) or oral glucocorticosteroid medication. After recruitment all were actively treated with continuous DMARDs (intramuscular gold, sulphasalazine or methotrexate) according to the so-called “saw tooth” strategy (Fries 1990). Serum samples taken at study entry and at every follow-up point were analysed for the present study. Sera were stored frozen at -20˚C until assays for ANCA.

1.3. Study III

Here the study population consisted of 50 patients (26 woman, 24 men) with spondylarthropathy (SpA). Of these, 25 had ankylosing spondylitis (AS) or sacroiliitis fulfilling the New york diagnostic criteria (Moll et al. 1973), 9 had chronic seronegative oligo- or polyarthritis according to the European Spondylarthropathy Study Group criteria for SpA (Dougados et al. 1991), and 16 had reactive arthritis, diagnosed as described (Lauhio et al. 1991). In 11 of the patients with reactive arthritis, the condition had been triggered by enteritis and in 5 patients by urethritis. Chronicity was defined as a duration of arthritis of ≥ 6 months. Ileocolonoscopy was performed in all cases for clinical indications of silent inflammation. Biopsy specimens were taken of any macroscopic changes observed in the ileocolonoscopy. In addition, biopsies were obtained from the terminal ileum, cecum, transverse and sigmoid colon and rectum even if macroscopically normal. Clinical and laboratory data were collected at the time of ileocolonoscopy, i.e. at the start of the study, and 6 and 12 months later. Sera for the current study were collected at the time of ileocolonoscopy and stored at -20˚C until analysed.

1.4. Study IV

This study population consisted of 15 patients (4 females, 11 males; mean age 33 years, range, 23-46 years) undergoing proctocolectomy for ulcerative colitis (UC). Thirteen patients had been treated with corticosteroids continuously or intermittently and two with sulphasalazine prior to the operation. Thirteen had total and two had left-sided UC. The mean duration of the disease before proctocolectomy was 7.3 years (range, 1-28 years). The indication for surgery was chronic active disease in 13 patients and dysplasia of the colonic mucosa in two. Fourteen patients had ileoanal anastomosis and one had undergone conventional ileostomy. A liver biopsy was taken during the proctocolectomy. Serum samples were taken immediately before and at a median of 23 months (range, 11-62 months) after the operation. Specimens were stored at -20˚C until analysed.

1.5 Study V

The study population here consisted of 73 consecutive patients (53 male, 20 female) with UC. The mean age of the patients was 34.1 years (range, 19-64 years), and the mean duration of UC 5.7 years (range, 1-26 years). They had already been found to be

refractory to conventional treatment, and 34 of the 73 (47%) were undergoing steroid treatment. They were randomized for treatment with either prednisone, mesalamine, and ciprofloxacin (n = 39) or prednisone, mesalamine, and placebo (n = 34). Therapy with ciprofloxacin or placebo was started after the initial colonoscopy at entry and continued until the colonoscopy at 6 months. Prednisone and mesalamine were administered to all subjects according to the regimen described in detail in Study V. Colonoscopy was performed at entry and after 3, 6, and 12 months. At each examination, two biopsy specimens were obtained from the terminal ileum and from eight locations in the large bowel: cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon and rectum. Endoscopic biopsy specimens from the diseased area of the colon were grown in a sterile mortar and cultured for intestinal pathogens. Disease activity was assessed at entry and at the visits after 1, 3, 6 and 12 months. Sera for the determination of ANCA were collected at study entry, and stored at -20˚C until analysed.

2. Methods

2.1. Detection of ANCA

Indirect immunofluorescence (IIF) utilizing ethanol- and formalin-fixed human granulocytes was used as the standard method (van der Woude et al. 1985, Wiik 1989) to detect ANCA. Briefly, granulocytes were isolated from healthy individuals using methylcellulose sedimentation without lysis of red blood cells. Cytocentrifuge slides were fixed with 1 % formalin or 99 % ethanol at +4˚C and incubated with test or control serum diluted 1:10 in phosphate buffered saline (PBS) for 1 hour in room temperature. The slides were washed three times with PBS, and bound antibodies were detected with a fluorescein isothiocyanate-conjugated polyvalent burro antibody (Studies I, III and IV) against human immunoglobulins or monovalent goat antibody (Studies II and V) against human IgG immunoglobulins (Kallestad, Austin, TX), diluted 1:80. The different ANCA staining patterns, i.e. cytoplasmic c-ANCA and perinuclear p-ANCA were identified. Positive sera were titrated to end-point. Dilutions of 1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000 were used for titration and titers $\geq$ 50 were considered positive. This cut-off titer corresponded to a < 5 % prevalence of p-ANCA in an apparently healthy control population evaluated for the present study. However, in

Study IV, titres ≥ 10 were considered positive. In all p-ANCA-positive patients, ANA were determined on multiblock cryostat sections of rat liver and kidney. If positive for ANA, a patient was considered to be p-ANCA-positive only if the titer of p-ANCA was more than 2 dilution steps higher than that of ANA (Wiik 1974).

Commercially available EIA kits were used for the determination of MPO and PR3 antibodies of IgG isotype (Euro-Diagnostica, Malmö, Sweden). Each kit included positive and negative controls and was used according to the manufacturer's instructions. Values exceeding that of the lowest standard (10 EU) were regarded as positive.

Antibodies against lactoferrin (LF), lysozyme (LZ), cathepsin G (CG) and human leukocyte elastase (HLE) were detected by in-house ELISA techniques. Briefly, 100 µl per well of enzyme antigen, LF from human milk (Sigma, St. Louis, USA) at 2.5 µg/ml, CG (Calbiochem, La Jolla, USA) at 0.005 U/ml, and HLE (Sigma, St. Louis, USA) at 0.010 U/ml in PBS, pH 7.4, was used to coat microtiter plates (Linbro^{R}, Flow Laboratories, United Kingdom). The plates were incubated overnight at room temperature. After 3 washes with PBS containing 0.05% Tween 20 (PBS-Tween), the plates were saturated with PBS-Tween containing 2 % casein from bovine milk (Sigma, St. Louis, USA) by incubation for 1 h at +37˚C. After another three washes, the serum samples were diluted in PBS-Tween containing 2 % casein. A dilution of 1:50 was used for the detection of anti-LF antibodies, and a dilution of 1:25 for the detection of anti-CG and anti-HLE antibodies. The sera were incubated in duplicate for 1 h at +37˚C. The plates were washed and bound IgG was detected by peroxidase-conjugated rabbit antibody against human IgG (Dakopatts, Copenhagen, Denmark) in PBS-Tween, diluted 1:6 000, by incubation for 1 h at +37˚C. After 3 final washes, *o*-phenylenediamine (Sigma, St. Louis, USA or Zymed, San Francisco, USA) in citrate-phosphate buffer, pH 5.0 was added. The reaction was stopped after 40 minutes for anti-LF, and after 60 minutes for anti-CG and anti-HLE antibodies by the addition of 100 µl of H_2SO_4 per well. The absorbances were measured at 492 nm (Labsystems Multiskan^{R} MCC/340, Helsinki, Finland).

For the determination of anti-LZ antibodies, 100 µl per well of LZ at 10 µg/ml in PBS, pH 7.4, was used to coat microtiter plates (Maxisorp^{R}, Nunc, Roskilde, Denmark). The plates were incubated overnight at room temperature. After 3 washes with PBS containing 0.5 %

Tween 20, the plates were saturated by incubation for 1 h at room temperature with PBS, pH 7.4, containing 1 % bovine serum albumin (Sigma, St. Louis, USA). After another 3 washes, the serum samples, diluted 1:50 in PBS, pH 7.4, containing 10 % fetal calf serum (ICN Biomedicals inc., Costa Mesa, USA), were incubated in duplicate for 1 h at +37˚C. The plates were washed and bound IgG was detected by alkaline phosphatase-conjugated rabbit anti-human IgG (Orion Diagnostica, Helsinki, Finland) in PBS, pH 7.4, by incubation for 1 h at room temperature. After 3 final washes, 4-nitrophenyl phosphate disodium salt (Boehringer Mannheim GmbH, Mannheim, Germany) 1 µg/ml in diethanolamine-$MgCl_2$ buffer, pH 10.0 (Oy Reagena Ltd., Kuopio, Finland), was added. The reaction was stopped after 90 minutes by addition of 100 µl of 1 N NaOH. The absorbances were measured at 405 nm.

The cut-off for positivity in the ELISAs was determined as the mean absorbance level + 2 SD of 50 apparently healthy blood donors.

IgG antibodies against human leukocyte elastase (HLE) in Study V were determined using a commercial ELISA kit (Shield Diagnostics, Dundee, UK). The assay was performed and the cut-off absorbance for positive and negative samples determined according to the manufacturer´s instructions.

In Study V, a commercial ELISA kit was used for the determination of bactericidal/permeability-increasing protein (BPI) IgG antibodies (DLD Diagnostika, GmbH, Hamburg, Germany). The assay was performed according to the manufacturer´s instructions except that the cut-off for positivity was set at 2 SD above the mean absorbance level in 50 apparently healthy blood donors.

2.2. Detection of rheumatoid factor, antiperinuclear factor and antikeratin antibodies

Rheumatoid factor (RF) was detected by an enzyme immunoassay (IgA-RF, IgG-RF, IgM-RF) (Teppo et al. 1986), by a quantitative immunoturbidic assay (Melamies et al. 1986), and by Rose-Waaler agglutination test, with titers $\geq$ 64 considered positive (Froelich et al. 1980). A patient was considered to be positive for RF if one of the assays used was positive (Study I). In Study II, the presence of RF was determined by the Rose-Waaler test.

In Study II, antikeratin antibodies were defined by IIF on the stratum corneum of rat esophagus, as previously described by Paimela et al. (1992). Antiperinuclear factor of buccal mucosa was defined by IIF according to the method described by Hoet et al. (1991).

2.3. Assessment of radiologic progression in patients with rheumatoid arthritis

The stage of radiologic progression was determined in Study I as described by Steinbrocker et al. (1949). In Study II, radiologic changes in hands and feet were graded according to the method of Larsen (Larsen et al. 1977). The joints evaluated included: wrists, MCP I-V, IP I, PIP II-V of both hands and the IP I and MTP I-V of the feet. Each joint was graded on a scale of 0-5 with the exception that the wrist score was multiplied by five. The grades of the individual joints were summed to form a score index. The maximum score was 210.

2.4. Assessment of clinical disease activity in patients with rheumatoid arthritis

In Study I, the class of functional capacity was determined as described by Steinbrocker et al. (1949). A Health Assessment Questionnaire (HAQ) was also used to evaluate functional capacity (Fries et al. 1980). Clinical disease activity was determined in Study II using the Ritchie articular index (Richie et al. 1968), number of swollen joints, and duration of morning stiffness and severity of pain (assessed on a visual analogue scale, VAS) (Scott et al. 1976).

2.5. Assessment of clinical disease activity in patients with ulcerative colitis

Clinical parameters used to assess clinical disease activity in Study V included number of liquid stools per day, presence of blood in the stools, urgency to defecate, fecal incontinence, abdominal pain, general well-being, fever, signs or symptoms of systemic illness, or extraintestinal manifestations.

2.6. Statistical analyses

Frequencies were compared by chi-square test. Means between two groups were compared with Student´s t-test or Mann-Whitney rank sum test. One-way analysis of variance was used when analysing differences between three or more groups. Analysis of variance and covariance with repeated measures was used to determine the difference in the progression rate of radiologic destruction between p-ANCA-positive and ANCA-

negative patients (Study II). Square root or logarithm transformation was used to obtain the normal distribution of data, where needed. ANCA titers were statistically analysed as log titers. A stepwise logistic regression analysis was used to determine the independent impact of selected demographic, clinical and laboratory findings (independent variables) on a certain event observed (dependent variable).

RESULTS

1. ANCA in rheumatoid arthritis

1.1. ANCA in patients with rheumatoid arthritis of long duration (I)

ANCA were found in 54 (22 %) of the 246 patients with RA of long duration. p-ANCA presented in 52 (96 %) of those with positive ANCA. Patients with clinically suspected or histologically proven nephropathy were significantly more frequently positive for p-ANCA than those without (30 % versus 7 %, respectively; $P < 0.00005$). Also mean titers of p-ANCA were significantly higher in patients with suspected or histologically proven nephropathy than in those without (103 versus 27; $P = 0.0011$). Additionally, the prevalence and the mean titer of p-ANCA depended significantly on whether patients had histologically proven nephropathy, clinically suspected nephropathy, or no symptoms or signs indicative of nephropathy. The same was observed when only patients originating from the same cross-sectional population-based study were considered.

The association of p-ANCA with histologically proven nephropathy was further corroborated by a logistic regression analysis which selected p-ANCA titer and elevated erythrocyte sedimentation rate (ESR) as having an independent and significant impact on the presence of nephropathy in patients with RA. However, positive p-ANCA were not associated with any single clinical manifestation or morphologic alteration of nephropathy in patients with RA.

p-ANCA-positive patients evinced significantly more intense inflammatory activity than ANCA-negative patients as estimated by mean ESR and mean blood hemoglobin concentration. Functional capacity as assessed by both Steinbrocker functional capacity scale and HAQ scale was significantly poorer in p-ANCA-positive patients than in those negative for ANCA.

Antigen specificity could be determined in 10 (19 %) of the 52 patients positive for p-ANCA, LF, MPO and LZ being the most frequent antigen specificities. Three p-ANCA-

positive patients had antibodies against more than one antigen, and 23 % of the patients classified on IIF as ANCA-negative had weak positive reactions in one or more of the ELISAs. No clinical associations were observed between patients positive or negative against any specific leukocyte antigen.

1.2. ANCA in patients with early rheumatoid arthritis (II)

ANCA were found in 43 (54 %) of the 80 serum samples available from 82 patients with early RA (i.e. duration of symptoms $\leq$ 12 months). The predominant ANCA pattern was p-ANCA, present in 40 (50 %) of the subjects studied.

During the seven-year follow-up period, radiologic destruction advanced more rapidly in p-ANCA-positive than in ANCA-negative patients as indicated by Larsen scores. The association between p-ANCA-positivity and radiologic progression was further corroborated in a stepwise logistic regression analysis which selected p-ANCA-positivity in early RA as having an independent and statistically significant impact on radiologic progression. Also the mean titer of p-ANCA in early RA was significantly higher in those RA patients who subsequently showed advanced radiologic progression than in those without such a disease course.

Fifty-four (66 %) patients with early RA were positive for rheumatoid factor (RF) at study entry. p-ANCA-positive patients were significantly more frequently positive for RF and for antiperinuclear factor (APF) than ANCA-negative patients. Likewise, p-ANCA-positive patients were more frequently positive for antikeratin antibodies (AKA), although the difference did not reach statistical significance.

At study entry, p-ANCA-positive and ANCA-negative patients did not differ by age or sex. During the follow-up period, no difference was seen in disease activity between p-ANCA-positive and ANCA-negative patients evaluated by ESR, C-reactive protein (CRP) or blood hemoglobin. Nor did they differ by number of swollen joints, joint tenderness assessed on the Ritchie articular index, duration of morning stiffness or severity of pain assessed by VAS. Similarly, positive p-ANCA determined at subsequent visits during the seven-year follow-up was not related to clinical and laboratory markers indicative of active inflammation.

Antigen specificity could be determined in 11 (28 %) of the 40 patients positive for p-ANCA at study entry, HLE and CG being the most frequent antigen specificities. Three p-ANCA-positive patients had antibodies against more than one antigen, and 38 % of those classified on IIF as ANCA-negative had weak positive reactions in one or more of the ELISAs. No clinical associations were observed between patients positive or negative against any specific leukocyte antigen.

During the seven-year follow-up, only 16 % of the patients with early RA were constantly positive for ANCA in all the determinations available, and 23 % were constantly negative for p-ANCA, respectively. Thus, ANCA findings varied during the seven-year follow-up in 61% of the patients.

2. ANCA in spondylarthropathy

In a 12-month follow-up study of patients with spondylarthopathy (SpA) (III), ANCA were found at entry in 12 (24 %) of the total of 50 patients. Positive ANCA was recorded especially frequently in SpA patients in whom the duration of symptoms was ≥ 6 months. No significant difference could be observed between HLA-B27 positive and negative patients.

Peripheral arthritis at 6 months and 12 months was significantly more frequent in the patients initially ANCA-positive than in those negative. Moreover, at 6 months, the initially ANCA-positive patients had a higher mean number of inflamed joints, higher mean ESR, and a higher serum concentration of CRP than negative patients. Thus, positivity for ANCA at commencement of follow-up was associated with the presence and persistence of symptoms and signs suggestive of chronic inflammatory arthritis. At 12 months, 7 patients had recovered and none of these had been initially positive for ANCA.

The progression of joint disease was clinically judged at the end of the 12-month follow-up. Those SpA patients in whom the joint disease had progressed were significantly more frequently positive for ANCA than those in whom the joint disease had not progressed. (42 % and 6 %, respectively; $P = 0.0092$). The same association

was seen when judgment of progression was confined to patients with peripheral arthritis. A stepwise logistic regression analysis also selected ANCA-positivity at the start of follow-up as a significant and independent predictor of progressive joint disease both when all SpA patients and when only SpA patients with peripheral arthritis were studied.

The specific ANCA antigen could be identified in 5 (63 %) out of 8 p-ANCA-positive patients and in one (25 %) of the 4 patients with atypical cytoplasmic ANCA. Two p-ANCA-positive patients evinced positive reactions to MPO only. The other 3 p-ANCA-positive patients reacted positively in 2 or more of the ELISAs. One patient positive for atypical ANCA proved positive for MPO.

ANCA-positive and ANCA-negative patients could not be differentiated by bowel symptoms or macroscopic findings in ileocolonoscopy. However, when the inflammatory activity in gut biopsies was histologically estimated, patients with chronic inflammation in the gut mucosa were more frequently ANCA-positive than those with normal or acutely inflamed gut mucosa.

3. ANCA in ulcerative colitis

3.1. ANCA in patients with ulcerative colitis before and after proctocolectomy (IV)

Prior to proctocolectomy, all 15 patients were positive for ANCA. Thirteen (87%) were positive for p-ANCA, one was positive for GS-ANCA and one for atypical c-ANCA. After proctocolectomy, 13 (87%) were positive for ANCA, 11 of them for p-ANCA. One patient tested positive for PR3 antibodies by ELISA preoperatively and showed a sixfold decrease in levels of this antibody after proctocolectomy. Simultaneously, the p-ANCA titer decreased from 200 to negative. None of the patients was positive for MPO antibodies before or after proctocolectomy.

The mean p-ANCA titers were significantly higher before proctocolectomy than after. The titers decreased in nine of the 13 patients; in two of these they turned negative. In three the titers remained the same and in one a twofold increase was observed after proctocolectomy. Also in the patient with positive GS-ANA before proctocolectomy the

titer decreased after the operation. In the patient with a low titer of c-ANCA preoperatively the titer remained the same after proctocolectomy. The clinical condition improved in all patients, irrespective of postoperative ANCA status.

Six patients yielded normal histology upon liver biopsy, two had mild steatosis, four had nonspecific reactive hepatitis, and three fibrous non-suppurative cholangitis. All patients with fibrous cholangitis or nonspecific reactive hepatitis upon liver biopsy were positive for p-ANCA. No significant difference in ANCA titers was observed between patients with different liver biopsy findings.

3.2. ANCA in patients with ulcerative colitis treated with long-term ciprofloxacin (V)

ANCA were found at study entry in 55 (75 %) of the 73 patients with UC. The predominant ANCA pattern was p-ANCA, present in 48 (66 %) of the patients at entry. Atypical ANCA occurred in 7 (9 %).

ANCA-positive and ANCA-negative patients did not differ from each other at study entry or respond differently to treatment in either treatment group when assessed by clinical or laboratory parameters. Nor did ANCA-positive and ANCA-negative UC patients differ from each other with respect to endoscopic or gut biopsy findings.

When all patients with UC were considered irrespective of treatment group, ANCA-positive patients ended up with treatment failure (the primary end-point in this study) significantly more frequently than ANCA-negative patients. In a closer analysis stratified by treatment group, the proportion of ANCA-positive patients among treatment failures was especially high in the ciprofloxacin group. The difference between ANCA-positive and –negative patients in the occurrence of treatment failures was seen both among those who ended up with treatment failure during the first 6 months and in those whose treatment failed later. Thus, ANCA-positivity (atypical or p-ANCA) in the ciprofloxacin group seemed to entail a risk of treatment failure. Irrespective of the treatment, the titers of ANCA were higher in patients who failed than in those who did not, but the difference did not reach statistical significance.

Antigen specificity could be determined in 28 (58 %) of the 48 patients positive for p-ANCA, and in six (86 %) of the seven patients positive for atypical ANCA at study

entry. Lactoferrin (LF) was the antigen specificity most frequently found, present in 24 (50 %) of the patients positive for p-ANCA, and in six (86 %) of those positive for atypical ANCA. Six patients positive for p-ANCA, and three patients with atypical ANCA had antibodies against more than one antigen. Five patients classed as ANCA-negative on IIF evinced positive reactions in ELISAs. In closer analysis, the patients in the ciprofloxacin group with positive ANCA (atypical or p-ANCA) and positive anti-LF antibodies ended up with treatment failure significantly more frequently than those negative for anti-LF antibodies. The difference was significant both at 6 months and at 12 months. In the placebo group, no difference in frequency of treament failures could be seen between anti-LF-antibody-positive and –negative patients.

A stepwise logistic regression analysis revealed anti-LF positive ANCA at study entry to have an independent and statistically significant impact on treatment failure in the ciprofloxacin group both at 6 months and at 12 months. In the placebo group, anti-LF-positive ANCA were not significantly associated with treatment failure. In contrast, in the placebo group ESR and previous prednisone treatment at study entry had an independent and significant impact on treatment failure at 6 and at 12 months.

Bacteriologic cultures of biopsy specimens taken at colonoscopy at entry showed no enteric bacterial pathogens in any patient. Nor could any pathogens been isolated in any patient during the treatment period or follow-up. During ciprofloxacin treatment, aerobic flora (mainly gram-negative coliforms) disappeared from stool cultures similarly in ANCA-positive and ANCA-negative UC patients. In stool cultures in the placebo group, no changes occurred in aerobic bacterial growth during the study period, and the bacterial flora was similar in ANCA-positive and ANCA-negative patients.

DISCUSSION

In patients with rheumatoid arthritis (I, II), the observed prevalence of ANCA and the predominance of p-ANCA as a staining pattern were in accordance with previous findings (Savige et al. 1991, Gross et al. 1991, Lassoued et al. 1991, Juby et al. 1992, Coremans et al. 1992, Braun et al. 1993, Mulder et al. 1993, Röther et al. 1994, Bosch et al. 1995, Afeltra et al. 1996, De Bandt et al. 1996, Braun et al. 1996). However, the prevalence of ANCA (22 %) in patients with RA of long duration in the current study was lower than that in patients with early RA (54 %). The difference observed in the prevalence of ANCA positivity between these populations might be explained by the high degree of selection of patients in the population of RA patients of long duration (I).

Radiologic joint destruction proved to be more pronounced in p-ANCA positive patients with early RA than in those negative for ANCA, although all patients were actively treated with DMARDs. The difference was seen both in the total scores of radiologic changes and in the rate at which the joint destruction progressed during follow-up. Rapidly progressing destruction was also accompanied by a higher mean titer of p-ANCA. The association was further corroborated by a logistic regression analysis which selected p-ANCA-positivity as an independent and statistically significant predictor of radiologic joint destruction. There have been few previous studies on the association of ANCA with radiologic joint destruction in patients with RA, and no follow-up studies have been published. De Carvalho and Graudal (1980) concluded that GS-ANA were associated with more progressive erosive disease. In the current study, GS-ANA and p-ANCA are considered indistinguishable on IIF and classified as p-ANCA according to a recent international consensus statement on ANCA determination (Savige et al. 1999). However, the strong association of p-ANCA with rapid radiologic progression suggests that positivity for p-ANCA in early RA might be used as a differentiating serologic marker to identify those patients who will benefit most from aggressive DMARD treament.

In patients with RA of long duration, p-ANCA positivity was shown to be an independent indicator of RA-associated nephropathy. Also the mean titers of p-ANCA were significantly higher in patients with histologically proven or clinically suspected

nephropathy than in those without renal disease. Thus, p-ANCA may serve as a serologic indicator and probably also as a predictor of RA-associated nephropathy. Nevertheless, p-ANCA were associated with a variety of morphological alterations, of which mesangial GN is probably related to RA itself (Helin et al. 1995). Thus, it seems unlikely that p-ANCA could have a direct etiologic or pathogenetic role in all of these different morphological alterations. Previous studies on ANCA in association with renal disease in RA are scarce. Savige et al. (1991) have reported on ANCA in RA patients with renal abnormalities. They found positive p-ANCA in 3 out of 8 RA patients with renal abnormalities in a study population of 55 patients. However, the number of RA patients with renal abnormalities in their study was too low for comparison with the results of the present series.

The association of ANCA with RF positivity in patients with early RA could not be seen in patients with RA of long duration, although the prevalence of RF positivity was similar in both populations. Previous findings have been controversial in this respect (Röther et al. 1994, Bosch et al. 1995). In many studies, again, this association has not even been studied (Savige et al. 1991, Mulder et al. 1993, Afeltra et al. 1996). The observed difference in the association of ANCA with RF positivity might be explained by patient selection and difference in disease duration (newly diagnosed RA versus mean duration of RA $\geq$ 10 years). Additionally, positivity for both RF and p-ANCA may be intermittent, as could be seen in the seven-year follow-up (II). Moreover, immunosuppressive treatment may have varying influence on the prevalence of both RF and ANCA. On the other hand, the observed discrepancy as to the association of ANCA with RF can be regarded as a strengthening argument for the independent role of ANCA as a predictive and indicative serologic marker in RA. In patients with early RA, ANCA were also associated with APF and AKA. To our knowledge, no previous studies on the association of ANCA with these serologic markers of RA have been reported.

ANCA were associated with clinical and laboratory markers indicative of severe disease in patients with RA of long duration, but not in those with early RA. Once again, patient selection may explain the observed difference between these populations. In previous studies, controversial results have been reported with regard to a possible association of ANCA with disease activity (Mulder et al. 1993, Röther et al. 1994, Bosch et al. 1995,

Afeltra et al. 1996). In most studies, no correlation between ANCA positivity and clinical disease activity in RA has been observed.

The antigen specificity of ANCA occurring in RA is for the most part unknown. Sera from patients with RA can be simultaneously positive for several antigen specificities, and the observed antibody levels against a specific antigen are usually low. Additionally, patients negative for ANCA by IIF may evince positive reactions in ELISAs (Afeltra et al. 1997, Brimnes et al. 1997). In this respect, the present results are in accordance with those in previous studies. Antigen specificity could be determined in 19 % of the p-ANCA-positive patients with RA of long duration and in 28 % of those with early RA. HLE was the antigen specificity of ANCA most frequently found in patients with early RA, whereas LF predominated as a target antigen in patients with RA of long duration. The clinical significance of this difference warrants further investigation. Previous studies have revealed variable target antigens of ANCA in RA (Savige et al. 1991, Gross et al. 1991, Lassoued et al. 1991, Juby et al. 1992, Coremans et al. 1992, Braun et al. 1993, Mulder et al. 1993, Röther et al. 1994, Bosch et al. 1995, Afeltra et al. 1996, De Bandt et al. 1996, Braun et al. 1996). The discrepancies are partly due to the lack of standardized solid-phase assays.

Previous data on the occurrence of ANCA in patients with spondylarthropathies (SpA) are scarce and controversial (Gross et al. 1991, Helsloot et al. 1995, Koh et al. 1995, Weinerth et al. 1996, Locht et al. 1995, Stoffel et al. 1996). However, the 24 % prevalence of ANCA observed in SpA in this series (III) is in accordance with previous reports (Gross et al. 1991, Helsloot et al. 1995). Also the occurrence of both p-ANCA and atypical ANCA is concordant with previous observations (Gross et al. 1991, Helsloot et al. 1995, Koh et al. 1995, Locht et al. 1995).

In the 12-month follow-up study on patients with SpA, ANCA were associated with chronic and persistent peripheral arthritis and predicted a progressive joint disease. The association of ANCA with inflammatory activity in patients with SpA has not been previously studied. Initially ANCA-positive patients were found to have a higher mean number of inflamed joints, higher mean ESR, and a higher mean serum concentration of CRP than ANCA-negative patients during the follow-up. Previously, Locht et al. (1995) have reported ANCA positivity to be associated with chronic reactive arthritis.

Spondylarthropathy is a spectrum of clinical manifestations of joint disease and is not generally regarded as ANCA-associated. This might explain the scarcity of studies on ANCA in patients with SpA. The results of the present study suggest that ANCA might be included in the evaluation of patients with SpA as a potential new marker of chronicity and progression of joint disease. The anticipated association of ANCA with "silent" bowel inflammation in patients with SpA could not be established (Leirisalo-Repo et al. 1993).

Among patients with ulcerative colitis (UC), ANCA (mostly p-ANCA) have been detected in the sera of 50-90 % of cases (Saxon et al. 1990, Rump et al. 1990, Duerr et al. 1991a, Duerr et al. 1991b, Cambridge et al. 1992, Oudkerk Pool et al. 1993, Hardarson et al. 1993, Hauschild et al. 1993, Kossa et al. 1995, Castellino et al. 1995, Hertervig et al. 1995). The observed prevalences of ANCA and the predominance of p-ANCA as a staining pattern on IIF here (IV, V) are concordant with previous observations. Atypical ANCA, observed in 9 % of UC patients in Study V, are also frequently found in patients with UC (Hertervig et al. 1995).

As in the case of ANCA occurring in RA, the target antigens in UC are mainly obscure. A variety of cytoplasmic leukocyte antigens for ANCA, namely CG, LF, LZ, HLE, BPI, are detected with variable frequency in patients with IBD (Halbwachs-Mecarelli et al. 1992, Peen et al. 1993, Kossa et al. 1995, Stoffel et al. 1996). Recently, nuclear antigens have also been documented as target antigens of ANCA in IBD (Eggena et al. 1997, Sobajima et al. 1997, Terjung et al. 1998). Here, UC patients undergoing proctocolectomy were only studied for anti-MPO and anti-PR3 antibodies, whereas those in Study V were examined for a wide variety of cytoplasmic leukocyte antigens. In Study V patients, anti-LF antibodies were found in 62 % of ANCA-positive patients.

Most previous studies have shown no correlation between presence or titer of ANCA and clinical disease activity, localization of disease or outcome in IBD (Cambridge et al. 1992 Oudkerk Pool et al. 1993, Kossa et al. 1995, Castellino et al. 1995, Bansi et al. 1996). Likewise, no demographic or clinical differences between ANCA-positive and ANCA-negative could be shown in the present study.

Positivity for ANCA has been reported to persist after colectomy (Oudkerk Pool et al. 1993, Reumaux et al. 1993). This was confirmed in the study where the effect of proctocolectomy on serum ANCA in UC was evaluated (IV). However, the titers of ANCA decreased significantly after the operation. Whether this resulted from the reduction of inflammation or the available (auto)antigenic material which modifies the immune disturbance related to UC is not known.

In Study V, the prognostic role of ANCA in UC patients treated with long-term ciprofloxacin was assessed. Interestingly, UC patients with positive ANCA treated with ciprofloxacin ended up with treatment failure significantly more frequently than those negative for ANCA. Further analyses revealed that especially patients with anti-LF-specific ANCA were prone to fail in the ciprofloxacin group. In the placebo group, no difference with respect to treatment failure could be seen between patients positive and negative for ANCA.

Elucidation of the biochemical backgrounds to the association of ANCA with treatment failure in UC patients treated with ciprofloxacin calls for further studies. Bacterial flora in stool cultures did not differ in patients positive or negative for ANCA at study entry, and no differences could be observed between these groups during the treatment period or follow-up in either the ciprofloxacin or the placebo group. Gram-negative coliforms disappeared similarly under the ciprofloxacin therapy in ANCA-positive and –negative patients. Whether some properties of aerobic bacteria protecting against inflammatory activity in UC were lost with ciprofloxacin treatment, is not known. Nevertheless, ciprofloxacin seemed to have a different effect on ANCA-positive and ANCA-negative patients, although as a whole, UC patients receiving ciprofloxacin seemed to benefit from the treatment, as previously reported (Turunen et al. 1998). Fluoroquinolones have been reported to exert immunomodulatory effects (Riesbeck et al. 1989, Rubinstein et al. 1993, Riesbeck et al. 1994), mainly on cytokine production. Thus, the cytokine profile in ANCA-positive and ANCA-negative patients with UC may be different. Heterogeneity in immunoregulation within disease entities in IBD is known to be marked (Dalekos et al. 1993, Fiocchi 1998, Facklis et al. 1999). It is also possible that ANCA-positive and ANCA-negative UC have different etiologies.

In conclusion, the results here confirm that ANCA are a common finding in patients with UC. ANCA do not differentiate UC patients with respect to clinical disease activity. However, the difference in the effect of fluoroquinolone treatment in ANCA-positive and ANCA-negative patients with UC is interesting. Antibiotic treatment is one of the factors known to be associated with relapses in UC (Onderdonk 1995). Whether UC patients relapsing due to antibiotic treatment are more frequently ANCA-positive than ANCA-negative is a matter warranting further studies. Nevertheless, the present results suggest that positivity for ANCA may entail a risk of treatment failure in UC patients treated with long-term fluoroquinolones. Determination of ANCA might therefore be of value before initiating long-term antibiotic treament for UC with fluoroquinolones.

SUMMARY AND CONCLUSIONS

ANCA are commonly found in patients with RA, SpA and UC, but the significance of a positive test result in these disorders is still for the most part unknown. The aim of the present study was to investigate the prevalence of, the target antigens for, the clinical associations of and the possible prognostic role of ANCA in patients with RA, SpA and UC.

The prevalence of ANCA in patients with RA of long duration was 22 %, and 54 % in patients with early RA. The predominant staining pattern was p-ANCA, present in about 95 % of RA patients positive for ANCA. In those with early RA, positivity for p-ANCA was also associated with immunologic markers of RA such as RF, APF, and AKA. In patients with RA of long duration, a significant association was documented between positive p-ANCA and RA-associated nephropathy. Additionally, p-ANCA in patients with RA of long duration were associated with clinical and laboratory findings indicating severe disease and increased inflammatory activity. This association could not been seen in patients with early RA, in whom p-ANCA seemed not to be a good marker of momentary inflammatory activity. On the other hand, in patients with early RA, positivity for p-ANCA proved to be an independent and significant predictor of rapid radiologic progression. HLE was the antigen specificity of ANCA most frequently found in patients with early RA, whereas LF predominated in those with RA of long duration. The significance of this difference warrants further investigation.

In conclusion, the results of this study suggest that ANCA may serve as a valuable serologic marker in patients with RA and be helpful in selecting those patients already in early RA who will benefit most from aggressive DMARD treatment.

The observed prevalence of ANCA in patients with SpA was 24 %. In a 12-month follow-up study of these patients, ANCA were shown to be associated with chronic and persistent peripheral arthritis and also to predict progression of joint disease. Additionally, patients positive for ANCA at commencement of follow-up had a higher mean number of inflamed joints, higher mean ESR, and higher mean serum concentrations of CRP than ANCA-negative patients during the follow-up.

Spondylarthropathy is a spectrum of clinical manifestations of joint disease not generally regarded as ANCA-associated. The results of this study suggest that ANCA might be included in the evaluation of patients with SpA as a potential new marker of chronicity and progression of joint disease.

The present results show that ANCA are commonly present in patients with UC. Here, 100 % of patients before and 87 % after proctocolectomy were positive for ANCA. In the other study population, the prevalence of ANCA was 75 %; 66 % for p-ANCA and 9 % for atypical ANCA. It appeared that ANCA do not differentiate UC patients with respect to clinical disease activity. Additionally, ANCA persist after proctocolectomy although the titers decrease significantly after the operation.

Interestingly, ANCA-positive patients with UC treated with long-term ciprofloxacin ended up with treatment failure significantly more frequently than those negative for ANCA. Further analyses revealed that especially patients with anti-LF antibody-positive ANCA predominated among treatment failures. In the placebo group, no difference could be seen between patients positive and negative for (anti-LF antibody-positive) ANCA with respect to treatment failure.

The results suggest that positive ANCA may be associated with a risk of treament failure in patients with UC treated with long-term fluoroquinolones. Determination of ANCA might thus be of value before initiating long-term antibiotic treatment with fluoroquinolones. The observation may be due to different genetic features of UC in patients positive and negative for ANCA, but further studies are needed to reveal the underlying immunologic mechanisms.

Taken together, the results of this series show that ANCA in inflammatory disorders other than vasculitis are of clinical significance. A positive ANCA result in these disorders would appear to select patients who might be in need of more aggressive treatment. Thus, after further confirmation of the present results, ANCA may serve as a useful serologic tool in clinical decision-making when treating patients with RA, SpA or UC.

YHTEENVETO

Neutrofiilien granulosyyttien sytoplasmavasta-aineita (antineutrophil cytoplasmic antibodies, ANCA) esiintyy yleisesti nivelreumaa ja spondylartropatiaa, samoin kuin haavaista paksusuolentulehdusta (colitis ulcerosa) sairastavilla potilailla. Positiivisen ANCA-löydöksen merkitys näissä taudeissa on kuitenkin ollut epäselvä. Tämän tutkimuksen tarkoituksena oli tutkia ANCA:n esiintyvyyttä, kohdeantigeeneja, kliinistä merkitystä ja mahdollista ennustearvoa reumaattisia nivelsairauksia ja haavaista paksusuolentulehdusta sairastavilla potilailla.

ANCA:a esiintyi 22 %:lla nivelreumapotilaista, joiden tauti oli kestänyt pitkään (yli 10 v) ja 54 %:lla potilaista, joiden tauti oli aivan tuore (oireiden kesto alle 12 kk). Pääasiallinen ANCA:n värjäytymiskuvio epäsuoralla immunofluoresenssitekniikalla tutkittaessa oli perinukleaarinen p-ANCA, jota esiintyi yli 95 %:lla ANCA-positiivisista potilaista.

Potilailla, joilla oli tuore nivelreuma, ANCA esiintyi yhdessä nivelreumalle tunnusomaisten laboratoriolöydösten, kuten reumafaktorin, antiperinukleaarifaktorin ja keratiinivasta-aineiden kanssa. Nivelreumapotilailla, joilla tauti oli kestänyt jo pitkään, p-ANCA-positiivisuus kytkeytyi nivelreumaan liittyviin munuaiskomplikaatioihin. Näillä potilailla p-ANCA liittyi lisäksi sellaisiin kliinisiin ja laboratoriolöydöksiin, jotka kuvastivat vaikeaa perustautia ja aktiivista tulehdusta. Samaa ei havaittu potilailla, joilla oli tuore nivelreuma ja joilla p-ANCA:n sen sijaan osoitettiin olevan merkittävä ja muista kliinisistä ja laboratoriolöydöksista riippumaton nopean niveltuhon ennustaja. Tuoreessa nivelreumassa tavallisin ANCA:n kohdeantigeeni oli leukosyyttielastaasi ja pitkään kestäneessa taudissa laktoferriini.

Nivelreumassa ANCA osoittautui serologiseksi markkeriksi, joka jo taudin alkuvaiheessa ennusti taudin etenemistä ja komplisoitumista. ANCA-määritystä voitaisiin siksi nivelreumassa käyttää esim. tunnistamaan aggressiivisesta lääkehoidosta eniten hyötyvät potilaat.

Spondylartropatiapotilailla ANCA:n esiintyvyys oli 24 %. Tehdyssä 12 kk:n seuranta-tutkimuksessa ANCA:n liittyi kroonistuvaan perifeeriseen artriittiin ja ennusti niveltaudin etenemistä. ANCA-positiivisilla potilailla oli seurannan aikana enemmän tulehtuneita niveliä, korkeampi lasko ja korkeampi CRP-arvo kuin ANCA-negatiivisilla. Spondylartropatia käsittää laajan joukon erilaisia niveltauteja, joita ei useinkaan pidetä tyypillisesti ANCA:aan liittyvinä. Tämän tutkimuksen tulokset kuitenkin osoittavat, että ANCA:a esiintyy ja että ANCA-määritys voitaisiin sisällyttää spondylartropatia-potilaiden arviointiin mahdollisena kroonistuvan ja etenevän niveltaudin osoittajana.

ANCA-positiivisuus on yleinen löydös myös haavaista paksusuolentulehdusta sairastavilla potilailla. Toisessa tutkituista aineistoista kaikki potilaat olivat ANCA-positiivisia ennen paksusuolen poistoa ja 87 % myös operaation jälkeen. Toisessa aineistossa, joka koostui lääkehoidossa olevista potilaista, ANCA:n esiintyvyys oli 75 %. Näissä aineistoissa ANCA-positiiviset ja ANCA-negatiiviset colitis ulcerosa -potilaat eivät eronneet toisistaan kliinisen tautiaktiviteetin suhteen.

Pitkäaikaista siprofloksasiinihoitoa saavilla colitis ulcerosa -potilailla hoito epäonnistui merkitsevästi useammin ANCA-positiivisilla kuin ANCA-negatiivisilla potilailla. Epäonnistumiset liittyivät nimenomaan laktoferriinivasta-ainepositiiviseen ANCA:an. Plasebolla hoidetussa ryhmässä (laktoferriinivasta-ainepositiivisten) ANCA-positiivisten ja ANCA-negatiivisten potilaiden hoitotulokset eivät poikenneet toisistaan. Havainto pitkäaikaisen siprofloksasiinihoidon erilaisesta vaikutuksesta saattaisi perustua ANCA-positiivisten ja -negatiivisten colitis ulcerosa –potilaiden erilaisiin immunogeneettisiin ominaisuuksiin. On myös mahdollista, että ANCA-positiivisten ja ANCA-negatiivisten potilaiden taudilla on eri aiheuttaja. Tulokset kuitenkin osoittavat, että ANCA:n määrittäminen saattaa olla hyödyllistä harkittaessa colitis ulcerosa –potilaiden pitkäaikaista antibioottihoitoa ainakin fluorokinoloneilla, koska ANCA-positiivisuus osoittautui riskiksi hoidon epäonnistumiselle.

Loppuyhteenvetona voidaan sanoa, että ANCA:lla on kliinistä merkitystä myös reumaattisissa nivelsairauksissa ja haavaisessa paksusuolentulehduksessa. ANCA-positiivisuus näissä taudeissa näytti valikoivan potilaita, jotka saattaisivat olla intensiivisemmän hoidon tarpeessa, mitä tietoa voidaan mahdollisesti jatkossa soveltaa käytännön hoitotyössä.

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