Review

New developments in the pathogenesis of ANCA-associated vasculitis

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ABSTRACT

In recent years there have been substantial developments in the understanding of the pathogenesis of ANCA-associated vasculitidies. Animal models have now been developed that finally prove a direct pathogenic role for ANCA, a subject fiercely debated since their original identification. We are also closer to understanding how ANCA exert their effects to cause disease. Progress has been made in elucidating how ANCA activate neutrophils, from how they bind antigen and where that antigen is located, to how antigen binding is translated into intracellular activity. The effects of ANCA activation on the effector functions of neutrophils and monocytes are being further dissected and the flow-based assay is allowing interactions with endothelium to be studied in more detail. Knowledge of the role of T cells has been enhanced by examining contributions to disease by differing subsets and their cytokine secretions. Defects in apoptosis playing a role in the initiation of other autoimmune diseases has prompted investigations into whether a similar pathogenesis is relevant in vasculitis, and various genetic polymorphisms have been discovered to be important in determining in whom vasculitis develops. This article reviews how recent research has helped in the understanding of the pathogenesis of small vessel vasculitis.

ANCA are pathogenic

Up until recently, the evidence that ANCA are pathogenic has been largely circumstantial. They are present in at least 90% of patients with pauci-immune vasculitis and their levels appear to reflect disease activity. *In vitro* evidence shows that ANCAcan bind to its antigens, the neutrophil serine proteases proteinase 3 and myeloperoxidase, when they are surface expressed following neutrophil priming. This priming can be achieved *in vitro* by TNF-α and probably occurs as a result of an intercurrent infection in patients. Binding via the Fab and Fc portions of ANCA to neutrophil Fcγ receptors IIa and IIIb, causes neutrophil activation with respiratory burst and release of granule enzymes and cytokines. These activated neutrophils then go on to induce endothelial cell damage by release of granule contents, superoxide products and nitric oxide, and recruit other inflammatory cells via cytokine release.

Early animal models

Early animal models have only been able to implicate ANCA as a co-factor in causing glomerulonephritis rather than as the sole causative factor. For instance, Kobayashi *et al.* induced rat nephrotoxic serum nephritis with subnephritic doses of anti-GBM antibody by the addition of rabbit anti-rat MPO (1). Heeringa *et al.* similarly showed that immunization of rats with human MPO with levels of anti-GBM that would normally give only mild disease, resulted in acute glomerulonephritis (2). Brouwer *et al.* perfused the kidneys of rats, which had previously been immunized with human MPO and developed both anti-human and anti-rat MPO antibodies, with human neutrophil extracts resulting in acute crescenteric glomerulonephritis (3). However, this was accompanied by glomerular immune deposits and was therefore distinct from human pauci-immune disease.

MPO-ANCA causes vasculitis development in mice

Xiao *et al.* have now produced convincing *in vivo* evidence of ANCA pathogenicity (4) (Fig. 1). MPO knockout mice were immunised with murine MPO and splenocytes from these animals transferred into recombinase-activating gene-2 (Rag2) deficient mice.

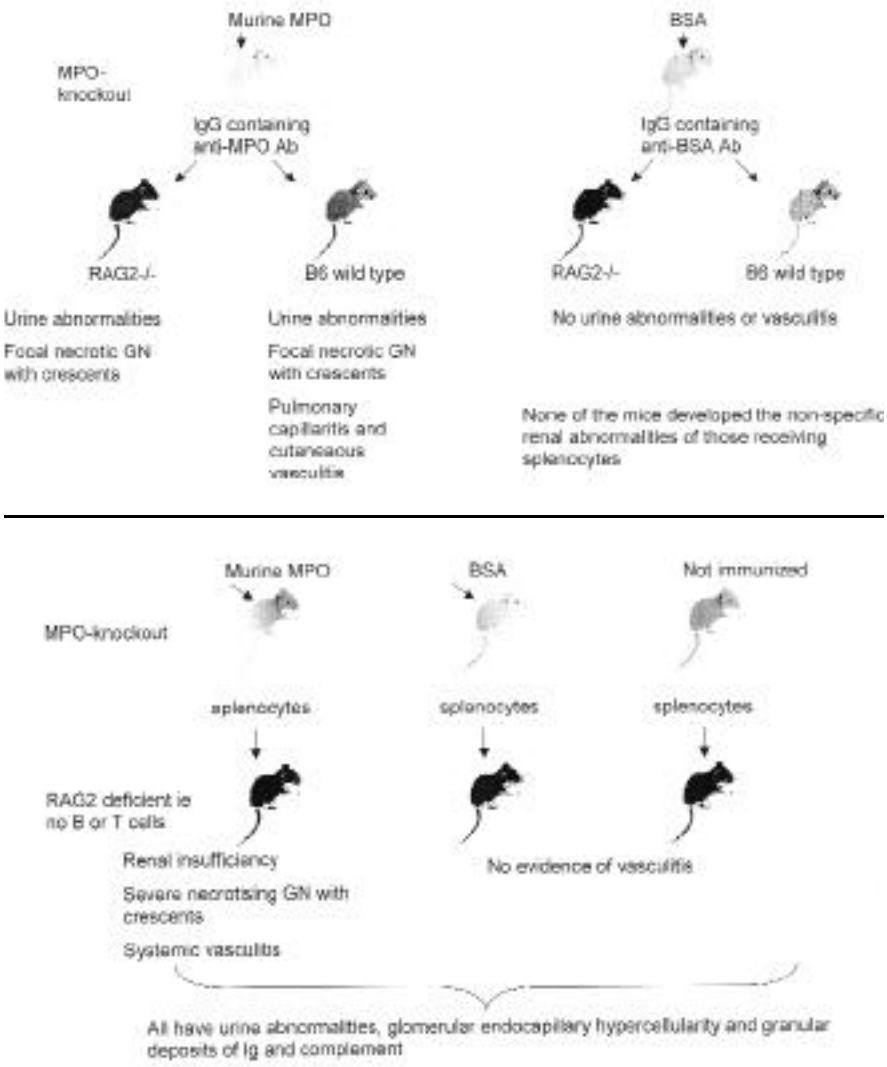

Fig. 1. MPO-ANCAare pathogenic – Xiao *et al*.

These recipient animals lack the ability to initiate V (D)J rearrangement and therefore do not produce functioning T or B lymphocytes. The Rag2$^{-/-}$ mice all developed anti-MPO antibodies within three days of receiving the anti-MPO splenocytes in a dose dependent manner. This was in contrast to mice receiving splenocytes from BSA immunised mice or control non-immunised mice, neither of whom developed anti-MPO antibodies. The Rag2$^{-/-}$ mice receiving the larger doses of anti-MPO splenocytes developed marked renal insufficiency and all were shown to have severe necrotising and crescenteric glomerulonephritis. Several of these mice also developed other features of systemic vasculitis including necrotising arteritis in the spleen and lymph nodes and a pulmonary capillaritis. None of the control mice, receiving either the anti-BSA splenocytes or non-immunised splenocytes developed either renal insufficiency or a necrotising, crescenteric glomerulonephritis. However, all mice receiving the higher doses of splenocytes developed urine abnormalities (proteinuria, haematuria and the presence of urine leucocytes) and a mild to moderate glomerular endocapillary hypercellularity. This was associated with granular glomerular deposits of immunoglobulins and complement. The cause of this immune complex glomerulonephritis is unknown but the authors speculate that it may be caused by introducing functioning lymphocytes into an animal without an adaptive immune system either through reaction of antibodies with exogenous antigen or via a graft-versus-host reaction.

To test the hypothesis that it was ANCA alone causing the vasculitic disease, anti-MPO or anti-BSA antibodies purified from the MPO knockout mice were given as a single intravenous injection to either Rag2$^{-/-}$ or wild-type B6 mice. Anti-MPO activity was confirmed by ELISA in the mice receiving anti-MPO antibodies and not in those receiving anti-BSAantibodies. By three days, those mice receiving the anti-MPO antibodies had developed urine abnormalities and on sacrifice at day six, were shown to have developed a focal, necrotising glomerulonephritis with crescents. In neither the mice given anti-MPO antibodies nor in the controls given anti-BSA antibodies, was there any evidence of other glomerular abnormalities or immunoglobulin deposition and in this way the renal lesion precisely matched that seen in human disease. In addition to the renal lesions, several of the wild-type mice receiving anti-MPO antibodies also developed pulmonary capillaritis and cutaneaous vasculitic lesions that were histologically identical to human ANCA-associated vasculitis. The renal lesions were not as extensive as in the mice receiving splenocytes or accompanied by severe renal dysfunction. This may be due to a role for Tcells in induction of the inflammation or may simply be as a result of the co-localisation of immune complexes seen in the mice receiving splenocytes. Indeed ANCA is known to exacerbate renal diseases caused by IgA nephropathy or lupus producing a crescenteric nephritis. Moreover, a recent report (5) suggests that the presence of glomerular immune deposits in patients with ANCA-associated glomerulonephritis is associated with greater proteinuria and a trend towards a worse outcome with regards to renal function and death.

This animal model is the first convincing evidence of the direct pathogenicity of ANCA. A different animal model has been developed by Smyth *et al.* (6). Wistar Kyoto rats were immunised with purified human MPO and over the following four weeks all rats developed

anti-MPO antibodies to both human and rat MPO confirmed on both immunofluoresence and ELISA. The majority went on to develop haematuria and mild proteinuria and histological examination revealed segmental inflammation in the glomeruli with tubular red cell casts and tubulo-interstitial inflammation. There were no deposits of IgG and scanty tubular C3. Examination of lung tissue showed evidence of pulmonary haemorrhage in 80% of the rats.

In order to investigate the addition of a local renal immune stimulus, in addition to MPO, a group were also given a sub-nephritogenic dose of rabbit anti-rat glomerular basement membrane antibody. These rats rapidly developed macroscopic haematuria and heavy proteinuria. Histological examination of kidney revealed much more extensive renal lesions with segmental inflammation in 100% and fibrinoid necrosis and crescents in 80%.

C-ANCA causes pulmonary vasculitis in rats

In addition, Weidebach *et al.*, were able to provoke pulmonary vasculitis in Wistar rats, following the injection of the C-ANCApositive IgG fraction isolated from the serum of three different Wegener's granulomatosis patients prior to treatment (7). Animals were sacrificed 24 hours after receiving the infusion and at this stage all rats had pulmonary vasculitis (although no fibrinoid necrosis) and some had granuloma-like structures. Such changes were absent in animals receiving IgG from patients with rheumatoid arthritis and from healthy controls. Moreover, there was a dose-dependent relationship between the cANCA concentration and degree of inflammatory response seen. ANCA pathogenicity was further implicated by the absence of vasculitis in animals receiving IgG from one of the initial patients who had become ANCA negative on remission.

These models provide the previously missing evidence proving a truly pathogenic role for ANCA and in addition should allow the further *in vivo* study of ANCA-associated vasculitis and glomerulonephritis.

How are ANCA produced?

How ANCA are produced initially and autoimmunity initiated is still not known. PR3 and MPO are intracellular enzymes, stored in granules inside neutrophils and if released on neutrophil activation they are rapidly scavenged by antiproteinases. Thus they are hidden from the immune response. However, following priming such as by TNFα, these enzymes are surface expressed on neutrophils (8). Apoptosis can occur in ageing, non-activated neutrophils or following exposure to inflammatory stimuli such as TNFα or reactive oxygen species so to limit tissue damage. Apoptosis induces cell membrane changes exposing molecules such as phophatidylserines which allow macrophages to recognise them as apoptotic and clear them in a non-inflammatory manner by releasing cytokines such as TGFβ. However, under certain conditions it appears that when apoptotic cells are taken up by dendritic cells, which are potent antigen presenting cells, cross-presentation of antigen can occur and T cells specific for antigen expressed on the surface of apoptotic cells can be activated (9, 10). This may be an important route to the production of autoimmunity and has been postulated to occur in systemic lupus erythematosus. Many of the autoantigens in lupus have been shown to be present in the blebs of apoptotic cells (11) and defects in apoptosis documented. In addition, mice immunised intravenously with syngenic apoptotic thymocytes have been shown transiently to develop antinuclear and anticardiolipin antibodies (12).

Do apoptotic neutrophils have a role in initial ANCA production?

Recent work has investigated this phenomenon in the production of ANCA. Patry *et al*. have shown that when Brown Norway rats are injected with syngenic apoptotic neutrophils, but not freshly isolated neutrophils, ANCAare produced (13). These were pANCA with the majority being specific for human leukocyte elastase (HLE). The sequence of HLE is largely conserved across species and closely related to proteinase 3, which has not yet been defined in rats. Despite the presence of ANCA, there was no histological evidence of glomerulonephritis or vasculitis in any tissues. Rauova *et al*. immunised C57BL/6J mice with either live or apoptotic human lymphocytes, or live, apoptotic, formalin fixed or lysed human neutrophils (14). Mice receiving intraperitoneal live or apoptotic neutrophils developed ANCA specific for lactoferrin or myeloperoxidase. Following a further intravenous infusion of apoptotic neutrophils, these mice developed PR3-specific ANCA. Again, no vasculitic lesions were found in mice developing ANCA. It was felt that the live neutrophils may have led to ANCAproduction as they underwent apoptosis following intraperitoneal injection. Although human neutrophils were used, the investigators argue against ANCA being produced as a xenogenic response, as there was no antibody response produced by the formalin-fixed neutrophils or against the neutrophil lysate that contained PR3 and MPO. It is interesting that in both these animal models, despite the presence of ANCA, there was no vasculitic disease. This may be because ANCA titres were not high enough or neutrophils not primed enough to allow ANCAinteraction and activation.

In our laboratory it has been shown that human apoptotic neutrophils can be taken up by immature dendritic cells and cause some DC maturation as evidenced by increased MHC-II and CD-83 expression (15). However, this was accompanied by down regulation of CD40, CD80 and CD86 and associated with reduced allogenic T cell activation in the mixed lymphocyte reaction (MLR). The addition of TNFα partially overcame this suppression and allowed proliferation in the MLR to occur at a level above that caused by DC alone. In neither of the two animal models of immunisation with apoptotic cells were adjuvants used along with the apoptotic neutrophils. However, Patry *et al*. (13) utilised a crude neutrophil extract that presumably contained a suitable DC maturation signal and Rauova *et al*. (14) speculate that inflammation occurred intraperitoneally following injection of cells. Since neutrophils are

undergoing apoptosis in their millions everyday, a second signal, such as TNFα, must be required to allow such cross-presentation of antigen. Development of vasculitis is often preceded by a viral-like illness and it could be speculated that under certain conditions, in a genetically susceptible individual, the correct second signal may be present to allow cross-presentation of self-antigen by DC to occur.

It is also of interest that neutrophils from patients with acute, active Wegener's granulomatosis have been shown to express MHC-II and CD80 and CD86, markers usually restricted to antigen presenting cells (16). This did not occur in patients with microscopic polyangiitis, patients on immunosuppressive treatment, healthy controls or patients with bacterial infections. Neutrophils from healthy controls were able to acquire these characteristics following incubation with T cells or the T cell derived cytokine IFN-γ and such neutrophils were able to present T cell antigens in a MHC-II restricted manner. It is not clear as to whether this mechanism of antigen presentation is pathogenic in Wegener's granulomatosis or simply reflects T cell activation. However, it is potentially another mechanism by which self-antigen could be presented to T cells to cause their activation.

How do ANCAact?

What epitopes does ANCA recognise?

The precise nature of the interaction of ANCAwith their antigens has been unclear. Initial attempts to define ANCA epitopes using the construction of peptides with pins was hampered by a high level of background binding to the peptides by healthy and disease control sera. Van der Geld used 50 overlapping peptides, fifteen amino acids in length with an overlap of ten amino acids, synthesised by automated simultaneous multiple peptide synthesis (17). It was shown that although sera from both WG patients and healthy controls recognised a restricted number of peptides, four of these were recognised significantly more strongly by patient than control sera. Two of these peptides were located near the active centre of PR3.

Griffith *et al*. used optical biosensor technology to show IgG samples from patients binding to whole PR3 and demonstrated that binding of IgG from any one patient inhibited the binding of IgG from other patients (18). Control IgG did not cause such inhibition therefore suggesting that ANCAfrom different individuals bind to similar regions of the intact PR3 molecule. Using the SPOT system, 111 10mer peptides spanning the length of PR3 and overlapping by two amino acids were synthesised onto cellulose membranes and restricted binding of cANCA containing IgG was demonstrated (18). Five areas were identified which were bound by ANCA from seven of eight patients. Normal and disease control sera did not bind to any of the peptides. Because of concerns over the recognition of conformational epitopes on the SPOT system, soluble peptides were also used in an inhibition assay to prevent binding of cANCA in patients' IgG preparations to PR3. The peptides causing inhibition were similar in all patients tested and confirmed results from the SPOT system (18). Subsequent modelling of PR3 demonstrated that all the five identified dominant epitopes were surface located and that one of the epitopes runs through the catalytic site and the three of the others are clustered together around the catalytic site (18). Previous functional studies had suggested involvement of the catalytic site by ANCA binding in that it has been shown that ANCA inhibits the enzymatic action of PR3 (19) and that it prevents complex formation of PR3 with its inhibitor α-1-antitrypsin (20), which is known to bind in the catalytic site. If ANCAbinds near the catalytic site it could modify PR3 activity either by preventing proteolytic activity, or by preventing inhibitor binding, thus allowing unregulated protease activity.

The above experiments used sera from patients with active disease. Some patients can remain ANCA positive in remission. Griffith *et al*. compared sera from an ANCA positive patient with active disease and then subsequent remission (18) and found that although the initial epitopes were still recognised, epitope spreading had occurred such that other peptides were also recognised. It may be that changes in avidity of binding to specific epitopes, along with such epitope spreading, allow changes in the pathogenicity of ANCA. Interestingly, Van der Geld *et al*. have found that ANCA present in remission still inhibit the cleavage activity of PR3 and are in fact more effective at this than ANCA obtained from patients with active disease (21). This suggests that the pathogenic ANCA present with disease activity may not be binding to PR3 to prevent proteolytic activity and may be binding elsewhere to PR3. Further investigation is obviously needed within this field, as establishment of important ANCA epitopes may allow for therapeutic immunoabsorbant techniques to be developed.

Are there defects in apoptosis and clearance of apoptotic cells?

Priming of neutrophils with TNF-α *in vitro* appears to be necessary for the surface expression of ANCA-antigens, allowing ANCA-induced activation of neutrophils to occur. However, TNF-α also causes accelerated apoptosis of neutrophils through a caspase 3 dependent process (22) and, although this induces surface expression of ANCA antigens, there is a down-regulated respiratory burst response to ANCA. As such TNF-α appears to have a dual role with regards to neutrophils, allowing priming and ANCA-induced activation along with apoptosis and diminished responsiveness to ANCA.

However, there may be defects in apoptosis of neutrophils in patients with vasculitis which contribute to an enhanced inflammatory environment. Once activated by ANCA, neutrophils undergo accelerated apoptosis, driven by reactive oxygen species, that appears to be deranged. There is delayed expression of surface phosphatidylserines, that usually allow recognition by macrophages and the non-inflammatory clearance of the apoptotic cells (23). This may result in delayed clearance of the ANCA-activated neutrophils allowing progression of the apoptotic cells to secondary necrosis, subsequent release

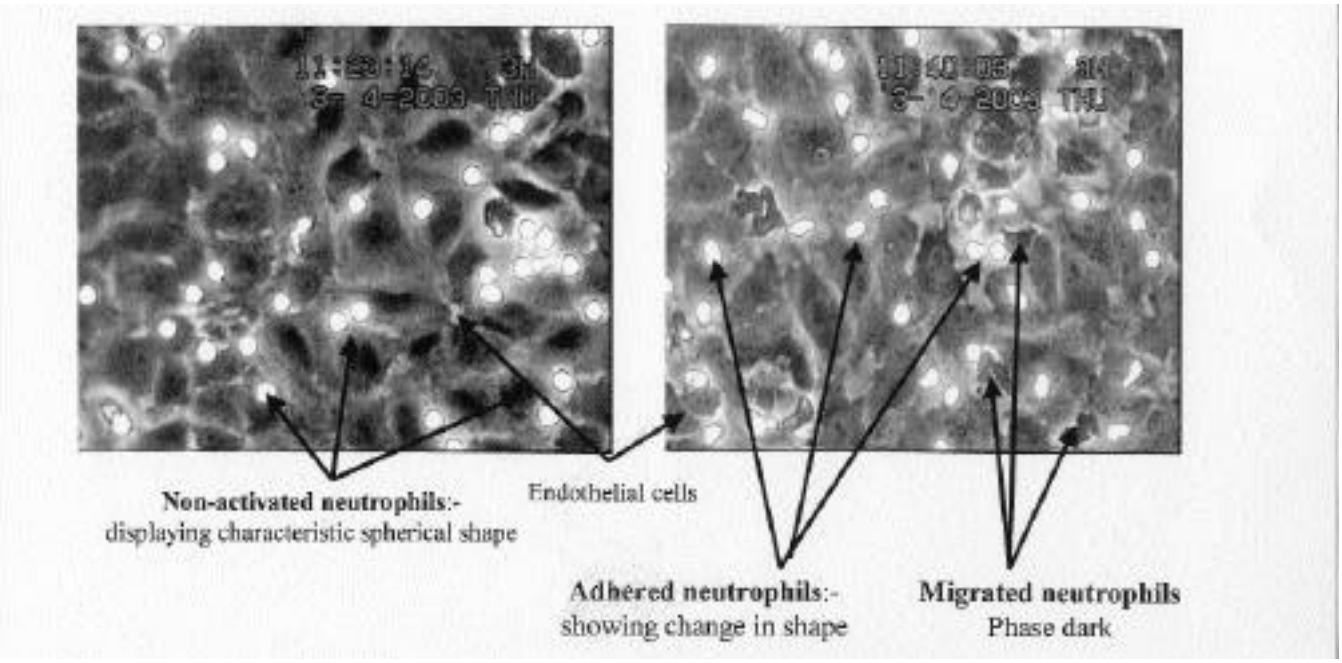

Fig. 2. Flow-based assay showing effects of ANCAon neutrophil migration

of inflammatory cell contents and further endothelial cell damage. As stated above, although apoptotic neutrophils express large amounts of surface ANCAantigen, binding by ANCAcannot cause further neutrophil activation. However, opsonisation of apoptotic neutrophils by ANCA and subsequent uptake by macrophages, causes the scavenger cells to produce inflammatory cytokines such as TNF-α (24, 25) rather than those such as IL-10 and TGF-1β that are usually produced by macrophages following phagocytosis of apoptotic cells. This serves to perpetuate any inflammatory damage.

Use of a flow-model to study ANCA-endothelial interactions

Use of a flow-model has allowed further understanding of the effect of ANCA on neutrophil-endothelium interactions (26) (Fig.2). HUVEC are grown to confluence on microslides and neutrophils perfused over the endothelial layer. When high doses (100 units/ml) of TNF-α were used to activate the endothelial cell monolayer, the perfused neutrophils were captured from flow, a few rolling over the endothelial cell layer and the majority transmigrating through the cell layer. When neutrophils were treated with ANCA prior to perfusion, none rolled on the TNF-α activated-endothelium, with the majority becoming firmly adhered and transmigrating. When a much smaller dose of TNF-α (2 units/ml) was used to pretreat the endothelial cells, the majority of untreated neutrophils rolled over the endothelium with few transmigrating and the number attached decreased with time during the wash-out phase. However, following treatment with ANCA-IgG prior to perfusion, adhesion was stabilized and the number of neutrophils transmigrating increased by ten-fold. Priming of neutrophils with TNF-α further increased stability of the neutrophil-endothelium interaction. These experiments show that ANCA is able to modify neutrophils to allow adherence to endothelium and further studies are now underway using this flow-model to dissect out the effects of ANCA on individual adhesion molecules. It has proven to be a useful model of the *in vivo* effects of shear stress that cannot be mimicked in a static *in vitro* system.

Other evidence of endothelial dysfunction

Studies measuring brachial artery vasodilatation and dermal microvascular responses to acetylcholine have revealed widespread and diffuse endothelial dysfunction in patients with primary systemic vasculitis (27). This effect appears to be independent of the target vessel size or ANCA association and unrelated to local disease expression (such as renal involvement). As such endothelial dysfunction has been proposed as a harbinger of future atheromatous disease and in the light of the excess mortality amongst vasculitis patients from atherosclerotic complications, further studies are needed to try and reverse such abnormalities.

Further evidence for endothelial dysfunction and destruction during active disease is provided by Woywodt *et al*. (28). Using Dynabeads coated with antibodies against the endothelial cell marker CD146, endothelial cells were removed from peripheral blood. Few circulating endothelial cells were found in healthy controls, patients with infection and those with non-ANCA associated glomerulonephritides. By contrast, large numbers of circulating endothelial cells were seen in patients with active ANCA-associated vasculitis and the number of cells fell with disease treatment implying a disease specific denudation of endothelial cells. These were generally of a necrotic and prothrombotic phenotype.

The role of monocytes

Monocytes are frequently found in the

vascular infiltrates in vasculitis but their role is less well characterised than that of neutrophils. Like neutrophils, monocytes also contain granules of PR3 and MPO and previous work has shown that ANCA are able to trigger the release of the cytokines MCP-1 (29) and IL-8 (30) from monocytes. Nowack *et al*. investigated the effect of ANCA on the cell surface molecules CD14 and CD18 (31) and found upregulation following incubation with ANCA for at least six hours. This upregulation did not appear to require Fc receptor ligation, as F $(ab)_2$ fragments had the same effect as the whole antibody. CD14 is a receptor for LPS, and once ligated by LPS bound to LPS-binding protein, cytokine production and upregulation of adhesion molecules is triggered. CD14 participates in TNF-α production by macrophages, thus contributing to the inflammatory response. CD18 is a β integrin induced by activation of monocytes and associating with the adhesion molecules, the α integrins CD11 a,b,c and d. It is involved in a variety of functions including cell migration, extravasation and phagocytosis. CD14 and CD18 are concomitantly upregulated and therefore increased expression of CD14 will enhance monocyte adhesion to activated endothelium. This causes production of proinflammatory cytokines and attracts further inflammatory cells that lead to the changes seen in vasculitis. Wikman *et al*. looked at the effect of ANCAon the expression of CD62L(L-selectin) and CD11b on monocytes (32). CD62L is involved in the initial rolling and tethering of leucocytes to endothelial cells and is down regulated on activation whilst CD11b is upregulated following activation. It was found that ANCA-positive sera augmented the down regulation of CD62L which occurred with enhanced metabolic activation as judged by hydrogen peroxide production. However, there was no concomitant CD11b upregulation, but the study focussed only on early changes and upregulation after a few hours, as described by Nowack, cannot be discounted.

Weidner *et al*. showed that ANCA can cause the formation of oxygen free-radicals from monocytes and that this was greatly reduced by the preincubation of monocytes with Fcγ receptor type II-blocking monoclonal antibodies (33). Hattar *et al*. found a similar requirement for the whole ANCA molecule in the production of the cytokines TNF-α, IL-1β and thromboxane, and that these cytokines acted as facilitators of the secretory response inducing the production of other inflammatory mediators (34). It may be that the F $(ab)_2$ portion of ANCAis sufficient to induce early changes in surface molecules of monocytes, but that effector functions such as the production of oxygen-free radicals and inflammatory cytokines requires the whole antibody.

Lamprecht *et al*. showed that monocytes from patients with acute vasculitis produced significantly more IL-12 and TNF-α than those from controls (35). IL-12 is an important cytokine in directing a TH1 response and thus IFN-γ production by T cells. Treatment with corticosteroids and cyclophosphamide rapidly reduced the production of these cytokines to control levels and as such removed an important drive for T cell effector function.

How do ANCA activate neutrophils?

ANCA interactions with target antigens and leukocyte cell surface receptors

The intracellular events that underlie leukocyte activation by ANCA have been examined in a number of recent reports. Whilst it is hypothesized that individual ANCA-IgG molecules ligate their target antigens on the plasma membrane of live leukocytes and "co-cross link" these with Fcγ receptors, this remains unproven. Determining the true nature of the interaction between ANCA and leukocytes should provide greater insight into how factors such as the activity of protease inhibitors and polymorphisms in β_2 integrin or Fcγ receptor genes, influence disease susceptibility and organ damage severity in ANCA associated vasculitis. Furthermore, if there are important differences between ANCA stimulated signaling events and signaling triggered through Fcγ receptors and β_2 integrins, it may be feasible to disrupt these in a targeted fashion, which does not disturb immunocompetency.

Very recently, an absolute requirement for ANCAinteraction with its target antigen in order to initiate neutrophil activation has been demonstrated by Reumaux *et al*. using neutrophils from two MPO deficient donors (36). Neutrophils from these donors were not activated by MPO-ANCA but responded normally to other stimuli.

A persisting controversy has been the differential capacities of intact ANCA-IgG and ANCA-F $(ab')_2$, which is devoid of its Fc moiety, to stimulate effector functions such as respiratory burst activity or cytokine production. The basis of the conflicting findings reported by different investigators remains unclear. However, in the last few years, data has accumulated to suggest that ANCA-Fc and ANCA-Fab are able to initiate distinct but overlapping events, which probably synergise to initiate leukocyte activation.

ANCAactivated intracellular signaling pathways

Previously, this laboratory reported that ANCA-IgG induce tyrosine phosphorylation of neutrophil proteins, rises in intracellular calcium concentration and membrane translocation of protein kinase $C\beta_{II}$ (a calcium sensitive PKC isozyme) (37,38). The role of tyrosine kinases was verified in a study by Ketteritz *et al*. who also demonstrated that the stress activated serine/threonine kinases, p38 MAPK and p42/p44 ERK, are involved in the ANCA induced respiratory burst (39). One function of tyrosine kinases and p38 MAPK is to facilitate translocation of PR3 and MPO to the plasma membrane during priming, which is consistent with their known functions in granule mobilization. However, both tyrosine and serine/threonine kinases are intimately involved in activation of NADPH oxidase in neutrophils stimulated by a variety of stimuli and they probably serve multiple functions in ANCA stimulated cells. The Kettritz group has subsequently demonstrated that ANCA induced respiratory burst activity is diminished in neutrophils incubated with statins, by a mecha-

nism that may involve ERK inhibition (40). However, statins have wide ranging inhibitory effects, partly relating to their capacity to inhibit protein prenylation and it is unlikely that they act via a single mechanism. Nonetheless, this simple observation is of clinical interest given that these drugs are already in widespread use. The potential therapeutic relevance of p38 MAPK in inflammatory glomerulonephritis has also recently been highlighted in a study using a rat model of anti-GBM antibody disease and examining biopsies from patients with post-infectious GN (41). Phosphorylated p38 MAPK was present within glomerular neutrophils. Furthermore, in the animal model, a systemically administered p38 MAPK inhibitor prevented neutrophil influx and preserved renal function.

Our laboratory has extended its investigations by showing that superoxide release from neutrophils stimulated by ANCA-IgG is sensitive to pertussis toxin as a result of the recruitment of $G_{i/0}$ GTPases at the plasma membrane (42, 43). Interestingly, GTPase activity was efficiently activated by both ANCA-IgG and ANCA-F $(ab')_2$ indicating that it did not depend upon Fcγ receptor ligation. The $G_{i/0}$ GTPases are not involved in conventional Fcγ receptor signaling and are typically recruited by heptahelical receptors for ligands such as the chemoattractant bacterial peptide, fMLP. $G_{i/0}$ GTPase activity contributes to the ANCA mediated downstream activation of the small GTPase, Ras, which has multiple signaling functions including NADPH oxidase activation. A direct target of Ras is phosphatidylinositol 3-kinase (PI3-kinase) and independent reports have identified PI3-kinase activation in neutrophils stimulated by ANCA (42, 44). Inhibitors of PI3-kinase markedly attenuated ANCA induced superoxide release. Furthermore, our observations suggest that unusually, ANCA do not activate the p85 PI3-kinase isozyme recruited by conventional Fcγ receptor engagement and may alternatively, activate PI3-kinaseγ. PI3-kinaseγ is stimulated by the βγ subunit of heterotrimeric GTPases such as $G_{i/0}$, which would be consistent with our other findings.

The serine/threonine kinase Akt/PKB, is a target of PI3-kinase and is activated by both TNFα priming of neutrophils and subsequent ANCA stimulation. Kettritz *et al*. have demonstrated that Akt exists in a complex with one of its substrates, p21 ras activated kinase 1 (PAK1) and that this association is enhanced by TNFα priming but unaltered during subsequent stimulation with murine monoclonal anti-MPO antibody (44). The functions of PAK1 include phosphorylation of NADPH oxidase subunits and of several proteins in the p42/p44 ERK-activating cascade. Cytokine priming of neutrophils is a ubiquitous phenomenon that is not restricted to ANCA pathophysiology. Priming involves many intracellular events and does not only concern expression of target antigens on the plasma membrane. We observed that Akt phosphorylation is sensitive to both tyrosine kinase inhibitors and pertussis toxin, implying that both ANCA-Fc and ANCA-Fab mediated signals converge upon the PI3-kinase/Akt pathway (43).

ANCA influence neutrophil chemotaxis and chemoattractant production

In the context of these findings, it is of interest that low concentrations of ANCA, which do not activate neutrophils, have profound effects on subsequent responsiveness to fMLP. Hattar *et al*. used anti-proteinase 3 antibodies to prime fMLP-induced chemotaxis, but found that at the same time, degranulation and SO production were inhibited (45). Thus ANCA could promote the influx of neutrophils into sites of infection, but prevent them from mediating bacterial killing. Thereafter, the inflammatory response to infection might be prolonged or amplified, further priming neutrophils and rendering them available for stimulation by higher concentrations of ANCA. An early study demonstrated that anti-PR3 monoclonal antibodies stimulate neutrophils to produce significant quantities of another chemoattractant, LTB_4, in the presence of arachidonic acid. LTB_4 also potentiated superoxide release and degranulation through an LTB_4 positive feedback loop (46). Recently, a family of lipoxygenase-generated eicosanoids, the lipoxins, has been shown to inhibit LTB_4 stimulated neutrophil-endothelial cell interactions and to promote non-phlogistic clearance of apoptotic neutrophils by macrophages (47, 48). The potential therapeutic significance of these observations is underscored by the existence of stable lipoxin analogues and by capacity of aspirin to promote endogenous lipoxin generation. It would be intriguing to know if lipoxins can abrogate the pro-inflammatory phagocyte phenotype stimulated by ANCA opsonised apoptotic neutrophils.

Microarray technology and ANCA activated leukocytes

Yang *et al*. used microarray technology and semi-quantitative PCR to demonstrate that in leukocytes from healthy volunteers, ANCA-IgG and ANCA-F $(ab')_2$ stimulate transcription of distinct subsets of genes and of a panel of shared genes (49). The study applied the same techniques to leukocytes obtained from patients with ANCA associated vasculitis and demonstrated that transcription of *DIF-2*, *COX-2* and *IL-8* genes was increased, as it was in ANCAstimulated leukocytes from healthy individuals. Furthermore, in a small number of patients, expression of *DIF-2* was correlated with disease severity assessed using a clinical scoring system and with ANCA titre. Disease severity and *DIF-2* protein levels also correlated. DIF-2 is of interest because its functions are thought to include a role in monocyte differentiation, a crucial event in the evolution and resolution of inflammation. Intriguingly, monocyte differentiation into macrophages actually diminishes PR3 and MPO expression, so limiting their capacity to be stimulated by ANCA.

Effects of neutrophil products on endothelial cells

Proteinase 3 induces endothelial cell apoptosis, adhesion molecule expression and chemoattractant production

Another area of recent interest has been the effect of neutrophil granule con-

stituents upon endothelial cells (EC). This is relevant to the pathogenesis of vasculitis because ANCA stimulated neutrophils are believed to become trapped and inappropriately activated within small vessels. Indeed, as described above, our laboratory has demonstrated that ANCA has marked effects on neutrophils flowing over activated endothelial cells. Concurrent production of reactive oxygen species and granule exocytosis by the activated neutrophils, may lead to "bystander" endothelial damage. Previously, PR3 was shown to promote EC apoptosis and induce IL-8 production by EC, providing a stimulus for further neutrophil recruitment (50). More recently, PR3 has been shown to induce EC expression of monocyte chemoattractant protein-1 (MCP-1) (51). Furthermore, PR3 increased expression of ICAM-1 on EC and slightly increased VCAM-1. This resulted in increased static adherence of neutrophils to PR3-activated EC via an ICAM-1/β2-integrin interaction. None of the effects described above appeared to absolutely depend upon enzymatic activity of PR3. Taekema-Roelvink *et al*. showed that exogenous PR3 binds to the surface of EC, via a 111-kDa receptor composed of 2 distinct subunits, although its exact nature remains unknown (52).

Proteinase 3 induced endothelial apoptosis involves NFkB cleavage and JNK activation

Preston *et al*. have characterized the pro-apoptotic signaling events in PR3 treated EC (53). Although their earlier report confirmed that enzymatically inactive fragments of PR3 were internalized by EC and induced apoptosis, they have now shown in some detail that active PR3 cleaves NFκB at a site distinct from those cleaved by elastase and caspases. Cleavage of NFκB decreases transcription factor activity and is pro-apoptotic although insufficient to induce apoptosis in its own right. C-Jun N terminal kinase (JNK) is another member of the MAPK family and is involved in pro-apoptotic signaling. Preston *et al*. identified increased levels of phospho-JNK 2 in PR3 treated EC and demonstrated that the MAPK inhibitor SB203580, was able to rescue EC from PR3 stimulated apoptosis. Elastase, a closely related serine protease with similar substrate specificities to PR3, also induced EC apoptosis, but did not increase phospho-JNK 2 and JNK inhibition appeared to be relatively ineffective at preventing elastase induced apoptosis. Proteinase 3-induced JNK phosphorylation was sensitive to a PI3-kinase inhibitor suggesting that the lipid kinase was upstream of JNK. Whereas intracellular cleavage of NFκB by PR3 presumably depends upon internalization of the protease it is not clear whether JNK phosphorylation also results from this event or alternatively, if it is the result of PR3 binding to a cell surface receptor and initiating a signaling cascade. MPO is also internalized by EC and although this does not induce apoptosis, production of reactive oxygen species does ensue, which might well contribute to localized EC damage (50).

Vasculitis, tissue factor and endothelial protein C receptor

Haubitz *et al*. demonstrated that elastase and PR3 induce production of the pro-coagulant tissue factor (TF) by EC (54). Alpha 1-antitrypsin did not inhibit TF production in response to PR3, suggesting that enzymatic activity may not be required, although it is difficult to be certain that PR3 was totally inhibited at the EC membrane in this system. As already described, these investigators have subsequently isolated circulating EC from patients with active vasculitis (28). These EC were predominantly apoptotic and stained positively for TF. Leukocytes may also contribute to TF production and a new report has demonstrated that anti-MPO antibodies are able to stimulate tissue factor production by a myeloid cell line (55).

Finally, whilst this laboratory has demonstrated that ANCA stimulated production of IL-8 may contribute to intravascular retention of neutrophils within the glomeruli, it is of interest that protein C has now been shown to inhibit neutrophil chemotaxis (56). Endothelial protein C receptor appears to be expressed by neutrophils but it is also possible that soluble EPCR released from activated EC, binds to neutrophils. Previously PR3 and β_2 integrins were found to be important for the binding of soluble EPCR to activated neutrophils (57). PR3 expressed upon the surface of activated neutrophils in the microcirculation of patients with vasculitis might stabilize EPCR and in turn, increase protein C binding and retard neutrophil emigration, amplifying endothelial damage. Soluble EPCR levels in the plasma of patients with WG have now been shown to correlate with disease activity and to rise prior to clinical relapse (58).

The role of T cells

That T cells are involved in the pathogenesis of ANCA-associated vasculitis is clear. ANCA are high-affinity, class-switched antibodies that will require T cell help in their production (59), levels of T cell activation markers (such as sIL-2R) are raised in active disease (60) and monoclonal antibodies directed against T cell markers are effective in disease treatment (61). In affected tissue, T cells are seen to accumulate, and in the kidney, their numbers correlate with renal impairment (62).

Antigen specific T cells are present in patients

Various investigators have shown the presence of ANCA-antigen specific T cells and their proliferation to PR3 and MPO (63-66). It has been shown that these cells are present in higher numbers in patients than in controls. Recent work has looked at these cells in more detail. Van der Geld attempted epitope definition using overlapping peptides in proliferation assays (67) and showed responses in both patients and controls with the dominant peptides being located in the signal sequence, propeptide or C-terminus. However, proliferation assays may not be the most sensitive method for assessing the presence of such cells and the employment of newer techniques is required for further investigation.

Cytokine profiles of T cells: TH1 or TH2?

The cytokine profile of the antigen-specific cells is also a subject of ongo-

ing investigation. Initial studies looking at the whole T cell population in vasculitis patients revealed a high level of activation of both peripheral and tissue based T cells as evidenced by raised levels of MHC II expression and high levels of IFNγ production following non-specific stimulation, thus implying a TH1 driven response (68, 69). The only exception to this was found by Balding *et al.* looking at nasal tissue, where higher levels of the TH2 cytokine IL-4 were found (70). Further studies of antigen-specific T cells from patients in remission have been undertaken. Balding *et al*. showed that PBMC from patients in remission produced mainly IFNγ following stimulation with PR3, as assessed by mRNA expression and concluded a TH1 response (70). When Popa *et al.* looked at PBMC in remission patients, proliferation to ANCA-antigens was described (71). However, little IL-2 or IFNγ was found in the supernatant by ELISA, with high levels of IL-6 and IL-10. This was described by the authors as a TH2 response. However, experimental conditions were probably not ideal for measuring cytokine responses as proliferation after seven days was the prime readout.

$CD28^-$ T cells

Several authors have investigated the presence of $CD28^-$ T cells in ANCA-associated vasculitis. CD28 is an important co-stimulatory molecule on T cells engaged by CD80 and CD86 on antigen-presenting cells. Without engagement of this molecule, T cells presented with antigen via the MHC II complex are probably made anergic. Initial studies in rheumatoid arthritis showed the presence of T cells lacking CD28, the number of $CD4^+$ $CD28^-$cells correlating with disease clinical activity (72). Giscombe described $CD28^-$ cells as being very prevalent in the expanded, activated T cell subset in vasculitis patients implying an effector role for these cells (73). Further studies have revealed high numbers of $CD28^-$ T cells in the peripheral circulation and bronchoalveolar fluid of patients (74, 75). These cells showed high levels of IFNγ and TNFα on intracytoplasmic analysis, in addition to the presence of intracytoplasmic perforin and surface CD18 all implying an effector phenotype (75). Further studies of this $CD28^-$ subset in viral infections has revealed that these cells tend to be end-differentiated and highly susceptible to activation induced apoptosis (76). As such, $CD28^-$ T cells in patients with vasculitis are probably the effector arm of the T cell response. Further study of this subset of T cells may increase understanding of the role of T cells in ANCA-associated vasculitis.

Increased expression of CTLA-4

CTLA-4 has sequence homology to CD28 and is also present on the surface of activated T cells. It binds to the same ligands on antigen presenting cells as CD28 but has an antagonistic function in that its role is essentially inhibitory. In addition, it appears to have a role in inducing TH1 cytokines and suppressing TH2 cytokines. Steiner *et al.* used potent signal enhancement to study CTLA-4 levels on the surface of PBMC of patients with Wegener's granulomatosis compared with levels in healthy controls (77, 78). There was significantly increased CTLA-4 expression on $CD4^+$ cells in patients and higher levels correlated with disease activity. This may be associated with the strong TH1 response seen in vasculitis patients. In contrast, following PHA stimulation (a non-specific T cell mitogen) high levels of CTLA-4 were seen on the surface of T cells in controls with a severe impairment of this response in patients. Polymorphisms in CTLA-4 expression have been revealed in patients with Wegener's granulomatosis with a decreased prevalence of the shortest allele in patients compared with healthy individuals. This may contribute to differences in CTLA-4 levels and T cell activation in vasculitis patients when compared to controls.

Despite the evidence for a role of T cells in the initial production of ANCA and in ongoing diseases, in some patients with active disease and high ANCA titres, it appears that circulating B cells are producing ANCA spontaneously, without the need of presentation of antigen and T cell help (79). These B cells may escape regulatory control *in vivo* and perpetuate inflammation in the acute disease setting.

Environmental induction of ANCA

Linkage with propylthiouracil and other anti-thyroid medication

There is now a well reported link with anti-thyroid medication, particularly propylthiouracil (PTU) [although cases with the closely related drugs carbimazole and methimazole have also been reported (80)], and ANCA-associated vasculitis (81). These cases tend to be MPO-ANCA associated, although ANCA of other differing specificities are sometimes present also. Several cross-sectional studies have shown raised ANCA levels, of varying specificities following antithyroid medication, with ANCApositivity rates of 20-60% (82, 83). Actual vasculitic disease is rare amongst this population. It appears that ANCAmay also be induced by thyroid disease itself, particularly Graves' disease, with patients being ANCA positive prior to treatment (84). It is not entirely clear as to why ANCA develop in this disease or with treatment but it is presumably related to the altered immune environment in which the thyroid disease occurs in the first place. T cell sensitisation to self-peptides has also been shown to occur following accumulation of a reactive intermediate of PTU in neutrophils (85). Authors advise the close observation of patients with thyroid disease and ANCA, particularly MPO-ANCA, with the withdrawal of drug treatment and definitive therapy if necessary (82).

Arole for silica?

Silica is another environmental insult associated with development of ANCA-associated vasculitis. Hogan *et al.* confirmed previous smaller case-control studies with a study in 65 patients (86). An odds ratio of significant silica dust exposure was 4.4 times greater in patients with ANCA-associated vasculitis compared with control subjects. These results have been confirmed in a study by Lane *et al*. (87), who also showed a significant association with farming. How silica causes vasculitis is

not known. However, following inhalation it is phagocytosed by macrophages, leading to a foreign body reaction and granuloma formation. It has been shown to induce apoptosis of alveolar neutrophils and macrophages and granuloma formation on intra-tracheal installation in Wistar rats (88). As such it may cause abnormalities in apoptosis and induction of disease via dendritic cell uptake as discussed above.

Table I. Genetic polymorphisms and their effects in ANCA-associated vasculitis.

Structure of PR3	Valine / Isoleucine at 119	Nil obvious [92]
Surface PR3 levels	? A-564G polymorphism in PR3 promoter region	Higher levels of surface PR3 in vasculitis patients [89-91]
α-1-antitrypsin levels	Over-representation of PiZ and PiZZ alleles	Lower levels of α-1-antitrypsin, the protease inhibitor of PR3 in patients [95, 96]
Fcγ receptor subtypes	FcγIIa-R131	More prone to developing severe renal disease [97]
CTLA-4	Decreased prevalence of shortest CTLA-4 allele	Possible effects on Tcell activation and polarisation to TH1 response [77]
Myeloperoxidase levels	-463 G/Apromoter polymorphism	Associated with disease in females Increased risk of relapse [98]

Table II. Evidence for a pathogenic role for ANCAin vasculitis.

Clinical	
ANCApresence and levels	Majority of patients with WG and MPAexpress ANCA and levels often rise prior to relapse
Animal models	
Xiao *et al.*	Rag2-/- or wild type mice developed vasculitic lesions when given splenocytes or IgG containing MPO-ANCAfrom MPO immunized mice
Smyth *et al.*	Wistar-Kyoto rats immunized with human MPO develop MPO-ANCAand vasculitis
Weidebach *et al.*	Wistar rats develop pulmonary vasculitis when injected with C-ANCApositive IgG fraction of human vasculitic patients
In vitro evidence	
ANCAbind to primed neutrophils and cause activation	Via Fab and Fc causing neutrophil activation and release of superoxide, nitrous oxide and other inflammatory mediators
ANCAactivation leads to disordered apoptosis	Delayed expression of surface phophatidylserine and therefore delayed clearance of apoptotic cells leading to secondary necrosis and enhanced inflammatory damage
Flow-based assay	ANCAallow neutrophils to adhere to and transmigrate through the endothelium
Monocyte activation	ANCAupregulate CD14 and CD18, allowing proinflammatory cytokine production and adhesion to activated endothelium

Genetics of PR3

Surface PR3 expression

PR3 has recently been found to be present on the surface of resting neutrophils, the amounts expressed varying between individuals. Some individuals have a uniform pattern of mPR3 (monomodal) whereas others have been shown to have bimodal mPR3 expression in that a proportion of neutrophils have low levels of surface PR3 (mPR3-) and the rest high levels (mPR3+). The mPR3 levels are stable over time in a given individual and show a wide variation between individuals (89). Data from several studies shows that patients with Wegener's granulomatosis have larger mPR3+ subsets than healthy controls (89-91) and that within the Wegener's granulomatosis patients, higher levels of mPR3 make relapse more likely (90). Intracellular levels of PR3 do not reflect surface levels and appear to be the same in either mPR3 subset (91).

Surface PR3 expression is probably genetically regulated with high concordance in twin studies (91), and an association with WG has been demonstrated for a polymorphism in the PR3 promoter region, affecting a putative transcription factor-binding site, which may allow PR3 overexpression (92, 93). It is proposed that this increased mPR3 expression may allow easier interaction with ANCA and thus increased neutrophil activation and endothelial damage. In addition, mPR3 has been shown to be enzymatically active and could therefore participate in accelerating tissue damage. Further, if the hypothesis about initial induction of ANCA being related to surface PR3 on apoptotic cells is correct, higher membrane levels could possibly potentiate this effect.

Previous studies on patients with active vasculitis have shown raised levels of PR3 in the circulation and suggested that this may predispose to autoimmunity (93). However, due to the highly activated inflammatory cascade in patients with active disease, part of which involves neutrophil activation and granule contents release, a study of patients in remission has been undertaken (94). PR3 levels were found to be significantly raised in comparison with healthy individuals and disease controls. This was independent of ANCA specificity. Raised levels were not due to impaired renal function, ongoing inflammation or neutrophil activation but could be related to genetic factors, defects in the reticuloendothelial system or selective neutrophil degranulation or leakage. Raised levels could have important pathophysiological consequences in vasculitis in causing mod-

Table III. Evidence against the pathogenicity of ANCA.

Evidence against the pathogenicity of ANCA	Possible explanations
Small vessel vasculitis with identical clinical and histopathological features can arise in patients who are ANCAnegative	ANCAnegative patients are unusual when rigorous testing is employed. Moreover, vasculitis results from inappropriate leukocyte recruitment and activation. In rare circumstances, other stimuli may substitute for ANCAin directing the recruitment and activation processes.
ANCAare detectable in patients with non-vasculitic illnesses	ANCAin patients without vasculitis are generally directed against antigens other than PR3 and MPO. Most of these atypical ANCAhave not been shown to activate leukocytes and therefore may not share the proposed pathogenic potential of anti-PR3 and anti-MPO antibodies. Occasional patients do have PR3- or MPO-ANCAwithout vasculitis, perhaps because other factors control the outcome ANCA-leukocyte interactions
ANCApersist in some vasculitis patients who enter remission and some studies have indicated that rising ANCAtitres do not predict clinical relapse.	Shifts in antibody subclass and epitope spreading may down-regulate the capacity of "remission" -ANCAto activate leukocytes. Furthermore, leukocytes may be unresponsive to ANCAin the absence of pro-inflammatory priming signals or in the presence of competing signals that promote inflammation resolution.
ANCAare not detectable within the glomerulus or other vasculitic lesions.	ANCAmay not form stable immune complexes that persist within lesions. ANCAare probably internalised after ligating leukocyte Fcγ receptors and then degraded. Alternatively, ANCAmay be cleared from vasculitic lesions as the leukocytes to which they bind undergo necrosis or apoptosis and are removed. Recently, ANCAtarget antigens have been identified within vasculitic lesions using new immunohistochemical techniques [99].

ulation of the inflammatory environment via cleaving of cytokines and increased uptake by endothelial cells allowing activation and neutrophil and monocyte recruitment.

Table I summarises the effects of other genetic polymorphisms on ANCA-associated vasculitis.

Summary

Over the past few years substantial steps forward have been made in research into the pathogenesis of ANCA-associated vasculitis. Animal models now provide good evidence for a directly pathogenic role for ANCA, a hotly debated subject since the original identification of the autoantibodies in pauci-immune glomerulonephritis. (Tables II and III). In addition, the manner in which ANCA act to cause disease is also better understood, with progress having been made various fields. We are now closer to understanding what ANCAbinds to, where the ANCAantigens are located and how binding is translated into intracellular activity. The effects of ANCA activation on neutrophils and monocytes with regard to their effector roles are being dissected and the flow-based assay is allowing interactions with endothelium to be studied in closer detail.

Interest in the initiation of autoimmunity has been provoked by the role of defects in apoptotic cell clearance in other autoimmune diseases, and the increasing number of associated genetic polymorphisms may point to in whom disease is likely to develop, or have a worse outcome.

With such advances in our understanding of pathogenesis, translation into clinical practice remains the ultimate aim. As with other autoimmune diseases, progress in treatment options for ANCA-associated vasculitis is desperately required to allow more specific disease therapies, with fewer side-effects, to be developed.

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