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Anti-neutrophil cytoplasmic antobodies in inflammatory bowel disease and autoimmune liver disease

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Chapter 11

The studies described in this thesis deal with the role of anti-neutrophil cytoplasmic antibodies (ANCA) in inflammatory bowel disease (**Chapters 2-7**) and autoimmune liver disease (**Chapters 8-10**). Three main questions were unsolved when we started the studies four years ago. First, what are the antigenic specificities of ANCA in these diseases? Secondly, what is their clinical value? And thirdly, do ANCA play a role in the pathophysiology of the diseases?

Chapter 1, the Introduction, explains why, based on the knowledge on ANCA in the systemic vasculitides, these questions were raised.
ANCA were first detected in patients with Wegener's granulomatosis, a disease characterized by necrotizing granulomatous inflammation of the upper and lower airways in conjunction with systemic vasculitis and necrotizing crescentic glomerulonephritis. The antigen recognized by ANCA in Wegener's granulomatosis is proteinase 3, a constituent of the azurophilic granules of the neutrophil granulocytes. Antibodies to proteinase 3 have become important tools in the diagnosis of Wegener's granulomatosis. A second type of ANCA, directed against myeloperoxidase, proved an important diagnostic tool to diagnose other types of systemic vasculitis, including microscopic polyangiitis, the Churg-Strauss syndrome, and the renal limited form of systemic vasculitis, that is idiopathic necrotizing crescentic glomerulonephritis.
Apart from their role in the diagnosis of the vasculitides, ANCA have been proven useful as prognostic markers in the follow-up of these diseases. ANCA titers are related to disease activity, and a rise in ANCA titer may precede a clinical relapse. Routinely performed serial quantification of ANCA titers may thus be useful for follow-up and treatment of patients with ANCA-associated vasculitides.
A role for ANCA in the pathophysiology of the systemic vasculitides has been suggested by *in vitro* and *in vivo* studies. *In vitro*, ANCA directed against proteinase 3 and myeloperoxidase have been shown capable to induce degranulation and oxygen radical production in cytokine-primed neutrophils. *In vivo* studies in animal models have shown that an autoimmune response to myeloperoxidase is able to induce vasculitis and glomerulonephritis in the presence of activated neutrophils and to aggravate subclinical glomerulonephritis into severe necrotizing glomerulonephritis. Based on these findings, it is now believed that ANCA alone do not induce vasculitis but, together with other, possibly exogenous, inflammatory stimuli result in the development of severe and persistent vasculitis.
Thus, the detection of ANCA has been a major step forward in the diagnosis and follow-up of the systemic vasculitides and in the understanding of their pathophysiology.

After the detection of ANCA in the systemic vasculitides, ANCA have also been detected in other inflammatory diseases, including inflammatory bowel disease and autoimmune liver

disease. The identification of ANCA in association with inflammatory bowel disease and autoimmune liver disease seemed to promise the development of a potential new marker for disease classification and possibly prognosis, analogous to the significance of ANCA in the systemic vasculitides. However, it became clear that ANCA in inflammatory bowel and liver diseases were different from ANCA in the vasculitides. The antigens were not proteinase 3 and myeloperoxidase, but included other granule proteins, among which lactoferrin, cathepsin G, and bactericidal/permeability-increasing protein (BPI), and unidentified antigens located in the cytosol and the nucleus of the neutrophil. Furthermore, the clinical value of ANCA testing for the follow-up of inflammatory bowel and autoimmune liver disease remained a matter of debate in the years following their first detection. Studies on the clinical significance of subgroups of antibodies with defined specificities had not been performed. Finally, the pathophysiological significance of ANCA in inflammatory bowel disease and autoimmune liver disease had not been studied in detail.
Based on the value of ANCA in the vasculitides, the detection of ANCA in inflammatory bowel disease and autoimmune liver disease raised the three questions that were addressed in this thesis: first, what are the antigenic specificities of ANCA in inflammatory bowel disease and autoimmune liver disease? Secondly, what is their clinical usefulness in these diseases? And thirdly, do they have a role in the pathogenesis and pathophysiology of these diseases?

Chapter 2 describes the prevalence and the antigenic specificities of ANCA in inflammatory bowel disease. In our study population, 58% of patients with ulcerative colitis and 19% of patients with Crohn's disease were positive for ANCA as detected by indirect immunofluorescence. To identify the antigenic specificities of these ANCA, we performed immunoblotting experiments, using crude extracts of neutrophil granulocytes as source of antigens. We observed reactivity of samples from patients with both ulcerative colitis and Crohn's disease with three particular proteins. The first protein, seen as a band of 80 kD on immunoblot, was lactoferrin. Antibodies to lactoferrin were present in 26% of patients with ulcerative colitis and in 11% of patients with Crohn's disease. The other two proteins, a 57/56 kD doublet and a 47 kD single band, could not be identified as known ANCA antigens. Partial purification by cation and anion exchange chromatography, followed by excision of the bands from an SDS-PAGE gel and internal amino acid sequence analysis, revealed that the 57 kD band was identical with catalase and the 47 kD band was identical with alpha-enolase. Antibodies to catalase were found in 38% of patients with ulcerative colitis and 26% of patients with Crohn's disease. Remarkably, reactivity with catalase on immunoblot was also seen in 10% of samples from healthy controls. Antibodies to alpha-enolase were detected in 10% of patients with ulcerative colitis, 18% of patients with Crohn's disease, and 6% of healthy controls.

Thus, ANCA in inflammatory bowel disease are mainly directed against lactoferrin, catalase, and alpha-enolase.

In **Chapter 3**, we analyzed the clinical significance of the presence of ANCA in inflammatory bowel disease. First, we searched for a correlation between ANCA as detected by indirect immunofluorescence and clinical features of inflammatory bowel disease. In ulcerative colitis, ANCA titers correlated with disease activity, and in Crohn's disease, ANCA were associated with colonic localization of the disease. No associations were found between ANCA and Crohn's disease activity, localization of ulcerative colitis, duration of disease, immunosuppressive medication, or previous bowel resection.
Secondly, since the association between ANCA and clinical features in vasculitis is based on ANCA directed against one single antigen, we analyzed the clinical significance of antibodies to lactoferrin, catalase, and alpha-enolase. Antibodies to lactoferrin were associated with colonic localization of Crohn's disease, but other associations were not found. Thus, definition of the antigenic specificities of ANCA did not significantly contribute to their clinical significance in inflammatory bowel disease.

In **Chapter 4**, we analyzed the relation between disease activity and ANCA titers in longitudinal samples from patients with inflammatory bowel disease. We were not able to detect any correlation between disease activity and ANCA titers both in ulcerative colitis and in Crohn's disease. Thus, in contrast to their significance in Wegener's granulomatosis, ANCA titers are not useful for monitoring disease activity in inflammatory bowel disease.

ANCA directed against proteinase 3 and myeloperoxidase have been shown to induce oxygen radical formation and degranulation in primed neutrophils *in vitro*. In **Chapter 5**, we investigated whether ANCA with other specificities were also able to activate neutrophils *in vitro*. Primed neutrophils were stimulated with monoclonal or polyclonal antibodies to lactoferrin, BPI, catalase, and alpha-enolase. Polyclonal antibodies to lactoferrin, BPI, and catalase were able to induce superoxide production in neutrophils. In addition, cells were stimulated with IgG fractions from patients containing autoantibodies to these four antigens. However, these autoantibodies were not able to induce superoxide production. Thus, ANCA directed against antigens different from proteinase 3 and myeloperoxidase do not activate neutrophils *in vitro*. This suggests that their possible pathophysiological role in the disorders associated with these ANCA is limited.

Chapter 6 tries to link the induction of ANCA in inflammatory bowel disease to genetic differences in the production of the cytokine interleukin-10 (IL-10). IL-10 is an important anti-inflammatory cytokine. IL-10 deficient mice have been shown to develop spontaneously

chronic enterocolitis, suggesting a protective role for IL-10 in the pathogenesis of inflammatory bowel disease. Since IL-10 is able to activate B cells, a role for IL-10 has been implicated in the induction of autoantibodies. Recently, a polymorphism in the promoter region of the IL-10 gene has been shown to influence the levels of IL-10 production. First, we studied whether the distribution of this polymorphism was different in inflammatory bowel disease compared to healthy controls. No differences in distribution were found. Secondly, we determined whether the IL-10 genotype was associated with the severity of the disease course. This was not the case. Finally, we studied whether the IL-10 genotype was related to the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA). No relation was found. Thus, this IL-10 polymorphism is not likely to contribute to the susceptibility for inflammatory bowel disease, to the severity of the disease course, and to the induction of ANCA.

Chapter 7 gives a summary of our own findings and the findings of others on ANCA in inflammatory bowel disease. ANCA are detected in 50-85% of patients with ulcerative colitis and in 10-20% of patients with Crohn's disease. Multiple target antigens have been identified. In addition to a number of granule proteins including lactoferrin and BPI, these include the cytosolic enzymes catalase and alpha-enolase and the nuclear proteins histone H1, HMG1/2, and lamin. Our own studies and studies of others have shown that the clinical significance of ANCA in inflammatory bowel disease is very limited, as the presence of ANCA is not related to clearly distinguishable clinical subgroups of disease and titers of ANCA are not related to disease activity. Determination of the antigenic specificities of ANCA does not lead to an increase of their clinical value. Furthermore, the absence of *in vitro* effects of these ANCA on neutrophils suggests a limited role in the pathophysiology of inflammatory bowel disease. Recent studies have indicated that ANCA in inflammatory bowel disease may be induced due to cross-reactivity towards bacterial antigens.
In conclusion, in inflammatory bowel disease, ANCA may just be an epiphenomenon of chronic inflammation, and their development may be triggered by dysregulation of the immune system together with accidental crossreactivity with environmental antigens.

The second part of this thesis deals with the role of ANCA in autoimmune liver disease. **Chapter 8** reports that 67% of patients with primary sclerosing cholangitis are positive for ANCA. Main target antigens for ANCA were BPI (46%), cathepsin G (23%), and lactoferrin (22%). ANCA as detected by indirect immunofluorescence were not significantly associated with clinical characteristics of the disease, including the presence of cirrhosis and the coexistence of inflammatory bowel disease. However, antibodies to BPI and cathepsin G were both associated with the presence of cirrhosis, and antibodies to lactoferrin were more frequently detected in patients with primary sclerosing cholangitis in conjunction with

ulcerative colitis. Thus, defined specificities of ANCA in primary sclerosing cholangitis may be related to particular clinical features of the disease.

Chapter 9 defines the prevalence, antigenic specificities, and clinical significance of ANCA in autoimmune liver disease. By indirect immunofluorescence, ANCA were present in 74% of patients with autoimmune hepatitis, 26% of patients with primary biliary cirrhosis, and 60% of patients with primary sclerosing cholangitis. In these diseases, ANCA were mainly directed against the novel ANCA antigens catalase and alpha-enolase, that we identified in Chapter 2, and against lactoferrin. ANCA as detected by immunofluorescence were associated with relapsing autoimmune hepatitis and with a decreased liver synthesis function in primary biliary cirrhosis and in primary sclerosing cholangitis. ANCA may thus be associated with a more severe course of autoimmune liver disease. In contrast to the findings for primary sclerosing cholangitis as reported in Chapter 8, definition of the antigenic specificities of ANCA did not significantly contribute to their clinical significance in this study.

Chapter 10 summarizes the literature on ANCA in autoimmune liver disease. ANCA have been detected in 25-90% of patients with primary sclerosing cholangitis, 40-96% of patients with autoimmune hepatitis, and 0-30% of patients with primary biliary cirrhosis. The antigenic specificities of these ANCA are not different from the antigens found in inflammatory bowel disease and include lactoferrin, BPI, cathepsin G, catalase, and alpha-enolase. The clinical value of ANCA has been studied most extensively in primary sclerosing cholangitis. ANCA may be related to the coexistence of ulcerative colitis, involvement of the intra- and extrahepatic biliary tree, and cirrhosis. Several studies have shown that ANCA may remain present after liver transplantation.
The occurrence of ANCA was also studied in liver diseases that have no autoimmune origin. Of particular interest are the hepatic disorders associated with alpha-1-antitrypsin (α1-AT) deficiency. α1-AT is the major plasma inhibitor of proteinase 3, the main target antigen for ANCA in Wegener's granulomatosis. Severe α1-AT deficiency is a risk factor for the development of panlobular emphysema and various hepatic disorders. Several studies have shown that patients with anti-proteinase 3 ANCA were more frequently deficient for α1-AT than healthy controls. The imbalance between protease and protease inhibitor may lead to increased levels of proteinase 3 in plasma, which may give rise to the formation of autoantibodies. However, studies determining the prevalence of ANCA in α1-AT deficient populations or in α1-AT deficient patients with hepatic disorders have not been performed so far.

At the end of this summary, let us return to the three questions that we asked when we started the studies described in this thesis. First, what are the antigenic specificities of ANCA in inflammatory bowel disease and autoimmune liver disease? Secondly, what is their clinical value? And thirdly, do ANCA play a role in the pathophysiology of the diseases?
To answer the first question, we have shown that ANCA in inflammatory bowel disease and autoimmune liver disease recognize a broad spectrum of antigens, including the novel cytosolic antigens that we identified, catalase and alpha-enolase, and the granule proteins lactoferrin, bactericidal/permeability-increasing protein, and cathepsin G.
Regarding the second question, we have shown that the diagnostic value of ANCA in inflammatory bowel disease and autoimmune liver disease is limited. Antibodies to the aforementioned antigens are not disease-specific, but have been detected in a variety of idiopathic inflammatory diseases, including ulcerative colitis, Crohn's disease, autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis, and rheumatoid arthritis. Antibodies to catalase and alpha-enolase have even been detected in a small percentage of healthy subjects. With respect to the value of ANCA as markers for defined clinical subsets in inflammatory bowel disease, besides their association with colonic involvement in Crohn's disease, no major associations have been detected. In autoimmune liver disease, ANCA are associated, albeit weakly, with more severe disease. Analysis of the antigenic specificities of ANCA does not contribute to their clinical significance.
With respect to the third question, the limited clinical value of ANCA in inflammatory bowel disease and autoimmune liver disease already suggests that their role in the pathophysiology of these diseases is restricted. Indeed, *in vitro* studies have demonstrated that ANCA from patients with these disorders are not able to induce activation of neutrophils.

In conclusion, ANCA in inflammatory bowel disease and autoimmune liver disease recognize multiple antigens, their clinical usefulness is limited, and a major role in the pathophysiology of these diseases is unlikely. In inflammatory bowel disease and autoimmune liver disease, ANCA have to be considered an epiphenomenon of chronic inflammation.