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Pathophysiology of ANCA-Associated Small Vessel Vasculitis

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Abstract Antineutrophil cytoplasmic autoantibodies (ANCAs) directed to proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA) are strongly associated with the ANCA-associated vasculitides—Wegener's granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome. Clinical observations, including the efficacy of B-cell depletion via rituximab treatment, support—but do not prove—a pathogenic role for ANCA in the ANCA-associated vasculitides. In vitro experimental studies show that the interplay of ANCA, neutrophils, the alternative pathway of the complement system, and endothelial cells could result in lysis of the endothelium. A pathogenic role for MPO-ANCA is strongly supported by in vivo experimental studies in mice and rats, which also elucidate the pathogenic mechanisms involved in lesion development. Unfortunately, an animal model for PR3-ANCA–associated Wegener's granulomatosis is not yet available. Here, cellular immunity appears to play a major role as well, particularly via interleukin-17–producing T cells, in line with granulomatous inflammation in the lesions. Finally, microbial factors, in particular *Staphylococcus aureus* and gram-negative bacteria, seem to be involved in disease induction and expression, but further studies are needed to define their precise role in disease development.

Keywords Wegener's granulomatosis · MPO-ANCA · Microscopic polyangiitis · hLAMP-2 autoantibodies · Churg-Strauss syndrome · *Staphylococcus aureus* · Necrotizing crescentic glomerulonephritis · FimH · ANCA-associated vasculitis · Animal models · Antineutrophil cytoplasmic autoantibodies · Th17 cells · ANCA · T-regulatory cells · Proteinase 3 · PR3-ANCA · Myeloperoxidase

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Introduction

The antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAVs) include Wegener's granulomatosis (WG); microscopic polyangiitis and its renal limited form, idiopathic necrotizing crescentic glomerulonephritis; and Churg-Strauss syndrome (CSS) [1]. These small vessel vasculitides are characterized by necrotizing inflammation of the vessel wall, particularly of small arteries, arterioles, capillaries, and venules, in conjunction with the presence of ANCAs. ANCAs in the AAV are directed to proteinase 3 (PR3) or to myeloperoxidase (MPO). In the right clinical context, sensitivity and specificity of PR3-ANCA and MPO-ANCA for the AAVs are extremely high, with the possible exception of CSS [1]. In CSS, however, a primarily vasculitic disease pattern is strongly associated with MPO-ANCA, whereas a disease presentation dominated by eosinophilic tissue infiltration generally is ANCA negative [2]. The strong association of PR3-ANCA/MPO-ANCA with the AAVs has led to the assumption that ANCAs are directly involved in the pathogenesis of these diseases.

In this review, I discuss current evidence indicating that ANCAs are involved in the pathogenesis of AAV. Data from clinical studies and from in vitro and in vivo experimental studies are presented. These data will provide insight into the pathophysiologic pathways involved in lesion development of the AAVs. Insight in these pathways

has led and will lead to more focused and specific methods of treatment.

Are ANCAs Pathogenic?

Evidence from Clinical Observations

As mentioned, PR3-ANCA and MPO-ANCA are strongly associated with AAVs. Furthermore, longitudinal observations showed a relationship between increases in levels of PR3-ANCA and the occurrence of relapses. In a study of 100 patients with WG and PR3-ANCA, Boomsma et al. [3] observed that 26 of the 33 relapses that occurred during the study period were preceded by a rise in PR3-ANCA as measured by enzyme-linked immunosorbent assay (sensitivity, 79%), whereas 12 of 38 increases were not followed by a relapse (specificity, 68%). Although the association between increases in ANCA and ensuing relapses in patients with AAVs has been confirmed by others, Finkielman et al. [4], using data from the Wegener's Granulomatosis Etanercept Trial [5], could not confirm this association. In their study, the increase in ANCA levels was not associated with a relapse, and the decrease in ANCA following induction treatment was not associated with a shorter time to remission, although titers of ANCA were significantly lower at the time of remission compared with titers at disease onset. A clinical argument for a pathogenic role of PR3-ANCA comes otherwise from observations that the persistence of ANCA after induction of remission in patients with WG is strongly associated with relapses. Stegeman et al. [6] showed that persistence of ANCA confers a relative risk of 9.0 for relapse. These findings, confirmed by others, have led to the question of whether maintenance treatment for AAVs should be continued for a longer period when ANCAs persist after induction of remission [7]. A randomized controlled trial to assess this issue is currently under way. Another argument for a pathogenic role of ANCAs in AAVs comes from two recently published papers describing the efficacy of rituximab, a B-cell–depleting monoclonal antibody, in patients with severe, active AAVs. The Rituximab for ANCA-Associated Vasculitis study showed that rituximab was not inferior to daily cyclophosphamide treatment for induction of remission in severe AAVs and may be superior in relapsing disease [8•]. The second study also showed the efficacy of rituximab as not being inferior to that of intravenous cyclophosphamide in severe AAVs with impending renal insufficiency [9•]. Although the effects of B-cell depletion may be more extensive than inhibiting antibody production, the efficacy of rituximab in AAV could be explained at least in part by its effect on ANCA production.

Finally, one clinical observation strongly suggests a pathogenic role for MPO-ANCA. Bansal and Tobin [10] reported a case of neonatal microscopic polyangiitis in a child born to a mother with MPO-ANCA. Taken together, current evidence from clinical studies suggests, but certainly does not prove, that ANCAs are involved in the pathogenesis of AAVs.

Evidence from In Vitro Experiments

A major breakthrough regarding the pathogenicity of ANCA was based on the discovery by Falk et al. [11] that ANCAs can activate primed neutrophils for the production of reactive oxygen species and the release of lytic enzymes. This process of activation occurs on a surface only, Fcγ receptors (in particular, FcγRIIa and FcγRIIb) are involved, and the signal transduction routes involved in activation have been largely delineated [12]. These in vitro data could open new possibilities for more focused treatment of AAVs. For instance, Schreiber et al. [13•] showed in vitro that phosphoinositol 3-kinase–γ mediates ANCA-induced degranulation and granulocyte-macrophage colony-stimulating factor stimulated migration in a transwell assay of isolated human neutrophils; a specific inhibitor of this enzyme abrogated these processes in vitro and protected mice against the development of necrotizing glomerulonephritis in a murine model of MPO-ANCA–associated glomerulonephritis.

In vitro data also demonstrate the role of complement in AAVs as an amplification loop for ANCA-induced neutrophil activation. Schreiber et al. [14•] also showed that supernatants from ANCA-activated neutrophils activate the complement system via the alternative pathway, resulting in the production, among others, of C5a. C5a was able to prime neutrophils for ANCA-induced activation, and blocking of the C5a receptor on neutrophils abrogated this process. The important role of complement in AAVs also has been pointed out in animal models of AAV, as discussed subsequently. In agreement with these experimental data, the complement components membrane attack complex, C3d, and factor B could be detected in diseased glomeruli of patients with AAVs. The alternative pathway component factor B colocalized with membrane attack complex, but the classical pathway component C4d could not be detected [15].

Necrotizing vasculitis in AAVs is characterized by neutrophil activation within the vessel wall requiring neutrophil endothelial interaction. In vitro studies have shown that ANCAs stimulate neutrophil-induced cytotoxicity toward endothelial cells [16]. In an in vitro flow system, Radford et al. [17] demonstrated that ANCAs induce conversion of rolling of neutrophils to firm integrin-mediated adhesion. Adhesion could be blocked by inhibiting monoclonal antibodies to the FcγRIIa receptor and to

CD11b. In vivo experimental studies have confirmed by intravital microscopy that MPO-ANCAs reduce leukocyte rolling over the endothelium and augment adhesion to and transmigration across the endothelium, a process that can be inhibited by blocking Fcγ receptors and β2 integrins [18]. One particular observation of note was made by Kessenbrock et al. [19•]. They showed that neutrophil activation by ANCA induces the generation of neutrophil extracellular traps (NETs), which contain PR3 and MPO. NETs can adhere to the endothelium and damage these cells. Furthermore, they can activate plasmacytoid dendritic cells, thus contributing to local production of ANCAs at the site of inflammation.

In conclusion, in vitro studies strongly support a pathogenic role for ANCAs in AAVs. The adaptive immune response manifested by ANCAs interacts with innate immunity, in particular neutrophils and the complement system. Together they target the endothelium, resulting in necrotizing vasculitis.

Evidence from In Vivo Experiments

The most convincing evidence for a pathogenic role of ANCA comes from in vivo experimental studies. After immunizing MPO-deficient mice with murine MPO, Xiao et al. [20] observed that the mice developed antibodies to murine MPO. Next, spleen cells from these immunized mice were transferred into immunodeficient or healthy mice. The recipients developed pauci-immune necrotizing crescentic glomerulonephritis (NCGN) and hemorrhagic pulmonary capillaritis. When they transferred only IgG from the immunized mice, the recipient mice developed pauci-immune focal NCGN, demonstrating the pathogenicity of MPO-ANCA. Addition of lipopolysaccharide to the IgG fraction augmented the lesions, and lesion development could be blocked in part by anti–tumor necrosis factor treatment [21]. Neutrophils are required for lesion development, as shown by neutrophil depletion [22]. Within neutrophils, the presence of MPO is essential, as shown by Schreiber et al. [23]. When $MPO^{-/-}$ bone marrow was transferred into irradiated $MPO^{+/+}$ mice, lesions did not develop. Conversely, transfer of $MPO^{+/+}$ bone marrow cells into $MPO^{-/-}$ mice with anti-MPO antibodies resulted in NCGN. Besides neutrophils, the complement system proved essential to lesion development. Complement depletion prevented disease development, and mice deficient in C5 or complement factor B did not demonstrate NCGN. Absence of complement factor C4, however, did not influence NCGN development [24]. These data show that activation of the complement system via the alternative pathway occurs in this animal model of MPO-ANCA–associated AAV. Indeed, blocking of C5a or the C5a receptor could prevent the development of vasculitis in this murine model [14•]. This opens new methods of treatment. Another method of treatment is suggested by recent experiments in which endoglycosidase S (Endo S) was used to modulate IgG glycosylation, thus abolishing Fc receptor–mediated activation of leukocytes and complement. Administration of Endo-S–treated anti-MPO antibodies to recipient mice strongly reduced neutrophil influx and crescent formation in the above-described model of MPO-ANCA AAV [25•]. The pathogenic potential of MPO-ANCA also was demonstrated in a rat model in which rats were immunized with human MPO [26]. Through use of intravital microscopy, the ability of MPO-ANCA to enhance leukocyte-endothelial interaction and to induce microvascular hemorrhage was demonstrated.

Whereas the aforementioned experimental studies strongly suggest or even prove that MPO-ANCAs are pathogenic in the AAVs, the in vivo evidence of a pathogenic role of PR3-ANCA is less clear. To establish an animal model for PR3-ANCA AAV, Pfister et al. [27] used the same approach as described above for a murine model of MPO-ANCA AAV. They immunized PR3-deficient mice with recombinant murine PR3. These mice developed anti-PR3 antibodies that recognized murine PR3 on the membrane of murine neutrophils. Next, the antibodies were passively transferred into naive mice. No signs of vasculitis developed in the kidneys or lungs. The only observation that they made was of an increased tumor necrosis factor–α–induced local inflammation in the skin of anti-PR3 transferred mice compared with control mice. Also, a rat model of PR3-ANCA AAV was not successful. Van der Geld et al. [28] immunized rats with human–mouse chimeric PR3. These rats developed antibodies that reacted with recombinant rat PR3 and selectively bound to rat granulocytes, but vasculitic lesions did not develop despite systemic administration of lipopolysaccharide. Why these efforts to establish an animal model of PR3-ANCA AAV have not been successful is currently not clear. As discussed subsequently, increasing evidence indicates that in addition to autoantibodies, effector T cells are involved in the pathogenesis of PR3-ANCA–associated WG. As WG is closely associated with PR3-ANCA, this may explain why autoantibodies alone are not sufficient to induce the clinical phenotype of PR3-ANCA–associated vasculitis.

Two other models related to AAV need attention. The first relates to the possibility that a protein complementary to PR3 may induce PR3-ANCA. A complementary protein is a protein translated from the antisense DNA strand encoding for the original protein. Pendergraft et al. [29] observed that some patients with PR3-ANCA AAV had antibodies to complementary PR3 (cPR3). Next, they immunized mice with cPR3 and found that these mice not only developed antibodies to cPR3, but also to PR3. As a complementary protein seems to be a mirror of the original

protein, they suggested that antibodies to cPR3 could induce an antibody response to PR3 via idiotypic–anti-idiotypic interaction. Interestingly, they noticed that cPR3 shows homology with several microbial proteins, including peptides derived from *Staphylococcus aureus*. This opens the possibility that infection with *S. aureus* underlies the development of PR3-ANCA, as discussed further subsequently. More recently, this group described T cells in the peripheral blood of patients with PR3-ANCA AAV reacting with cPR3 [30].

The second interesting observation was recently published by Kain et al. [31•]. In 1995, they described a novel class of ANCA directed to the lysosomal membrane glycoprotein hLAMP-2 [32]. This antigen is present not only on the membrane of neutrophil granules but also on other cells, such as endothelial cells. They observed that 78 of 84 (93%) patients with active ANCA-associated NCGN had detectable anti–hLAMP-2 antibodies in their sera, whereas only 6 of 84 (7%) were positive during remission. The antibodies were not detectable in healthy controls or diseased controls. To show the pathogenic potential of anti–hLAMP-2, they raised anti–hLAMP-2 IgG class antibodies in rabbits and injected these antibodies into rats. Rats developed pauci-immune focal necrotizing glomerulonephritis. To explain the pathogenicity of anti–hLAMP-2, they further showed that in vitro, the antibodies were able to activate neutrophils and to kill human microvascular endothelial cells. Most interestingly, they found that eight of nine amino acids of the immunodominant epitope of hLAMP-2 are identical to the P72-80 peptide of FimH, an adhesion molecule of fimbriae from gram-negative bacteria. Next, they immunized rats with FimH, which resulted in antibodies cross-reacting with hLAMP-2, which in turn induced pauci-immune glomerulonephritis. These data, which still need to be confirmed by others, strongly suggest a pathogenic role for anti–hLAMP-2 [33].

Taken together, in vivo experimental studies support, if not prove, that MPO-ANCAs are pathogenic for necrotizing vasculitis/glomerulonephritis. This is not as clear for PR3-ANCAs. A pathogenic role for anti–hLAMP-2 antibodies has been strongly suggested but awaits further study.

Besides Autoantibodies, is Cellular Immunity Involved in the Pathogenesis of ANCA-Associated Vasculitis?

As mentioned, granulomatous inflammation is present in WG associated with PR3-ANCA. In persisting localized WG, ANCAs are not detectable in about 50% of patients [34]. This suggests involvement of cellular immune effector mechanisms. Indeed, Abdulahad et al. [35] described increased levels of effector memory T cells in the peripheral blood of patients with WG during remission. Immune effector cells have been observed in granulomatous tissue in patients with AAV [36]. Surprisingly, effector memory cells disappeared from the peripheral blood during active disease in AAV. Interestingly, however, these cells could be detected in the urine during active disease with renal involvement [37•]. These data suggest that even during remission, the immune system is activated in patients with WG, and that these activated cells migrate to the target organs during active disease. The phenotype and cytokine pattern of the effector memory cells in WG have been further defined. Both $CD8^+$ and $CD4^+$ T cells are present, but $CD4^+$ T cells producing interleukin (IL)-17 seem to be more prominent. Analysis of the intracellular cytokine pattern of peripheral blood cells stimulated with the autoantigen PR3 in WG showed that the ($CD4^+$) T cells proliferating upon interaction with PR3 produced—in the vast majority—IL-17 [38]. Indeed, Nogueira et al. [39] reported elevated levels of IL-17 and IL-23, as well as autoantigen-specific T-helper type 17 (Th17) cells in the peripheral blood of AAV patients. In an animal model of anti-MPO glomerulonephritis, Gan et al. [40•] found that Th17 cells were instrumental in orchestrating renal injury. Thus, T-effector cells, in particular Th17 cells, seem to play a major role in the pathogenesis of AAVs. Whereas most of the studies mentioned relate to the $CD4^+$ subset of T cells, a recent study described a transcription signature of $CD8^+$ T cells that predicts poor prognosis in patients with AAVs and systemic lupus erythematosus (SLE). McKinney et al. [41•] found that genes involved in the IL-7 receptor pathway and T-cell–receptor signalling and genes expressed by memory T cells were enriched in the $CD8^+$ subset of patients with AAVs and SLE with a poor prognosis. These data also point to a major role for T cells in the pathogenesis of AAVs. A hypothetical scheme of T-cell involvement in AAV is shown in Fig. 1.

How are humoral and cellular responses in AAVs regulated? Increasing attention has been paid to T-regulatory cells (Tregs) in controlling autoimmunity. The phenotypic and functional characterization of Tregs is still being discussed. Abdulahad et al. [42] showed that $CD4^+$ T cells highly expressing CD25 on their surface—considered to be natural Tregs—were increased in the peripheral blood of AAV patients; however, their capacity to suppress responses of $CD25^-$ $CD4^+$ effector cells was highly deficient. Functional impairment of Tregs in patients with WG was also described by others [43]. Thus, deficient function of Tregs may at least in part underlie the autoimmune response in AAVs.

Role of Microbes in the Pathogenesis of AAVs

The etiology of AAVs is still unknown. Genetic factors have been suggested but are not particularly strong [44].

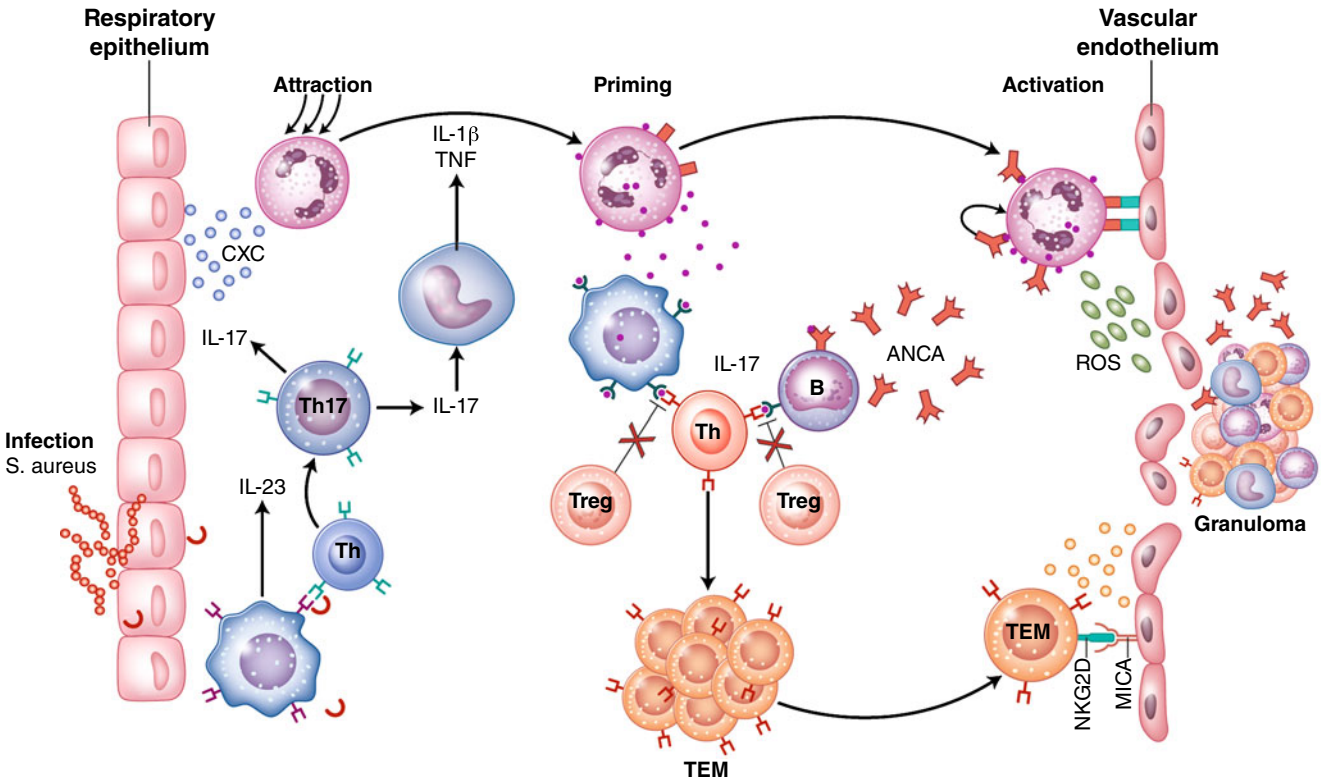

Fig 1 A proposed model representing innate and adaptive immune mechanisms supposedly involved in the pathogenesis of antineutrophil cytoplasmic autoantibody (ANCA)-associated systemic vasculitis. Superantigens and peptidoglycans from *Staphylococcus aureus* stimulate antigen-presenting cells (APCs) in the respiratory tract to produce interleukin (IL)-23, which then induces proliferation of T-helper (Th) type 17 cells and release of IL-17. IL-17 acts further on respiratory epithelium and tissue macrophages. In response to IL-17, bronchial epithelial cells secrete CXC chemokines that attract neutrophils to the infected tissue, whereas macrophages release proinflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α. These inflammatory cytokines cause priming of neutrophils (membrane expression of proteinase 3 [PR3]) and upregulation of adhesion molecules on their surface as well as on the vascular endothelium. Subsequently, primed neutrophils adhere to the endothelial cells. Released PR3 can be processed and presented by APCs to Th cells. As T-regulatory cells (Tregs) fail to inhibit this autoimmune response in Wegener's granulomatosis, autoreactive T cells might undergo repeated stimulation by PR3-pulsed APCs, resulting in a pool of effector memory T cells (TEMs). Furthermore, PR3-stimulated Th cells act on B cells and enhance the production of ANCAs. Subsequently, ANCAs activate neutrophils that adhere to endothelial cells, resulting in local production of reactive oxygen species (ROS) and release of proteolytic enzymes that damage vascular endothelial cells. Moreover, the expanded population of $CD4^+$ TEMs resulting from persistent activation of Th cells by PR3 upregulate their NKG2D protein and migrate to the peripheral blood and remain in the circulation during remission. When the disease becomes active, MICA protein will be upregulated on several vascular endothelial cells (especially in the kidney), which attract TEMs to the inflammatory areas. The MICA protein on the target cells can bind to NKG2D on the TEMs, which in turn enhances their cytotoxic function to kill the target cell in a perforin- and granzyme-dependent way, ending up in vasculitis. (*From* Abdulahad et al. [48]; with permission.)

Interaction of genetic and extrinsic factors seems likely in the etiology of AAVs. With respect to extrinsic factors, toxic materials—in particular silica exposure—have been incriminated [45]. More attention has been given to microbial factors. Stegeman et al. [6] first noted that 63% of patients with WG were chronic nasal carriers of *S. aureus* and that *S. aureus* carriage was associated with a higher relapse rate, with an adjusted relative risk of 7.16. Furthermore, they showed that prophylactic treatment with cotrimoxazole could prevent the occurrence of relapse (60% reduction) [46]. The mechanisms underlying the relationship between *S. aureus* and AAVs have not been fully elucidated, but superantigen stimulation of B and T cells and neutrophil activation have been proposed [47]. As mentioned previously, Pendergraft et al. [29] presented evidence that complementary PR3, which shows homology with certain *S. aureus*–derived peptides, may induce antibodies to PR3. As such, *S. aureus* carriage may, by molecular mimicry, lead to the development of PR3-ANCA. More studies are needed, however, to elucidate how *S. aureus* carriage relates mechanistically to AAVs.

A recent study also points to the role of microbes in the etiopathogenesis of AAVs. As mentioned previously, Kain et al. [31•] described autoantibodies to hLAMP-2 as a sensitive and specific marker for ANCA-associated pauci-immune glomerulonephritis. They also showed cross-reactivity between an immunodominant epitope of hLAMP-2 and FimH. Immunization of rats with FimH

resulted not only in antibodies to FimH but also in antibodies to hLAMP-2 and pauci-immune glomerulonephritis. Furthermore, they noticed in a small group of patients that most had acquired an infection with gram-negative bacteria before the onset of AAV (accompanied by hLAMP-2 antibodies). These data, which must be extended and confirmed, suggest that infection with gram-negative bacteria may lead, in a susceptible individual, to the development of AAVs.

Conclusions

The close association between AAV and PR3-ANCA/MPO-ANCA suggests that the autoantibodies are involved in the pathogenesis of these diseases. Clinical data support, but certainly do not prove, this suggestion. In vitro experimental data point to the pathogenetic pathways involved in lesion development, in which, in addition to the autoantibodies, neutrophils, the alternative pathway of the complement system, and endothelial cells play a major role. In vivo experimental studies strongly support a pathogenic role for MPO-ANCA, but an animal model for PR3-ANCA is lacking. In PR3-ANCA–associated WG, T cells seem to play a major role as well. Finally, microbial factors seem to be involved in disease induction and possibly in disease expression. Although great progress has been made in the understanding of AAVs, further studies are needed to fully elucidate the etiopathogenesis of these diseases.

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