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LABORATORY STANDARD IN THE DIAGNOSIS AND THERAPY MONITORING OF AUTOIMMUNE DISEASE: VASCULITIS

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7.1 Antineutrophil cytoplasmic antibodies (ANCA)

Antineutrophil cytoplasmic antibodies (ANCA) are a heterogeneous group of circulating autoantibodies directed against proteins of cytoplasmic granules or other cytoplasmic and nuclear constituents of neutrophils. ANCA were first described in a few patients with segmental necrotizing glomerulonephritis. Later, ANCA were described in patients with primary systemic small vessel vasculitides-SVV (Table 1).

Table 1. *Classification of primary vasculitides according to the Chapel Hill Consensus Conference*

Type of vessels (primarily) involved	Disease
Large vessels	Giant cell arteritis Takayasu's arteritis
Medium size-vessels	Polyarteritis nodosa Kawasaki disease
Small vessels	
ANCA-associated	Wegener`s granulomatosis (WG) Churg-Strauss syndrome (CSS) Microscopic polyangiitis (MPA)
Not ANCA-associated	Henoch-Schönlein purpura Essential cryoglobulinemic vasculitis Cutaneous leukocytoclastic angiitis

Subsequently, ANCA was also described in a wide range of connnective tissue disease (CTD), inflammatory bowel disease (IBD), autoimmune liver diseases and infectious diseases (Table 2.). Accumulated data support the hypothesis that ANCA and their target antigens may be implicated in the pathogenesis of at least primary small vessel vasculitides.

Table 2. *Disorders that are different from the ANCA-associated small vessel vasculitides for which positive results for ANCA by IIF and/or ELISA have been described*

Connective tissue disorders	Non-ANCA associated vasculitides
Systemic lupus erythematosus Systemic sclerosis Mixed connective tissue disease Sjögren syndrome Dermatomyositis Antiphospholipid syndrome Rheumatoid arthritis Felty syndrome Juvenile chronic arthritis Reactive arthritis Ankylosing spondilitis	Takayasu vasculitis Giant cell arteritis Kawasaki disease Polyarteritis nodosa Schönlein-Henoch Purpura Behcet disease Cryoglobulinemic vasculitis
Gastrointestinal disorders	**Infectious disorders**
Ulcerative colitis Crohn disease Autoimmune hepatitis Primary biliary cirrhosis Primary sclerosing cholangitis	Tuberculosis Pseudomonas in cystic fibrosis Bacterial septicemia Subacute bacterial endocarditis Amoebiasis Malaria Influenza HIV Hepatitis C
Neoplasia	**Miscellaneous disorders**
Lymphoid neoplasia Lymphomatoid granulomatosis Monoclonal gamopathies Myeloproliferative disorders Carcinomas	Sweet syndrome Poststreptococcal glomerulonephritis IgA nephropathy Sarcoidosis Nonspecific interstitial pneumonia
Drugs	
Thiamazole Propythiouracil, benzythiouracil Methimazole Minocycline Hydralazine	

7. 2 ANCA test methodology and their target antigens

Currently, three basic assay principles are applied for the detection of ANCA.

Indirect immunofluorescence (IIF) is the original and the most widely used method of ANCA detection. IIF tests are difficult to standardize, interpret and do not identify with certainty the specific antigen responsible for ANCA immunofluorescence patterns. Image analysis is an automated alternative to conventional IIF. The technique quantitates fluorescence in a single dilution of a patient sample in comparison with the intensity of standardized calibrators. Readings correlated well with ANCA levels as measured by IIF, direct enzyme-linked immunosorbent assay (ELISA) and capture ELISA. However, multicenter comparative studies are lacking.

Enzyme-linked immunosorbent assay (ELISA) is used for the determination of ANCA specific target antigen (s). Two types of such solid-phase assays were used. The target antigen can be coated directly onto plastic microtiter plate (standard ELISA) or it can be linked to the reaction well via target antigen-specific mouse monoclonal or rabbit polyclonal antibodies ("capture" ELISA or sandwich ELISA). Because the purification of native proteinase 3 (PR3) and myeloperoxidase (MPO) is laborious and requires large amounts of granulocytes, recombinant PR3 and MPO were used as target antigen in "capture" ELISA. However, ANCA assays based on these recombinant antigens have not been subjected to rigorous standardization procedures and, besides local applications, have not yet been routinely used. Other detection methods, such as immunoblotting (IB) or immunoprecipitation are not widely used for routine ANCA testing.

The "International consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)" advocates that all laboratories screen for ANCA by IIF on ethanol-fixed human neutrophils, and that any sera with ANCA fluorescence should be tested for both of the major ANCA specificities, PR3 and MPO, by ELISA.

According to the statement, ANCA recognized four different immunofluorescence patterns: a coarse granular cytoplasmic fluorescence with accentuation between the nuclear lobes - classical cytoplasmic or C-ANCA (Fig 1A, e, f); a diffuse (flatter) cytoplasmic fluorescence without accentuation of the interlobular fluorescence or glanular staining - atypical C-ANCA (not shown); a typically perinuclear fluorescence with some nuclear extension (Fig 1B, e) and granular cytoplasmic fluorescence on formalin-fixed neutrophils (Fig 1B, f)- perinuclear or P-ANCA; pronounced nuclear rim fluorescence with the center of nucleus unstained (Fig 1C, e) and non-reactivity with formalin-fixed neutrophils (Fig 1C, f) -very perinuclear or "atypical" P-ANCA and atypical ANCA which include all other IIF reactivity, most commonly a combination of cytoplasmic and perinuclear fluorescence (not shown).

Relations between ANCA staining patterns and their most common molecular targets are summarized in Table 3. Classical C-ANCA staining patterns are found characteristically in sera from most patients with WG, but also to a lesser extent in the sera from other necrotizing vasculitides. The target antigen recognized by most C-ANCA positive sera has been identified as PR3, a neutral serine protease present in the azurophilic granules of neutrophils. Proteinase 3 has been cloned and was shown to be a 29 kD glycoprotein of 228 aminoacids. Human antibodies to PR3 (PR3-ANCA) appeared to recognize conformational determinants on the molecule.

Very rarely, sera with PR3-ANCA reactivity can also cause P-ANCA fluorescence pattern, and vice versa, MPO-ANCA sometimes can give rise to a similar C-ANCA staining pattern makes testing with anti-PR3 as well as anti-MPO-ELISA relevant. A C-ANCA staining pattern may also be seen when ANCA directed to bactericidal/permeability-increasing protein (BPI) are present. In the case of chronic infections (subacute bacterial endocarditis or cystic fibrosis) testing for BPI-ANCA may explain positive IIF results and alleviate concerns about primary SVV.

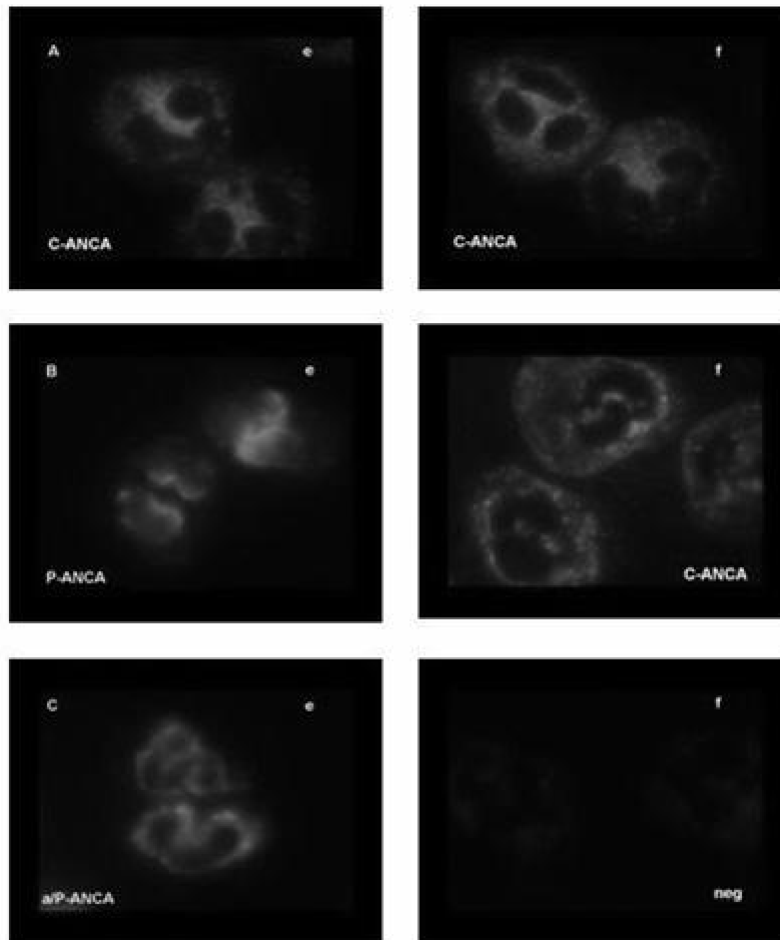

Figure 1. *Characteristic fluorescence patterns of ANCA on ethanol (e) and paraformaldehyde (f) fixed human neutrophil cytospin preparations. (A) a coarse granular fluorescence with accentuation between nuclear lobes (e and f) – C-ANCA; (B) a typically perinuclear fluorescence with some nuclear extension (e) and a granular cytoplasmic fluorescence on formalin fixed neutrophils (f) – perinuclear P-ANCA; (C) a pronounced nuclear rim fluorescence with the center of nucleus unstained (e) and negative fluorescence on formalin fixed neutrophils (f) – «atypical» perinuclear – a/P-ANCA or very perinuclear P-ANCA.*

P-ANCA staining pattern is the result of a redistribution of cationic hydrophilic substances such as MPO, elastase and lysozyme onto the oppositely charged nucleus after permeabilization of cells by ethanol. This ANCA reactivity was found in most patients with MPA and iNCGN, some patients with CSS and a few WG patients. "Atypical" P-ANCA staining patterns with different frequency were found in patients with IBD, autoimmune liver diseases, infectious diseases and connective tissue diseases (CTD) such as SLE and RA. MPO represents the P-ANCA target antigen with the greatest clinical utility because of the frequent association of MPO-ANCA with SVV. All serum samples should be assayed in PR3-ANCA and MPO-ANCA ELISAs, since about 5% of serum samples are positive only by ELISA. However, many sera that produce P-ANCA or "atypical" P-ANCA staining pattern on ethanol-fixed neutrophils do not contain autoantibodies to MPO or PR3 as tested by antigen-specific assays. Recent studies show

that a number of these sera contain autoantibodies directed against a multiplicity of neutrophil constituents. In particular, autoantibodies to human leukocyte elastase (HLE), cathepsin G (CG), lactoferrin (LF), lysozyme (LZ), azurodicin (AZ), α-enolase, catalase, actin, tropomyosin, high motility groups of non-histone chromosomal proteins 1 and 2 (HMG1 and HMG2), bactericidal/permeability-increasing protein (BPI), lamin B1, histone H1 and 50 kD nuclear envelope membrane protein (Table 3.) Recently, a new term it has been proposed for this autoantibody population, namely "neutrophil-specific autoantibodies-NSA".

Table 3. *Antineutrophil cytoplasmic antibodies (ANCA) staining patterns and associated target antigen in patients with systemic vasculitides and nonvasculitic disorders*

Immunofluorescence pattern	ANCA target antigen	Associated disease
C-ANCA	PR3	WG
P-ANCA	MPO	MPA, CSS
P-ANCA	HLE	UC, CD, PSC, SLE
P-ANCA	α-enolase	
P-ANCA	catalase	
P-ANCA	azurodicin (AZ)	
P-ANCA (atypical)	lactoferrin (LF)	UC, CD, PSC
P-ANCA (atypical)	cathepsin G (CG)	UC, CD, PSC, SLE, RA
P-ANCA (atypical)	lysozyme (LZ)	UC, CD, PSC
C-ANCA, P-ANCA (atypical)	actin	AIH
C-ANCA, P-ANCA (atypical)	BPI	UC, CD, PSC, AIH, SLE
P-ANCA (atypical)	HMG1/2	UC, SLE, RA
P-ANCA (atypical)	lamine B1	UC, CD, SLE
P-ANCA (atypical)	histone H1	UC
P-ANCA (atypical)	50 Kd	UC, AIH

WG-Wegener's granulomatosis; MPA-microscopic polyangiitis; CSS-Churg-Strauss syndrome; UC-ulcerative colitis; CD-Crohn's disease; PSC-primary sclerosing cholangitis; SLE-systemic lupus erythematosus; RA-rheumatoid arthritis; AIH-autoimmune hepatitis; HLE-human leukocyte elastase; BPI-bacteridical/permeability-increasing protein; HMG1/2-high mobility group of non-histone chromosomal proteins 1 and 2

7.3 ANCA as diagnostic markers

Diagnostic significance of both PR3-ANCA and MPO-ANCA for SVV is not questionable (Table 4). The diagnostic relevance of ANCA depends on the clinical ordering guidelines for ANCA testing. The positive predictive value (PPV) of IIF ANCAs for SVV was very low (55-59%), with negative predictive value (NPV) between 84% and 99%, during ANCA testing in a routine clinical setting. Adherence to clinical ordering guidelines for ANCA testing restricted to patients with a reasonably high likelihood of SVV (Table 5) was reported to reduce the number of false-positive tests by 27%, without missing any cases of SVV. The total number of tests performed may thus be reduced by more than 20%.

The most clear-cut association of a disease with ANCA directed against a specific target antigen is the association between WG and PR3-ANCA. Between 80% to 95% of all ANCA in WG is C-ANCA. The use of more sensitive PR3-ANCA methods (capture ELISA) of detection has confirmed that the C-ANCA in WG is almost always associated with anti-PR3. An estimated 5-20% of ANCA in WG may be P-ANCA, which are mostly directed against MPO and only rarely directed against human leukocyte elastase (HLE). The sensitivity of C-ANCA/PR3-ANCA for WG is related to the extent, severity and activity of disease. In a meta analysis of C-ANCA in WG, the pooled sensitivity was 91% for the subset of patients with active disease compared to 63% for those with inactive disease. The specificity and positive predictive value of C-ANCA/PR3-ANCA for WG are very high (Table 4.). Most patients with MPA, iNCGN and CSS are ANCA positive, either with specificity for MPO or PR3. Other target antigens for ANCA in patients with SVV, such as BPI and AZ, may simultaneously occur with PR3-ANCA and MPO-ANCA. Whereas ANCA

Table 4. *Disease associations of C-ANCA (PR3-ANCA) and P-ANCA (MPO-ANCA) in primary systemic small vessel vasculitides*

Patients with	Auto Ab	Auto Ag	Test	Sensitivity (%)	Specificity (%)	PPV (%)
WG	C-ANCA		IIF	80-95	95-98	94-100
		PR3	ELISA	85	98-100	97-100
	P-ANCA		IIF	5-20	81-94	5
		MPO	ELISA	10-20	81-94	17
MPA	C-ANCA		IIF	35-45	92-97	97
		PR3	ELISA	26	86-89	
	P-ANCA		IIF	45-65	94	97
		MPO	ELISA	58	99	100
iNCGN	C-ANCA		IIF	30-40	95	
		PR3	ELISA	50	86-89	
	P-ANCA		IIF	45-65	81	
		MPO	ELISA	64	91	
CSS	C-ANCA		IIF	25-30	94	
		PR3	ELISA	33	94	
	P-ANCA		IIF	25-30	92	
		MPO	ELISA	50	99	

PPV-positive predictive value

Table 5. *Clinical ordering guidelines for ANCA testing.*

Glomerulonephritis, especially rapidly progressive
Pulmonary haemorrhage, especially pulmonary renal syndrome
Cutaneous vasculitis with systemic features myalgias, arthralgias or arthritis
Multiple lung nodules

Chronic destructive disease of the upper airways
Long-standing sinusitis or otitis
Subglottic, tracheal stenosis
Mononeuritis multiplex or other peripheral neuropathy
Retroorbital mass

as detected by IIF is found frequently in other inflammatory diseases, PR3-ANCA and MPO-ANCA are only rarely detected in disorders other than SVV. MPO-ANCA is also found in patients with anti-GBM disease. Futhermore, false-positive MPO-ANCA may be found occasionally in patients with SLE or RA and in other inflammatory disorders such as autoimmune liver disease and inflammatory bowel disease. These false positive results, however, can be avoided by using a capture ELISA to detect MPO-ANCA. For PR3-ANCA, the capture ELISA system does not seen to differ much from a direct ELISA system with respect to specificity but the capture ELISA seems to be more sensitive. Even though the presence for WG, MPA and/or CSS, a positive ANCA result should always be interpreted with consideration of the clinical setting since the presence of specific clinical patterns plays a major role in determinig the diagnostic probability of vasculitis.

7.4 Prognostic value of ANCA during follow-up

ANCA-associated vasculitis has a 1-year survival of at least 80-90%. Treatment consists generally of a combination of prednisolone and cyclophosphamide, or other immunosuppressive drugs. Since all drugs that are used produce toxic side effect, medications are generally tapered and eventually eliminated in most cases. However, during folow-up, up to 80% of the patients in remission experience relapses. Patients with WG relapse more frequently than patients with MPA or renal limited vasculitis. In addition, patients with PR3-ANCA have more frequent relapses than patients with MPO-ANCA. This is also true when patients are subdivided into groups according to their diagnosis. So, patients with either WG and MPA who are PR3-ANCA positive have a higher relapse rate than patients with MPO-ANCA associated with the respective disease type. Thus, ANCA testing is not only a highly sensitive and specific test for making a diagnosis of WG, MPA or CSS, but ANCA antigen specificity has also a prognostic value with respect to the development of relapses during follow-up. Patients with PR3-ANCA may be at higher risk of death and patients with MPO-ANCA may be at higher risk for renal failures. Futhermore, relapses in PR3-ANCA positive patients are much more fulminant than relapses in MPO-ANCA positive patients. The causes of progression of renal failure differ between PR3 and MPO-ANCA positive patients. In patients with PR3-ANCA renal function is stable during remission, but declines with every relapse. In patients with MPO-ANCA, a slowly progressive course is often observed during follow-up without signs of clinically active disease. In these patients, proteinuria is the most important risk factor for renal failure during follow-up. In addition to ANCA specificity, ANCA levels at diagnosis and during follow-up have been shown to be predictive for patients renal and disease-free survival. A high PR3-ANCA level in capture ELISA at diagnosis is a risk factor for poor patient and renal survival and a constantly elevated MPO-ANCA level is a risk factor for poor renal survival. During induction therapy ANCA levels fall and become negative in many patients within the

first few months. Persistent or recurring C-ANCA during the first year is significantly related to subsequent relapse. More than 80% of the patients who were ANCA positive at diagnosis and who experience a relapse, are test positive for ANCA at the time of relapse. So, the patients persistently negative for ANCA have a very low risk of develop relapse, although relapses localized to the respiratory tract can occur in these patients.

7.5 Prediction of disease activity by serial measurement of ANCA levels

Relapses have a major impact on disease outcome in patients with SVV. Renal relapses during follow-up have recently been shown to be the most important predictor of long-term renal survival. Therefore, it is extremely important to identify patients at risk of relapse. The usefulness of serially measuring ANCA titers in predicting disease activity is at present still controversial. Several studies, most of them restrospective, have been published in which the relation between rises in ANCA levels as measured by IIF or by ELISA and disease activity of ANCA associated vasculitis was studied (Table 6 and 7).

Table 6. *Relationships between increases in ANCA as determined by IIF and relapse of ANCA-associated small vessel vasculitis as reported by different studies*

Number of patients	ANCA pattern on IIF (%)	ANCA rise prior or at the time of relapse (%)	ANCA rise followed by relapse (%)
35	C-ANCA	100	77
58	C-ANCA	90	75
68	C-ANCA	24	56
37	C/P-ANCA	43	23
85	C-ANCA	52	57

Table 7. *Relationships between increases in ANCA as measured by ELISA and relapse of ANCA-associated small vessel vasculitis as reported by different studies*

Number of patients	ANCA antigenic specificity	ANCA rise prior or at the time of relapse (%)	ANCA rise followed by relapse (%)
56	extract	41	62
60	N.R.	74	79
17	PR3	33	59
19	MPO	73	79
25	MPO	100	80
85	PR3	81	71
15	MPO	75	100
10	PR3 (direct)	79	92
10	PR3 (capture)	100	83
48	MPO/PR3 (direct/capture)	61	100
100	PR3 (capture)	74	60

N.R. - not reported

7.6 Pathogenic potential of ANCA

Although direct evidence for the pathogenicity of ANCA in vasculitis is lacking, ample evidence from in vitro and in vivo studies support a potential role of ANCA in the development of vascular lesions.

PR3-ANCA and MPO-ANCA are able to inhibit enzymatic activity and prevent the inactivation of PR3 and MPO by its natural inhibitor alfa-1 antitrypsin and ceruloplasmin, respectively. In vitro, sera or purified IgG from ANCA-positive patients, as well as, monoclonal antibodies directed against MPO or PR3, have been found to induce an oxidative burst and degranulation in healthy human neutrophils pretreated with inflammatory cytokines such as tumor necrosis factor-α, IL-1β or bacterial lipopolysaccharide (LPS). Furthermore, ANCA-activated PMN are capable of damaging cultured endothelial cells. Indeed, freshly isolated and untreated PMNs from patients with ANCA-associated vasculitis are found to produce significantly more superoxide than PMN from normal control subjects. ANCA have been shown to activate monocytes to production of reactive oxygen species, IL-8, a potent attractant for PMN, and monocyte chemoattractant protein 1 (MCP-1), even without prior priming. Immunopathological studies have shown that inflammatory infiltrate is composed mainly of activated T lymphocytes, the majority of which are CD4+ and macrophages. T-lymphocytes isolated from WG patients proliferate in response to a crude neutrophil extract containing PR3 and MPO.

Although all of the aforementioned mechanisms may be operative in vivo in idiopathic vasculitis, conclusive evidence for the pathogenicity of ANCA awaits, however, a convicing animal model of ANCA-induced vasculitis. In a rat model of autoimmunity, the administration of mercuric chloride (HgCl2) to Brown Norway (BN) rats leads to a syndrome characterized by the presence of autoantibodies against a variety of antigens, including DNA, collagen, thyroglobulin, glomerular basement components and MPO. On pathologic examination of the animals, moderate acute tubular necrosis and lymphocytic infiltration in the interstitium and perivascularly can be observed. Autoantibodies against human MPO that cross-react with rat MPO, can be observed in BN rats immunized with human MPO. The autoimmune response alone does not result in clinical lesions. However, upon administration of human MPO and its substrate H202, these rats develop necrotizing glomerulonephritis with interstitial tubulonephritis, pulmonary and gastrointestinal vasculitis. Recently, it has been shown that Wistar-Kyoto rats immunized with purified human MPO in CFA develop alveolar lung haemorrhage and a mild glomerulonephritis. Moreover, recent studies on mouse models provided elegantly prove that MPO-ANCA alone induce pauci-immune glomerulonephritis and vasculitis. MPO deficient mice were immunized with mouse MPO and circulating anti-murine MPO antibodies were developed. Adoptive transfer, either of splenocytes or purified IgG derived from the MPO-immunized MPO-deficient mice, resulted in the development of crescentic glomerulonephritis and systemic vasculitis mimicking the human disease.

Schematic presentation of an integrative view of ANCA-mediated vascular tissue damage is shown in Figure 2. The model is based on four prerequisities for endothelial cell damage by ANCA: 1) the presence of ANCA; 2) expression of target antigens for

ANCA on primed neutrophils and monocytes; 3) the necessity of an interaction between primed neutrophils and endothelium by means of β2-integrins and 4) activation of endothelial cells.

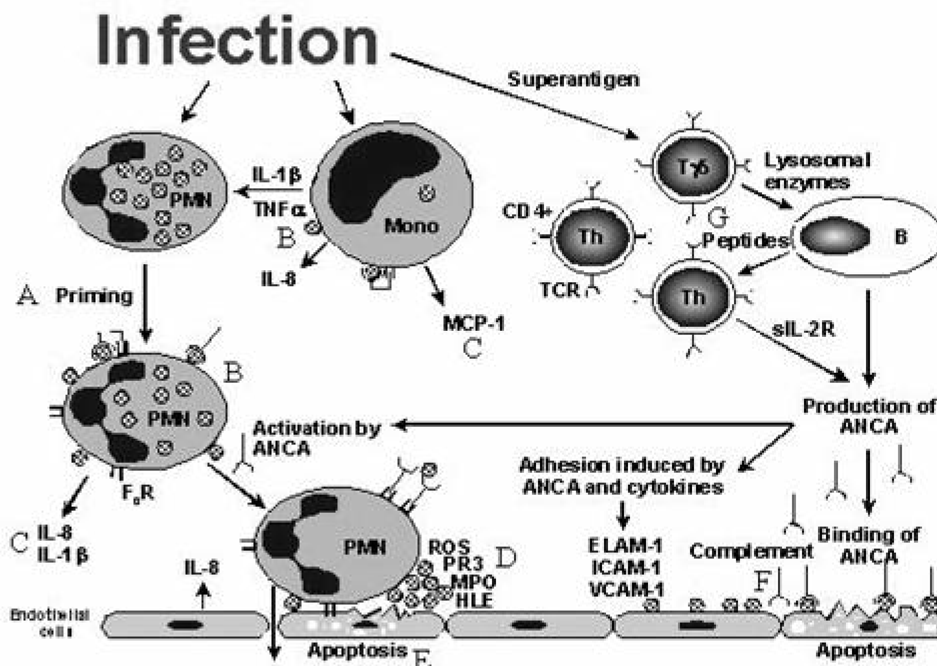

Figure 2. Schematic presentation of an integrative view of the immune mechanisms involved in the pathogenesis of ANCA-associated vasculitis. The cytokines released due to infection or other tissue injury cause the priming of neutrophils and/or monocytes (A) and upregulation of adhesion molecules (ELAM-1, ICAM-1, VCAM-1) on the endothelium. Circulating primed neutrophils and/or monocytes express ANCA antigens (PR3, MPO), adhesion molecules (LFA-1, VLA-4) and FcγR on the cell surface (B). The binding of ANCA to primed neutrophils and/or monocytes induces the release of cytokines such as IL-8, IL-1β, MCP-1 and possibly other factors that are strong chemoattractants for more inflammatory cells possibly leading to granuloma formation (C). Adherence of primed neutrophils and/or monocytes to the endothelium followed by activation of these cells by ANCA. Activated neutrophils and monocytes release reactive oxygen species (ROS), which leads to endothelial cell injury and eventually to necrotizing inflammation (D). PR3 and MPO from ANCA-activated neutrophils and/or monocytes result in the endothelial cell activation, endothelial cell injury, or even endothelial cell apoptosis (E). PR3 and MPO serve as planted antigens resulting in in situ immune complexes, which in turn attract other neutrophils (F). The mechanism by which ANCA production is triggered and perpetuated remain unclear. However, T-cells are thought to play a significant role in mediating the production of ANCA by plasma cells, which are derived from antigen-specific B-cells (G).

7.7 Concluding remarks

ANCA directed against PR3 and MPO can be detected in patients with WG, MPA including renal limited vasculitis and CSS. These ANCAs are highly specific for SVV and are the only ANCAs with clearly documented clinical relevance. Both PR3-ANCA and

MPO-ANCA as tested by ELISA, but not ANCA detected only by IIF, are important diagnostic markers for these forms of vasculitis. Changes in levels of PR3-ANCA and possibly also MPO-ANCA, are related to changes in disease activity, although this correlation is far from absolute. Treatment decisions should be based on the clinical presentation of the patient and histologic findings and not exclusively on the results of ANCA testing. Immunosuppressive treatment of patients with SVV should not be guided by sequential changes in ANCA titers.

However, a rapid increase in ANCA titers or the reappearance of ANCA after a period of ANCA negativity should alert the clinician to the possibility of a relapse and thus may lead to further diagnostic procedures or shorter intervals between follow-up visits. Both in vitro and in vivo experimental data strongly support a pathogenic role for ANCA in SVV. In vivo experimental mouse model has demonstrated that MPO-ANCA directly induces glomerulonephritis and vasculitis.

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