Introduction

Despite intensive research over the last 15 years, the etiology and pathogenesis of vasculitides associated with anti-neutrophil cytoplasmic antibodies is still far from understood. The close association with ANCA and the clinical observation that rises in the level of these autoantibodies often precede relapses in disease activity has prompted the hypothesis that ANCA are intimately involved in the pathogenesis of these vasculitic disorders. *In vitro* studies have provided some clues with regard to the immunopathogenic mechanisms involved. Most importantly, these studies have shown that ANCA are capable of activating neutrophils pretreated (primed) with low doses of TNFα leading to the release of lysosomal enzymes and the production of reactive oxygen species. As such, ANCA-mediated neutrophil activation may cause tissue injury and it has indeed been shown that ANCA-activated neutrophils adhere to and induce lysis of endothelial cells. Although these data suggest that ANCA are of pathogenic importance their *in vivo* relevance is not clear. In the present thesis several studies are described in which the pathogenic potential of ANCA in rats was explored.

Experimental anti-MPO associated necrotizing crescentic glomerulonephritis: the role of complement and antibodies.

As previously described, experimental anti-MPO associated necrotizing crescentic glomerulonephritis (NCGN) can be induced in MPO-immunized Brown Norway rats by renal perfusion of neutrophil lysosomal enzymes including MPO and its substrate hydrogen peroxide. The histopathological features of this experimental model resemble those observed in human anti-MPO associated NCGN and are characterized by a marked influx of PMNs and mononuclear cells, formation of giant cells, crescent formation, vasculitis and paucity of immune deposits at the time of full blown NCGN. However, in the early phase, i.e. 4 hours and 24 hours after perfusion, marked glomerular deposits of complement and IgG are observed. In **chapter 2** we investigated the relative contributions of anti-MPO antibodies and complement to the development of NCGN in this model. Therefore, anti-MPO associated NCGN was induced in MPO-immunized complement depleted Brown Norway rats and in naive rats passively transferred with anti-MPO antibodies. It was found that in MPO-immunized complement depleted Brown Norway rats no NCGN developed suggesting an essential role for complement in the induction phase of this model. It is known that activation of the complement cascade leads to the release of chemotactic factors which cause the recruitment of neutrophils.

Thus, depletion of complement may have attenuated neutrophil influx thereby inhibiting the inflammatory response and preventing the development of NCGN. In rats passively transferred with anti-MPO antibodies derived from MPO-immunized rats necrosis of the glomerular capillary wall was observed at 4 days after perfusion. However, severe lesions such as crescent formation and vasculitis did not develop. These results suggest that renal lesions in experimental anti-MPO associated NCGN are only partly mediated by the anti-MPO antibodies and indirectly suggest that T-cell mediated mechanisms are involved as well. Further studies are needed to clarify the role of T-cells in the development of experimental anti-MPO associated NCGN. These may include T-cell depletion studies and the transfer of MPO-specific T-cells to non-immunized rats.

Expression of eNOS, iNOS and, peroxynitrite-modified proteins in experimental anti-MPO associated necrotizing crescentic glomerulonephritis.

One of the characteristic histopathological features of the experimental model of anti-MPO associated NCGN is marked glomerular and interstitial infiltrates of neutrophils and monocytes. These inflammatory cells may contribute to tissue injury by the release of proteolytic enzymes and the production of reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals. Besides ROS, activated neutrophils and monocytes have been shown to produce NO radicals through an enzyme known as inducible nitric oxide synthase (iNOS) which is expressed in these cells upon stimulation with several inflammatory cytokines. It has been recognized that NO radicals may contribute to tissue injury especially through their reaction with superoxide anions which leads to the formation of peroxynitrite. Peroxynitrite is a very reactive oxidant which can induce lipid peroxidation and modifies proteins by nitration of tyrosine residues (nitrotyrosine) causing loss of structure and function of these proteins. Nitric oxide radicals are also produced by endothelial cells through a constitutively expressed enzyme known as endothelial NOS (eNOS). NO radicals generated by eNOS are physiologically important and play a role in maintaining basal vascular tone. In addition, NO-radicals inhibit platelet aggregation and platelet and leukocyte adhesion to the endothelium. These anti-inflammatory properties have mainly been attributed to eNOS derived NO radicals. Thus, in inflammatory conditions NO radical production may have beneficial as well as harmful effects possibly depending on the NOS isoform generating the NO radicals. In **chapter 3**, the expression of eNOS and iNOS in conjunction with platelet aggregation, leukocyte influx , superoxide anion production and nitrotyrosine formation was studied in the experimental

model of anti-MPO associated NCGN. At 24 hours after perfusion of the neutrophil lysosomal extract and hydrogen peroxide a marked decrease was found in the expression of eNOS in glomerular capillaries and interstitial blood vessels which was associated with massive aggregation of platelets. At later time points, eNOS expression was completely absent in severely damaged glomeruli associated with a marked influx of inflammatory cells. Although expression of iNOS was observed in neutrophils and monocytes at all time points, these cells were most abundantly present at 4 days after perfusion and were predominantly located in the glomeruli. This peak in iNOS expression coincided with the peak of superoxide anion producing cells. In addition, large numbers of nitrotyrosine positive cells were observed which, by double labeling experiments, were found to express iNOS and to produce superoxide anions. Taken together, these results are compatible with a protective role for eNOS derived NO radicals whereas NO radical production by iNOS may contribute to tissue injury through the reaction with superoxide radicals and the subsequent formation of peroxynitrite. However, to prove this suggested differential role for NO radicals further studies are needed in which the NO radical production by either eNOS or iNOS is inhibited. In this respect, interesting information could be derived from experiments in which experimental anti-MPO associated NCGN is induced in iNOS and eNOS knock-out mice both of which have recently been developed.

Anti-myeloperoxidase associated lung disease: an animal model.

In ANCA-associated vasculitides the lungs are a common target. A similar pathogenic mechanism may underlie the occurrence of disease manifestations in both kidneys and lungs. To test this hypothesis, experiments were performed in which, in analogy to the experimental model of anti-MPO associated NCGN, the left lungs of MPO- and control immunized Brown Norway rats were perfused with a neutrophil lysosomal enzyme extract consisting of proteolytic enzymes and MPO. As described in **chapter 4**, severe inflammatory lesions developed in the left lung of MPO-immunized rats upon perfusion of the neutrophil lysosomal extract characterized by pulmonary capillaritis sometimes accompanied by alveolar hemorrhage, marked influx of neutrophils and monocytes and, the presence of multinucleated giant cells. Although less extensive, inflammatory lesions were also observed in left lungs of control immunized rats with similar characteristics as those found in MPO-immunized rats. These results suggest a direct toxic effect of the neutrophil lysosomal extract inducing pulmonary inflammation which in the presence of anti-MPO antibodies may become more severe. A remarkable finding in these studies

was the observation that also in the non-perfused right lungs of MPO-immunized rats severe inflammatory changes developed. In contrast, no inflammation was observed in right lungs from control immunized rats. The mechanisms underlying the development of pulmonary inflammation in the right lungs of MPO-immunized rats are not known. However, we speculate that a combination of systemic effects of inflammatory cytokines released from the inflammatory lesions in the left lung, ischemia induced influx of PMNs, and the presence of auto-antibodies directed against rat MPO caused the development of pulmonary injury in the right lungs of MPO-immunized rats.

Systemic injection of products of activated neutrophils leads to necrotizing vasculitis in the lungs and gut.

In systemic ANCA-associated vasculitides the respiratory tract, the lungs, and the kidneys are the principal target organs. However, disease manifestations can be observed in virtually any organ in the body. The pathogenic mechanisms underlying the development of vasculitis may be similar, irrespective of the organ involved. In **chapter 5**, we hypothesized that release of products of activated neutrophils in the presence of an anti-MPO directed immune-response leads to systemic vasculitis. To test this hypothesis, a neutrophil lysosomal enzyme extract and H_2O_2 were systemically injected via the jugular vein in MPO-immunized Brown Norway rats and several organs were examined for the presence of inflammatory and vasculitic lesions. At 7 and 14 days after injection, patchy inflammatory lesions were observed in the lungs of MPO-immunized rats. These lesions were associated with vasculitis, granuloma formation, scattered multinucleated giant cells, and foci of alveolar hemorrhage whereas at 14 days early signs of fibrosis were observed. In addition to the lungs, a prominent leukocytoclastic vasculitis had developed in the small intestine of MPO-immunized rats characterized by fibrinoid necrosis of vessel walls and an extensive neutrophilic infiltrate. No inflammatory changes were found in the other organs studied (heart, liver, spleen, and kidneys) nor in any organ of control immunized rats. These results indicate that the development of vasculitis in the lungs and gut is dependent on the release of products of activated neutrophils in the presence of an anti-MPO response. Although it is not clear why vasculitic lesions in these studies were confined to the lungs and gut, we speculate that the susceptibility of these organs to bacterial infections might have played a role.

Autoantibodies to myeloperoxidase aggravate mild anti-glomerular-basement-membrane-mediated glomerular injury.
The experimental model of anti-MPO associated NCGN together with the experimental models described in chapters 4 and 5 suggest that anti-MPO antibodies are involved in the development of vascular injury in anti-MPO associated vasculitic disorders. However, a major drawback of these models is the use of heterologous (human) MPO and the necessity to infuse or perfuse heterologous (human) neutrophil lysosomal enzymes. Therefore, these models can not be regarded as genuine auoimmune models for anti-MPO associated vasculitis. However, upon immunization with human MPO Brown Norway rats develop antibodies to human MPO which cross-react with rat MPO. In **chapter 6**, we investigated the pathogenic potential of these autologous anti-MPO antibodies by evaluating the course of mild anti-glomerular-basement-membrane (GBM) mediated glomerular injury in MPO-immunized Brown Norway rats. Mild anti-GBM disease was induced in control and MPO-immunized rats by intravenous injection of a subnephritogenic dose of rabbit-anti GBM antibodies. In control immunized rats, this led to the development of mild glomerular injury reflected by the occurrence of a slight proteinuria and a moderate influx of monocytes at 10 days after anti-GBM antibody administration. In contrast, severe glomerulonephritis developed in MPO-immunized rats characterized by the early occurrence of hematuria and marked proteinuria at day 10 after administration of the anti-GBM antibodies. Histopathologically, crescent formation and fibrinoid necrosis of capillary loops associated with massive glomerular deposition of fibrin were found at day 10. In the interstitium, tubular necrosis and atrophy, and a marked mononuclear inflammatory infiltrate were observed. These studies demonstrate that autoantibodies directed against MPO are able to aggravate mild anti-GBM disease and suggest that anti-MPO antibodies may be a pathogenic factor in rendering mild glomerular injury into clinically overt disease although the exact underlying mechanisms are not known. However, considering the ability of human anti-MPO antibodies to activate primed neutrophils and monocytes *in vitro*, one might speculate that similar pathogenic mechanisms are operative in this model. This would suggest that the mild glomerular inflammation induced by the anti-GBM antibodies is amplified by the autologous anti-MPO antibodies through activation of (primed) neutrophils and/or monocytes.

Recombinant Proteinase 3 (Wegener's autoantigen) expressed in Pichia pastoris *is functionally active and recognized by patient sera.*
Proteinase 3 (Pr3) has been recognized as the major target antigen of ANCA in WG. For diagnostic and research purposes large amounts of highly purified Pr3 are needed. Conventional methods to purify Pr3 from neutrophils are laborious and require large amounts of blood with relatively low yields. Therefore, we produced a recombinant form of Pr3 and explored whether it could be used for the detection of Pr3-ANCA in WG patients as described in **chapter 7**. Early studies attempting to produce recombinant Pr3 in bacteria, insect or mammalian cells showed that the expression cloning of Pr3 is often accompanied by the loss of enzyme activity and epitopes reactive with WG sera. Therefore, we employed an inducible expression system in the methylotrophic yeast *Pichia pastoris* which, when grown on methanol as the sole carbon source, produces large amounts of the inducible alcohol oxidase 1 (*AOX1*) gene product. Here, the strong inducible *AOX1* promoter was used to drive the expression of Pr3. A transfer vector was made that introduced the human Pr3 gene together with a 6-histidine tag into *Pichia pastoris* to construct a recombinant that secreted Pr3 into the medium and which could be isolated by affinity chromatography. Using the *Pichia pastoris* expression cloning system, a recombinant form of Pr3 (rpPr3) was secreted into the medium which could be isolated by immobilized metal chelate affinity chromatography and yielded 613 ± 122 μg rpPr3 per liter induced culture supernatant. By gel electrophoresis, rpPr3 migrated as a 32 kD protein. By western blot and catching ELISA, rpPr3 was recognized by a polyclonal antibody and a monoclonal antibody raised against native human Pr3. rpPr3 was also found to be proteolytically active since it converted the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide. Furthermore, 7 out of 10 sera from WG patients recognized rpPr3 in a catching ELISA system but only one of them reacted with rpPr3 directly coated to ELISA plates. These results suggest that the folding of rpPr3 approaches that of native human Pr3. However, since not all sera form WG patients recognized rpPr3 it cannot replace native human Pr3 for diagnostic purposes at this stage. Studies are in progress to increase the yield of rpPr3 and its immunological reactivity with WG sera.

Elastase, but not proteinase 3 (Pr3), induces proteinuria associated with loss of glomerular basement membrane heparan sulfate after in vivo renal perfusion in rats.
Proteinase 3 (Pr3) and elastase are cationic neutral serine proteinases localized in the azurophilic granules of polymorphonuclear cells (PMN) and lysosomes of monocytes. It has long been recognized that release of these enzymes from

activated PMNs and monocytes may contribute to tissue injury through their proteolytic activity. *In vitro* studies have indeed shown that Pr3 and elastase are capable of degrading several constituents of the extracellular matrix and *in vivo* elastase induces proteinuria upon renal perfusion in rats. However, unlike elastase, little is known about the *in vivo* effects of Pr3. In **chapter 8**, we investigated whether Pr3, like elastase, induces proteinuria after perfusion of the left kidney in Brown Norway rats. As previously shown by other investigators, we found that elastase induced a dose-dependent proteinuria as measured at 24 hours after renal perfusion of the active enzyme. Since inactivated, but equally cationic, elastase did not induce proteinuria, it was concluded that the induction of proteinuria is dependent on the proteolytic activity of the enzyme. Histopathologically, no morphological abnormalities such as disruption of the glomerular basement membrane or influx of PMNs or monocytes were found. However, by immunohistology it was observed that elastase induced proteinuria was associated with a strong decrease in the intraglomerular expression of heparan sulfate proteoglycans (HSPG). Especially, the expression of the heparan sulfate glycosaminoglycan chains of HSPG was found to be greatly reduced. These results suggest that elastase-induced proteinuria is mediated by the proteolytic cleavage of HSPG since these extracellular matrix components are considered to be the main determinants for the charge-dependent permeabilitiy of the glomerular capillary wall, primarily due to their negatively charged glycosaminoglycan chains. In contrast to elastase, renal perfusion of proteinase 3 with equal proteolytic activity did not induce proteinuria and did not reduce the intraglomerular expression HSPG. *In vitro* studies showed that, in comparison with elastase, Pr3 binds to a lesser extent to HSPG-coated ELISA plates but is capable of degrading HSPG. From these studies it was concluded that the inabillity of Pr3 to induce proteinuria relates to its weaker binding to the GBM due to its lower isoelectric point (9.1 for Pr3 versus >11 for elastase). Taken together, these results indicate that proteolytic cleavage of HSPG by elastase is a contributing factor in PMN-mediated glomerular injury and suggest a minor role for Pr3.

Concluding remarks: Animal models of anti-neutrophil cytoplasmic antibody associated vasculitis

In **chapter 9**, the international literature concerning animal models of ANCA-associated vasculitis, including some of the models described in these thesis, was reviewed in the context of *in vitro* studies and observations in patients concerning the pathogenic potential of ANCA. It was concluded that, thus far, none of the models described prove that ANCA are pathogenic. In addition,

none of the models completely mirrors human ANCA-associated vasculitis. Perhaps the most important observation in these animal models of ANCA-associated vasculitis is that ANCA in itself are not pathogenic since the presence of these autoantibodies alone does not result in vascular injury. Additional factors, inducing priming or activation of neutrophils and/or monocytes, are needed initially after which the inflammatory response may be amplified by ANCA. In this respect, the *in vivo* models do mirror the situation in man in which high levels of ANCA can be present without any signs of disease activity. Thus, future studies should be aimed at elucidating these initial factors triggering ANCA-associated vasculitis.

Future perspectives: Where do we go from here?

Since the discovery of ANCA as a group of autoantibodies associated with idiopathic systemic vasculitides, the question has been raised whether these autoantibodies are involved in the pathogenesis of their associated diseases. Following, a vast number of studies have been performed to investigate the pathogenic potential of ANCA. As described in the general introduction, these studies have indicated that autoantibody (ANCA)-mediated, (T) cell-mediated mechanisms, infection related mechanisms or combinations thereof may be involved in the development of ANCA-associated disorders but , thusfar, the primary, initiating factor that causes ANCA-associated vasculitis has not been identified. Although it is generally assumed that ANCA play a role in the pathophysiology of ANCA-associated vasculitis, several arguments can be given which plea against this contention. First, the association between ANCA and certain forms of idiopathic vasculitis is not absolute.[1-4] Even in WG, cases have been described with biopsy proven WG but without the presence of ANCA in the serum. Second, high levels of ANCA can be present in patients without any signs of disease activity.[1-4] The animal studies on ANCA-associated vasculitis described seem to confirm this observation by showing that the mere presence of ANCA is not sufficient to induce vasculitis.[5,6] Based on the aforementioned arguments, it may be suggested that ANCA are not directly involved in the pathophysiology of their associated diseases but are just a diagnostically helpful epiphenomenon. However, although ANCA are probably not the initiating factor of vasculitis, the *in vitro* studies and the animal studies concerning ANCA-associated vasculitic disorders reported sofar, strongly suggest that ANCA are involved in the effector phase of their associated disease and can amplify the inflammatory response through several mechanisms. Therefore, it seems

justified to further study the pathogenic potential of ANCA. In this respect, it is interesting to note that recent studies in WG patients have shown that not only quantitative changes but also qualitative changes in anti-Pr3 antibodies are related to disease activity in WG. It was demonstrated that especially changes in the levels of IgG3-anti-Pr3 antibodies are related to disease activity in WG.[7] Moreover, a positive correlation was found between the relative level of IgG3-anti-Pr3 antibodies and the capacity of sera to activate primed neutrophils *in vitro*.[8] In addition, earlier studies have shown that the functional capacity of anti-Pr3 antibodies to interfere with the irreversible inactivation of Pr3 by its natural inhibitor a1-anti-trypsin, correlated with disease activity rather than the anti-Pr3 antibody titer.[9,10] Together, these studies indicate that in the course of the disease anti-Pr3 antibodies may recognize different epitopes on their target antigen, some of which are pathogenic and some are not. Thus, recently, we have started to characterize the different epitopes on Pr3 recognized by anti-Pr3 antibodies in the course of the disease. Similar studies are being performed by others with regard to the epitope specifities of anti-MPO antibodies.[11] In addition to autoantibody-mediated mechanisms, the contribution of (T)cell-mediated mechanims in the pathophyiology of ANCA-associated vasculitides needs further investigation. As mentioned in the general introduction, indirect evidence exists suggesting an important pathogenic role for T-cells in these diseases including the presence of large numbers of T-cells in vascular lesions and elevated levels of T-cell activation markers (e.g. soluble IL-2 receptor levels) in patients with active disease.[12-14] So far, *in vitro* studies attempting to identify T-cells autoreactive for the known ANCA-antigens Pr3 and MPO have not provided convincing evidence for the presence of such T-cells.[15-17] However, the results of these studies may have been biased by the use of peripheral blood lymphocytes. Therefore, an alternative approach might be to use T-cells derived from lesional tissue. Although such studies will be technically demanding they could throw some light on the pathogenic role of T-cells in ANCA-associated vasculitis.

The link between infections and ANCA-associated vasculitis is well established now. Infections often precede relapses of disease activity and antibacterial treatment can reduce the incidence of disease exacerbations in WG.[18-21] Infectious agents may contribute to ANCA-associated vasculitis through several mechanisms. Infections may induce the production of pro-inflammatory cytokines such as TNFα and IL-1β thereby creating the environment in which ANCA-mediated neutrophil and monocyte activation can occur. Also, bacterial components may act as superantigens inducing autoreactive B-cells to produce ANCA.

Although ANCA are closely associated with a range of primary vasculitides, it has become increasingly recognized that ANCA are not specific for vasculitic syndromes but can also be detected in a number of non-vasculitic diseases.[22] Most of these diseases are inflammatory or infection-related such as inflammatory bowel disease and cystic fibrosis.[22] ANCA in these diseases are usually not directed against MPO and Pr3. Instead, a number of proteins have been identified as novel ANCA antigens including bactericidal/permeability-increasing protein (BPI), lactoferrin and defensins.[25] It is interesting to note that the majority of ANCA antigens are essential components of the microbicidal activity of neutrophils. This has led to the hypothesis that autoimmunity directed against neutrophilic constituents initially develops in an inflammatory environment and may be driven by microbial agents possibly by forming complexes with these neutrophilic components.[25] In this theory, activation of the immune response directed against the infectious agent may also induce an autoimmune response against the neutrophilic constituents. This interesting hypothesis further supports the involvement of infections in ANCA-associated diseases but clearly needs further investigation in particular focused on the underlying infectious agents and their interaction with ANCA antigens.

An essential step in proving that ANCA are of pathogenic importance in their associated diseases is the development of an animal model which closely mimics the clinical and histopathological features of its human counterpart. Although the animal models for ANCA-associated vasculitis described thusfar can be useful in the study of certain aspects of the inflammatory process, all have some major drawbacks. Recently, experiments have started that focus on the role of *Staphylococcus aureus* in the development of WG. As part of this project, in vivo studies will be performed in which the effects of *Staphylococcus aureus* on the development of ANCA-associated glomerulonephritis will be explored. Hopefully, these studies will provide more evidence for the *in vivo* pathogenic potential of ANCA and the role of infections in ANCA-associated vasculitis.