

Pathogenesis of Vascular Inflammation by Anti-Neutrophil Cytoplasmic Antibodies

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The reports of a newborn who developed glomerulonephritis and pulmonary hemorrhage after transplacental transfer of anti-neutrophil cytoplasmic antibody (ANCA) IgG with specificity for myeloperoxidase (MPO) is compelling clinical evidence that ANCA are pathogenic. *In vitro* studies indicate that ANCA activate cytokine-primed neutrophils and monocytes through both direct Fab'2 binding and Fc receptor engagement. Neutrophils that have been activated by ANCA release oxygen radicals, lytic enzymes, and inflammatory cytokines and adhere to and kill endothelial cells. A murine model caused by passive administration of mouse anti-mouse MPO IgG provides convincing evidence that ANCA IgG alone in the absence of antigen-specific T cells can cause necrotizing glomerulonephritis and vasculitis. This pathogenic process is enhanced by synergistic inflammatory factors, probably through priming of neutrophils. Immunization of rats with human MPO induces antibodies that cross-react with rat MPO and cause glomerulonephritis and vasculitis. These ANCA act in concert with chemokines to cause adherence of leukocytes to the walls of small vessels with subsequent injury. To date, animal models of disease that is induced by anti-proteinase 3 are less robust. Clinical and experimental data suggest but do not prove that the ANCA autoimmune response is initiated by an immune response to an antisense peptide of the ANCA antigen or its mimic that may be introduced into the body by an infectious pathogen. This antibody response elicits anti-idiotypic antibodies that cross-react with ANCA antigens. The pathogenesis of ANCA disease is multifactorial, with genetic and environmental factors influencing onset of the autoimmune response, the mediation of acute injury, and the induction of the chronic response to injury.

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Anti-neutrophil cytoplasmic antibodies (ANCA) occur in >80% of patients with active, untreated, necrotizing, small-vessel vasculitis that has an absence or paucity of Ig deposited in vessel walls (1). The major clinicopathologic expressions of ANCA-associated small-vessel vasculitis are Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, and renal-limited vasculitis.

In 1936, Wegener suggested that the disease that now bears his name was induced by an allergic (*i.e.*, immunologic) reaction to an infection (2). In 1954, Godman and Churg (3) concluded that Wegener's granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome had the same pathogenesis and that this most likely involved a "hypersensitivity" (*i.e.*, immunologic) mechanism. In 1978, Fauci *et al.* (4) contended that "most of the vasculitic syndromes are caused by, or closely associated with, deposition of immune complexes in blood vessel walls." However, immunohistologic evaluation of vessels from patients with Wegener's granulomatosis, microscopic

polyangiitis, and renal-limited vasculitis demonstrated an absence or paucity immunoglobulins (5,6). Therefore, pauci-immune small-vessel vasculitis and glomerulonephritis seemed to have a pathogenesis that differed from typical immune complex disease.

The first hint of the cause of pauci-immune small-vessel vasculitis was reported by Davies *et al.* (7) in 1982, when he observed that eight patients with pauci-immune segmental necrotizing and crescentic glomerulonephritis had circulating "anti-neutrophil antibodies" that reacted with the cytoplasm of normal neutrophils. These anti-neutrophil antibodies usually disappeared after immunosuppressive therapy and disease remission and reappeared with disease recurrence. Seven of the eight patients had serologic evidence for Ross River virus infection, suggesting a possible role for infection in disease induction.

Davies's observation was confirmed and extended by two publications in 1985 (8,9). Fokko van der Woude *et al.* (9) and a European consortium were the first to emphasize that ANCA were closely associated with Wegener's granulomatosis and that the titer correlated with disease activity. During the ensuing 5 yr, the major antigen specificities for myeloperoxidase (MPO-ANCA) and proteinase 3 (PR3-ANCA) were determined (10–14). The possibility that ANCA are not only a serologic

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marker for pauci-immune small-vessel vasculitis but also the primary pathogenic factor has been the subject of numerous investigations in the past 20 yr.

Clinical Evidence for the Pathogenicity of ANCA

The high prevalence of ANCA in patients with active untreated pauci-immune small-vessel vasculitis does not prove that ANCA cause the disease, because the same pattern would occur if ANCA were a secondary epiphenomenon. The correlation between ANCA titers and disease activity also could be either a cause or a result of the disease. The apparent induction of circulating ANCA by certain drugs, such as propylthiouracil and hydralazine, with subsequent development of pauci-immune necrotizing and crescentic glomerulonephritis and vasculitis also suggests a pathogenic link between ANCA and vasculitis (15); however, once again, the induction of the ANCA could be merely a nonpathogenic process that occurs concurrent with the primary events that are causing the vasculitis.

The most compelling clinical evidence that ANCA cause disease are the reports of a newborn child who developed glomerulonephritis and pulmonary hemorrhage 48 h after delivery from a mother with active MPO-ANCA microscopic polyangiitis (16,17). The infant's blood contained MPO-ANCA IgG. Corticosteroid therapy and plasma exchange controlled the glomerulonephritis and pulmonary hemorrhage and eliminated the ANCA. A reasonable conclusion is that transplacental transfer of MPO-ANCA IgG caused glomerulonephritis and vasculitis in this neonate, but how does ANCA IgG mediate vascular inflammation?

Neutrophil and Monocyte Activation by ANCA *In Vitro*

Experimental studies from many laboratories have shown that both PR3-ANCA and MPO-ANCA IgG activate neutrophils *in vitro* to release mediators of acute inflammation (18,19). Figure 1 illustrates a hypothetical sequence of pathogenic events that could result in ANCA-mediated vascular inflammation (20).

Most MPO and PR3 are in the cytoplasm of unstimulated neutrophils; however, there are small amounts of antigen on the surface of circulating neutrophils. The proportion of circulating neutrophils that has ANCA antigens on the cell surface may be genetically determined, and higher levels seem to be a risk factor for development of ANCA vasculitis (21,22). Neutrophils express more ANCA antigens at their surfaces when they are stimulated by a variety of proinflammatory factors such as cytokines and microbial products. For example, exposure of neutrophils to low levels of TNF- α causes increased surface PR3 and MPO (23,24). Incubation of TNF-primed neutrophils with ANCA IgG causes the release of toxic reactive oxygen species and lytic and toxic granule enzymes, including PR3 and MPO (23,24). This neutrophil activation is mediated by both Fc receptor engagement (25,26) and Fab'2 binding (27,28). The precise intracellular signaling pathways through which ANCA IgG mediates neutrophil activation are not completely

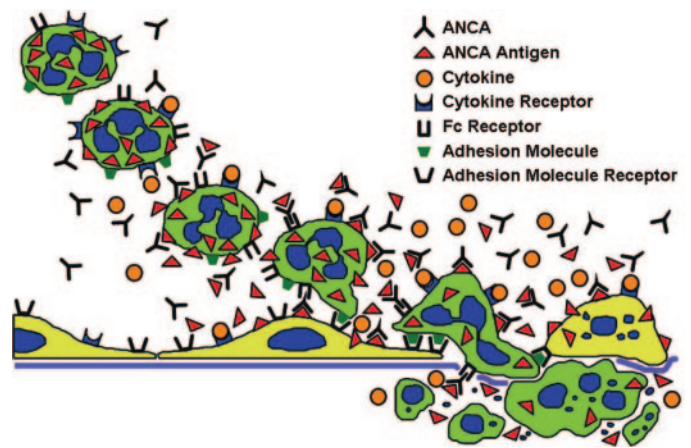


Figure 1. Events in the pathogenesis of anti-neutrophil cytoplasmic antibody (ANCA) small-vessel vasculitis that have been observed *in vitro*. Beginning in the top left and moving to the right, cytokines or other priming factors induce neutrophils to express more ANCA antigens at the cell surface, where they are available for binding to ANCA, which activates neutrophils by both Fc receptor engagement and direct Fab'2 ligation. Neutrophils that have been activated by ANCA interact with endothelial cells *via* adhesion molecules and release toxic factors that cause apoptosis and necrosis. Adapted from reference (20) with permission.

elucidated, but they seem to be different from the pathways that are involved in conventional immune complex-mediated activation of neutrophils (29).

Small amounts of immune complexes probably form on the surface of cells and exposed tissue matrix in the microenvironment where ANCA-induced inflammation occurs, because MPO and PR3 are cationic proteins that bind well to endothelial cells and matrix (30,31). Therefore, in some respects, this is an immune complex-mediated process; however, it is very different from classical immune complex-mediated vasculitis that requires gross accumulations of immune complexes in vessel walls and typically does not result in the severe degree of necrotizing injury that is characteristic of ANCA vasculitis. In addition to binding to the surface of endothelial cells, both PR3 and MPO are internalized into endothelial cells, where they have different pathologic effects (31). For example, after internalization, PR3 causes endothelial cell apoptosis, whereas MPO causes generation of intracellular oxidants (31). These differences in MPO and PR3 interaction with endothelial cells could influence the patterns of tissue injury that is induced when these antigens react with ANCA at the endothelial cell surface. Therefore, the clinicopathologic expression of disease, which correlates to a degree with ANCA antigen specificity, might be influenced by ANCA antigen-dependent differential effects on endothelial cells.

Neutrophils that have been activated by ANCA IgG kill cultured endothelial cells (32,33). Close proximity of the activated neutrophils to endothelial cells is required because the endothelial toxicity is inhibited by antibodies to $\beta 2$ integrins (34). Savage and associates (35–37) showed that activation of

neutrophils by ANCA causes integrin- and cytokine receptor-mediated adherence to cultured endothelial cells and transmigration across the endothelial layer. In addition, activation of neutrophils with ANCA causes a conformational change in β integrins that enhances ligand binding (37). A role for adhesion molecules in the interaction between ANCA-activated neutrophils and vessels also is supported by the immunohistologic evidence for upregulated adhesion molecules in glomerular lesions in renal biopsy specimens from patients with ANCA disease (38).

The target antigens for ANCA are not only in neutrophils but also in monocytes (9,10), which is an important consideration when contemplating the pathogenesis of ANCA disease. Incubation of monocytes with ANCA IgG causes activation with release of inflammatory mediators such as toxic oxygen metabolites (39), monocyte chemoattractant protein-1 (40), and IL-8 (41). MPO and PR3 are lost as monocytes transform into macrophages (42); therefore, it is unlikely that ANCA IgG can react with mature macrophages.

Induction of Glomerulonephritis and Vasculitis by Passive Administration of Anti-MPO IgG to Mice

The pathogenicity of ANCA IgG is supported by a mouse model that has been developed by Xiao and associates (43–45). This model is induced by the passive transfer of anti-MPO IgG (MPO-ANCA) or anti-MPO lymphocytes into recipient mice. The anti-MPO IgG or anti-MPO lymphocytes are derived from MPO knockout mice that have been immunized with mouse

MPO. Intravenous injection of anti-MPO IgG into either immune-competent mice or Rag2^{-/-} mice that have no functioning T or B cells causes pauci-immune crescentic glomerulonephritis and small-vessel vasculitis that is remarkably similar to human ANCA disease (43). Within 6 d of the intravenous injection, all mice develop focal segmental fibrinoid necrosis and crescents in glomeruli, whereas no mice that receive control anti-BSA IgG develop glomerular lesions (Figure 2). Some but not all mice develop systemic vasculitis, including leukocytoclastic angiitis, necrotizing arteritis, pulmonary capillaritis, and necrotizing granulomatous inflammation (Figure 3). Immunofluorescence microscopy reveals only a paucity of Ig and complement in glomeruli. Neutrophils are concentrated at sites of segmental necrotizing injury, and macrophages are concentrated in crescents (44). T lymphocytes are rare in acute glomerular lesions.

The severity, histologic appearance, and tissue distribution of disease that is induced by anti-MPO IgG is no different in immune-deficient Rag2^{-/-} mice compared with wild-type mice. Rag2^{-/-} mice have a genetic defect that prevents the recombination events that are required for the production of functioning Ig molecules and T cell antigen receptors. The induction of disease in Rag2^{-/-} mice demonstrates that antigen-specific T cells are not required to induce the acute injury. However, this does not address whether antigen-specific T cells are involved in the immunogenesis of human ANCA disease or in the progression or modulation of injury. For example, because the ANCA immune response is predominantly IgG, T

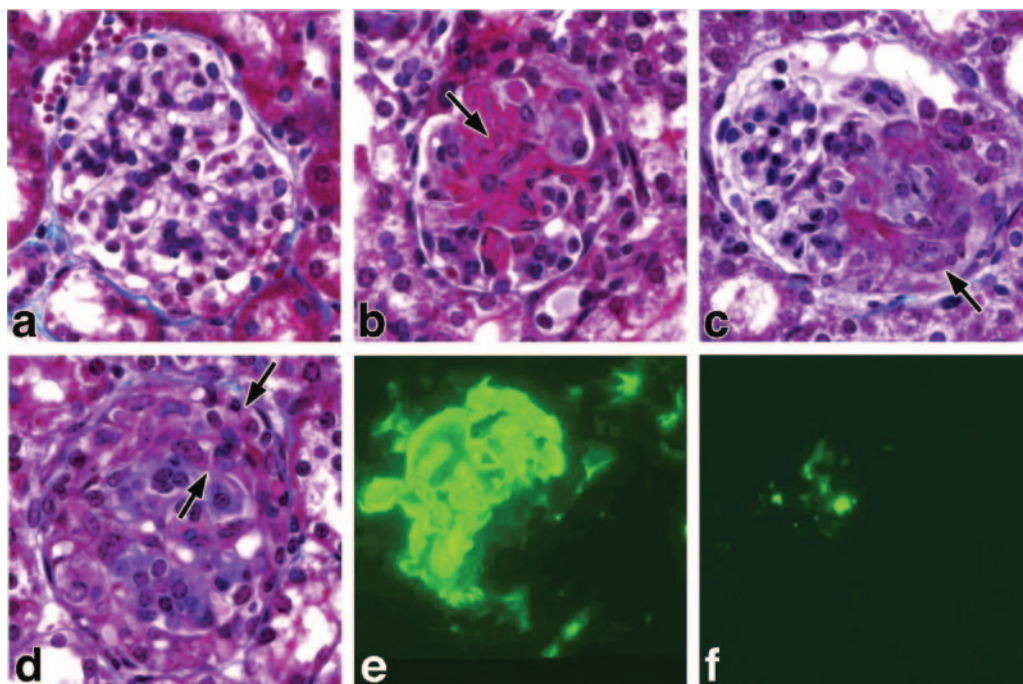


Figure 2. Glomeruli from a Rag2^{-/-} mouse 6 d after intravenous injection of anti-myeloperoxidase (anti-MPO) IgG showing no histologic lesion (a), segmental fibrinoid necrosis (arrow; b), segmental fibrinoid necrosis with a small cellular crescents (arrow; c), large circumferential crescent (arrows; d), fibrin in a crescent by immunofluorescence microscopy (e), and a paucity of segmental IgG by immunofluorescence microscopy (f). Reprinted from reference (42) with permission.

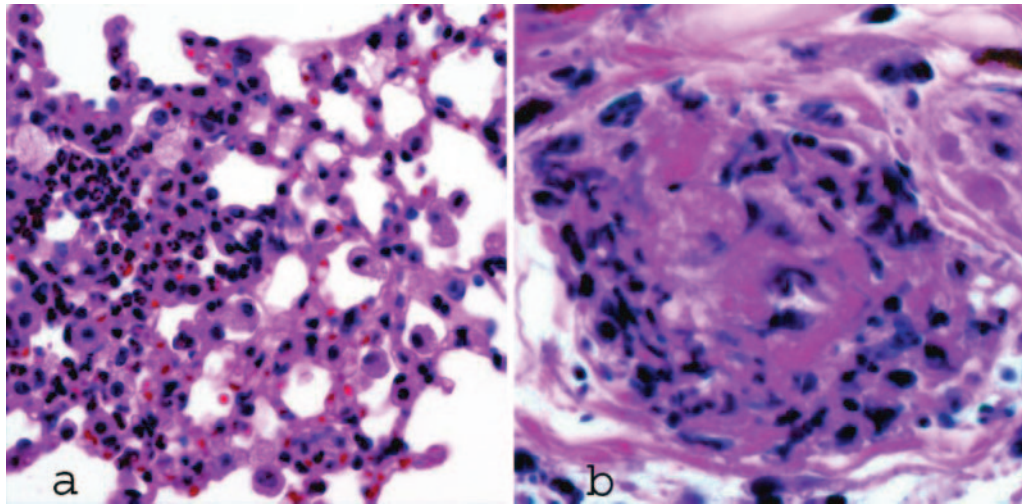


Figure 3. Vasculitic lesion from a wild-type mouse 6 d after intravenous injection of anti-MPO IgG, including pulmonary alveolar capillaritis with septal infiltration of neutrophils (a) and necrotizing arteritis with leukocytoclasia in the dermis of the ear (b). Adapted from reference (42) with permission.

lymphocytes must be involved in the immunogenesis of this response to facilitate isotype switching.

NIMP-R14 rat mAb that selectively depletes mouse neutrophils was used to investigate the importance of neutrophils in the pathogenesis of anti-MPO glomerulonephritis (44). Depletion of neutrophils completely blocks the induction of glomerulonephritis by injection of anti-MPO IgG, which indicates a pivotal role for neutrophils in this model. These blocking studies do not rule out a contributory role by monocytes, but any activation of monocytes was not adequate to cause identifiable disease.

Although all mice that received an adequate dose of anti-MPO IgG develop focal necrotizing glomerulonephritis, only approximately 15% of glomeruli have lesions (43). As reviewed earlier, *in vitro* experiments indicate that ANCA are more effective at activating neutrophils that have been primed by inflammatory stimuli. Therefore, a synergistic proinflammatory stimulus should exacerbate anti-MPO-induced disease. This was tested by treating mice with bacterial LPS (45). The LPS caused increased circulating levels of TNF- α and a dose-dependent increase in the severity of glomerulonephritis. Administration of antibodies to TNF- α prevented the LPS effect. These experiments support the hypothesis that neutrophil priming facilitates the induction of glomerulonephritis by ANCA. Differences in the onset and severity of ANCA disease in humans is influenced by infectious (46) and noninfectious (47,48) environmental influences that augment the responsivity of neutrophils to ANCA.

In summary, this mouse model of ANCA disease strongly supports a primary pathogenic role of ANCA IgG. The experimental animal data also suggest that the induction of disease is facilitated and modified by synergistic proinflammatory events. This model also shows that glomerulonephritis and small-vessel vasculitis that is remarkably similar to human ANCA disease can be induced by anti-MPO IgG in the absence of antigen-specific T lymphocytes.

Induction of Glomerulonephritis and Vasculitis by Transfer of Anti-MPO Lymphocytes to Mice

Severe necrotizing and crescentic glomerulonephritis and systemic vasculitis can be induced by the passive transfer of splenocytes from MPO $^{-/-}$ mice that have been immunized with murine MPO (43). Splenocytes consist of B lymphocytes, T lymphocytes, and other cells. Lymphocyte transfer requires that the recipient mice be immune deficient (*e.g.*, Rag2 $^{-/-}$ mice) because autoreactive anti-MPO lymphocytes will be eliminated in immune-competent mice. Glomerulonephritis that is caused by injection of anti-MPO in the absence of any synergistic inflammatory stimulus typically causes necrosis and crescents in <25% of glomeruli, whereas intravenous injection of anti-MPO splenocytes causes necrosis and crescents in approximately 80% of glomeruli even though the circulating titer of anti-MPO antibodies is similar (43). However, this lymphocyte transfer model is complicated by the fact that the transfer of immune-competent lymphocytes into the immune-deficient Rag2 $^{-/-}$ recipients results in immune complex deposits in glomeruli. The immune complex deposits are no different after transfer of normal control splenocytes or anti-MPO lymphocytes and therefore are not caused by the anti-MPO immune response. Mice that receive control splenocytes develop no necrosis or crescents even though they have the glomerular immune deposits. This background of glomerular immune complexes may be responsible for the more severe disease after anti-MPO splenocyte transfer compared with anti-MPO IgG transfer because of a priming effect on neutrophils in glomeruli. Alternatively, the splenocyte transfer could be more pathogenic because of the additive effect of anti-MPO T lymphocytes.

To test the importance of anti-MPO T lymphocytes, we transferred splenocyte preparations with different numbers of anti-MPO T cells into Rag2 $^{-/-}$ mice (49). Unfractionated splenocytes contained approximately 25% T cells and 65% B cells. Two

different preparations that were enriched for T cells were injected, one with approximately 80% T cells and 10% B cells and the other with >99% T cells. Injection of the unfractionated splenocytes caused crescents and necrosis in approximately 80% of glomeruli, whereas the preparation with 80% T cells caused necrosis and crescents in only 5% of glomeruli and the >99% pure T cell preparation caused no crescents or necrosis. These data do not support a pathogenic role for anti-MPO T cells in the induction of acute injury in this experimental model. The lymphocyte transfer experiments add further support to the importance of a synergistic inflammatory stimulus in augmenting ANCA-induced inflammation (in this instance, background immune complex deposition) and do not support a role for antigen-specific T cells in the induction of acute injury.

Induction of Glomerulonephritis and Vasculitis by Active Immunization of Rats with Human MPO

Little *et al.* (50) caused focal segmental pauci-immune glomerulonephritis and focal pulmonary capillaritis in rats by immunization with human MPO, which induced anti-MPO antibodies that cross-react with human and rat MPO. Intravital microscopy of mesenteric vessels was used to observe directly the interaction of leukocytes with the walls of small vessels. Application of CXCL-1 chemokine to the mesentery of rats with circulating anti-MPO resulted in increased firm adherence and transmigration of leukocytes. This same effect was observed in unimmunized rats after injection of IgG from rats that had been immunized with human MPO. Both induction of circulating anti-MPO by active immunization and passive transfer caused focal hemorrhage in the mesenteric microvasculature at sites of chemokine application. This rat model demonstrates that, in the presence of synergistic factors (*e.g.*, chemokines), anti-MPO IgG (MPO-ANCA) activates leukocytes and induced them to adhere to and injure small vessels *in vivo*.

Enhancement of Dermal Inflammation in Mice by Injection of Anti-PR3

Pfister *et al.* (51) knocked out the genes for PR3 and elastase in mice. Immunization of these mice with recombinant murine PR3 resulted in circulating anti-PR3 antibodies that reacted with the cytoplasm of mouse neutrophils; however, the mice did not develop glomerulonephritis or vasculitis. Intravenous injection of anti-PR3 into mice, even into mice that had been primed with LPS, produced only equivocal “incipient” glomerulonephritis and pulmonary capillaritis. However, intravenous injection of anti-PR3 enhanced dermal inflammation at sites of TNF- α injection. Pfister *et al.* (51) concluded that this observation supported the pathogenic potential of anti-PR3. Nevertheless, the injury that is induced by anti-PR3 in this model is not as convincing as the injury in the anti-MPO models. This suggests that anti-MPO and anti-PR3 antibodies have different mechanisms of action in animal models, which is in line with the clinically and pathologically different expression of disease in humans with MPO-ANCA *versus* PR3-ANCA.

Immunogenesis of the ANCA Autoimmune Response

The clinical and experimental data that have been reviewed strongly support a primary pathogenic role for ANCA but do not explain why ANCA develop. Pendergraft *et al.* (52) reported intriguing observations that suggest but do not yet prove a role for peptides that are mimics of the antisense complementary peptides of the autoantigen in the immunogenesis of the antibody response to the sense peptides of the autoantigen. Some but not all patients with PR3-ANCA glomerulonephritis and small-vessel vasculitis have antibodies that react with a peptide translated from the middle portion of the antisense DNA strand of PR3 (complementary PR3). Although not yet demonstrated experimentally, it is possible that patients who do not react with the middle portion of complementary PR3 do react with the N-terminal or C-terminal portions of complementary PR3. In patients with anticomplementary antibodies, these anticomplementary antibodies bound to anti-PR3 antibodies, indicating that the anticomplementary PR3 and anti-PR3 antibodies form an idiotypic and anti-idiotypic pair. Furthermore, mice that are immunized with complementary PR3 peptide produce not only antibodies to antisense PR3 peptide but also antibodies to sense PR3 peptide. These observations are the basis for the hypothesis that anti-PR3 autoantibodies (PR3-ANCA) are generated by an immune response that initially is mounted against a peptide that is antisense or complementary to the autoantigen. This could be an endogenously derived antisense peptide or an exogenously derived mimic of the true antisense peptide, such as a complementary peptide mimic introduced by an infectious pathogen. The antibody response to the complementary peptide or its mimic induces anti-idiotypic antibodies that cross-react with the sense peptides of the autoantigen and thus function as autoantibodies.

Chronic nasal infection with *Staphylococcus aureus* is associated with Wegener’s granulomatosis and is a risk factor for relapses (46). Of interest with respect to the complementary peptide hypothesis, *Staphylococcus aureus* contains a protein with an amino acid sequence that mimics the antisense sequence of PR3. Two other infections that are known to be associated with PR3-ANCA disease also are caused by pathogens with peptide that mimic the complementary peptide of PR3: Ross River virus and *Entamoeba histolytica* (52).

The theory of autoantigen complementarity is far from proved as the primary cause for ANCA disease; however, the preliminary evidence warrants further study of this possibility. Additional support for this theory derives from a variety of other autoimmune diseases that have autoantigen epitopes that have complementary sequences that are mimicked by infectious pathogens that are incriminated in susceptibility to these diseases (53).

Modulation of ANCA Disease

The induction of ANCA small-vessel vasculitis and glomerulonephritis is a multifactorial process that is influenced by genetic susceptibility and environmental factors that can influence not only the initial onset of disease but also the evolution

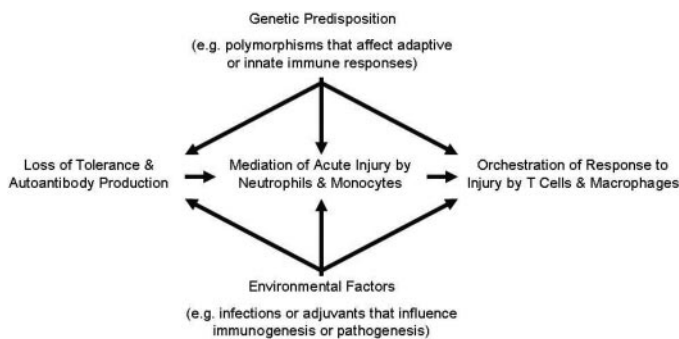


Figure 4. Pathogenic events during the evolution of ANCA disease. Loss of tolerance is required for the production of pathogenic amounts of ANCA. ANCA activate neutrophils and monocytes, resulting in acute inflammation and necrosis, which elicits a response that is orchestrated by macrophages and T cells. Multiple genetic and environmental factors modulate each pathogenic step from onset to outcome.

of disease over time (Figure 4). Genetically determined factors include differences in the expression of ANCA antigens by neutrophils, defects in antiproteinase alleles, and polymorphisms in genes that produce proteins that are involved in adaptive or innate immune responses, such as adhesion molecules, chemokine receptors, and lymphocyte immune regulatory molecules (54). Environmental factors such as infections (*e.g.*, *Staphylococcus*), drugs (*e.g.*, propylthiouracil), and phlogogenic irritants (*e.g.*, silica) seem to be adjuvants if not primary stimuli for the ANCA autoimmune response. Experimental data provide strong evidence that neutrophils are the principal effector cells that are responsible for mediating ANCA-induced acute necrotizing vascular inflammation. However, all destructive acute inflammatory injury engenders a response by the innate immune system that is orchestrated by macrophages and T cells and often results in sclerosis or fibrosis. This response to acute injury may be extremely important in the clinical manifestations and ultimate outcome of a disease process. Little currently is known about this late phase in the pathogenesis of ANCA disease.

In the 20 years since the discovery of ANCA, there have been tremendous advances in the knowledge of the pathophysiology of ANCA-associated small-vessel vasculitis and glomerulonephritis. In the coming 20 years, this knowledge should be valuable in guiding the development of more effective treatment strategies and possibly even preventive measures.

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