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**The Devil in the Details: The Emerging Role of Anticitrulline Autoimmunity in Rheumatoid Arthritis**

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Rheumatoid arthritis is a chronic inflammatory autoimmune disease of unknown cause. The immune response against citrullinated Ags has recently become the prime suspect for disease pathogenesis. Immunity against citrullinated Ags is thought to play a pivotal role in the disease for several reasons: 1) citrullinated Ags are expressed in the target organ, the inflamed joint; 2) anti-citrullinated protein Abs are present before the disease becomes manifest; and 3) these Abs are highly specific for rheumatoid arthritis. In this review, data from clinical, genetic, biochemical, and animal studies is combined to create a profile of this remarkable autoantibody response. Moreover, a model is proposed of how the anti-citrullinated proteins response is generated and how it could eventually lead to chronic inflammation. The Journal of Immunology, 2005, 175: 5575–5580.

Autoimmune diseases are classified according to the organs and tissues that are damaged by the immune response. There is an autoimmune disease directed against almost every organ in the body, involving, usually, an immune response to an Ag expressed in that organ (1). Autoimmune diseases may be defined using Witebsky’s postulates. These postulates require that 1) an autoimmune reaction is identified in the form of autoantibody or cell-mediated immune reaction, 2) the corresponding Ag is known, and 3) an analogous response causes a similar disease in experimental animals (2, 3).

Rheumatoid arthritis (RA) is considered an autoimmune disease, but specific immune responses against joint Ags have been difficult to demonstrate conclusively in the past. Recent data indicate that the immune response against citrullinated Ags is an attractive candidate for the fulfillment of the three Witebsky postulates. There has been considerable interest in recent years in the observation that a very high proportion of patients with RA have IgG Abs to citrulline-containing proteins. Interestingly, these Abs appear early in RA and are rarely found in healthy people or patients with other diseases. Moreover, recent data show that citrullinated Ags themselves are expressed in the inflamed joint. This leaves the third Witebsky postulate open, although recent data suggests that immunization with citrullinated peptides or proteins could lead to a significant T cell response in rodents (our unpublished data and Ref. 4).

As the response against citrullinated Ags is likely the first to meet all criteria in Witebsky’s postulates, it is very tempting to speculate that the immunity to citrullinated Ags is intimately involved in the pathogenesis of RA. We propose here a model for the production of Abs against citrullinated Ags and the subsequent induction of chronic inflammation in the joint.

**Pathogenic mechanisms of autoantibodies**

In autoimmune diseases, autoantibodies provide diagnostic criteria, serve as surrogate markers for disease activity, and play a requisite role in pathogenesis. Although most autoantibodies are not known to be pathogenic and are mainly used for diagnosis, several autoantibodies have been shown to be involved in development of autoimmune diseases. For instance, autoantibodies against type IV collagen in the glomerular basement membrane of the kidney and lung cause nephritis and lung hemorrhage in Goodpasture’s syndrome, and autoantibodies to the acetylcholine receptor cause muscle weakness in myasthenia gravis (5, 6). Autoantibodies contribute to disease by directly binding to end-organ tissue Ags, with triggering of immune-effector mechanisms, such as FcRs and the complement system, as a result (7).

RA is a chronic disease, with inflammation and deformities of the joints as the most striking features. The role of autoantibodies has been well established in several experimental arthritis models, but their role is less clear in the human disease (8). For one, most autoantibodies found in RA can also be detected in individuals without RA. One such example is the IgM rheumatoid factor (RF). RFs are Abs directed to the Fc fragment of IgG molecules and are found in the majority of RA patients. However, patients with other chronic inflammatory diseases, infectious diseases, as well as many healthy individuals, also produce RF, indicating that RF is not very specific for RA and that its mere presence is insufficient for a chronic inflammatory response (9).

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2 Abbreviations used in this paper: RA, rheumatoid arthritis; RF, rheumatoid factor; APF, antiperinuclear factor; AKA, anti-keratin Ab; ACPA, anti-citrullinated protein/peptide Ab; PAD, peptidylarginine deiminase; SE, shared epitope; GPI, glucose-6-phosphate isomerase.
Autoantibodies against the unusual amino acid citrulline

In 1964, Nienhuis and Mandema (10) discovered a novel Ab, the antiperinuclear factor (APF), while investigating the occurrence of autoantibodies in patients with various rheumatic diseases. Later on, also anti-keratin Abs (AKAs) (11) were identified by using rat or human esophagus cryostat sections for detection. Despite the fact that several research groups have recognized the clinical and diagnostic value of these Abs, especially because of their high specificity for RA (12–15), APFs and AKAs never became very popular. This was mainly a consequence of the fact that testing their presence was more laborious than the RF test, which soon became the standard laboratory test for RA. The first step toward identifying the Ag recognized by these Abs was done by the group of Serre (16) in 1993 when it became clear that the Ag recognized by the so-called AKAs is actually filaggrin.

More than 30 years after its initial description, a major breakthrough was made through the pioneering work of the groups of Serre and Van Venrooij (17, 18). They showed that the APF is an Ab directed against proteins containing the unusual amino acid citrulline. In addition, they demonstrated that the APF and AKA are directed to the same Ag and that other RA-specific autoantibodies such as anti-Sa Abs are also directed against citrulline-containing proteins (19, 20). Although the specificity of these Abs might differ and is most probably influenced by the citrulline-flanking residues, citrulline is the critical constituent of the antigenic determinant recognized by these Abs because its absence leads to lack of recognition (17, 18, 21). Thus, although the nomenclature (APF, AKA, anti-filaggrin, and anti-cyclic citrullinated peptide Abs) of the several Ab responses might differ as a result of the different Ags used for detection, they all recognize citrulline as common antigenic entity and will, therefore, be named in this review as anti-citrullinated protein/peptide Abs (ACPAs).

Citrullination is the posttranslational modification of protein-bound arginine into the nonstandard amino acid citrulline and results in a small change in molecular mass and the loss of a positive charge in the modified proteins (Fig. 1). Although the physiological role of citrullination remains to be elucidated, it has been proposed that citrullination plays an important role in preparing intracellular proteins for degradation during apoptosis (22, 23), as well as in regulation of transcription through citrullination of histones (24, 25).

Five mammalian peptidylarginine deiminases (PADs), PAD1–4 and PAD6, each with a defined tissue distribution, mediate citrullination of arginine in the presence of sufficient concentrations of Ca$^{2+}$ (reviewed in Ref. 26). Interestingly, PAD enzymes were found in monocytes (PAD4) and macrophages (PAD2 and PAD4) in synovial fluid (27), indicating that they could be involved in citrullination of synovial proteins once they become activated. Indeed, it has been shown that citrullination of synovial proteins is an active process during inflammation (28, 29) and that several citrullinated proteins, such as fibrin (30), can be found in the RA synovium. Together with citrullinated proteins in the inflamed joint, B cells actively secreting ACPAs have been detected in synovial fluid and synovium from RA patients (31, 32) but not in peripheral blood or in healthy controls. The presence of IgM ACPA-secreting B cells in synovial fluid is indicative of a continuous activation of B cells specific for citrullinated Ags from naive precursors, suggesting an Ag-driven proliferation and maybe local differentiation of these cells.

The ACPA response became prime suspect in the pathogenesis of RA once it became clear that, while being found in 60–70% of patients with RA, ACPAs display a unique specificity for RA and are rarely detected in other diseases or in healthy controls (13, 33–36). Remarkably, using recently developed ELISAs containing cyclic citrullinated peptides, ACPAs of the IgG isotype have been detected up to nine years before symptoms of RA occurred (37, 38). A similar observation was made in patients with undifferentiated arthritis (or unclassified arthritis). Although ~40% of these patients will eventually progress to RA, the remaining patients experience remission or develop other rheumatic diseases. Unfortunately, physicians are currently not able to predict which patients will progress to RA and which patients will have a more favorable outcome of disease. We have recently reported that patients with undifferentiated arthritis with ACPA have a chance of >90% to progress to full-blown RA within 3 years (39). This not only shows that detection of ACPAs is a powerful tool in predicting RA, but again points to the highly specific nature of the ACPA response.

The second observation, indicating that the immune reaction against citrullinated Ags is involved in the pathogenesis of RA, comes from genetic association studies. A haplotype of the gene encoding one of the citrullinating enzymes, PADI4, was shown to be associated with susceptibility to RA (Ref. 40 and our unpublished observations). Although it could not be confirmed in two other studies in Caucasians (41, 42), possibly as a result of differences in the haplotype structure between different ethnic populations (43), this haplotype was shown to be associated with an enhanced stability of PADI4 transcripts. Therefore, it was postulated that the more “stabile haplotype” leads to an increased production of PAD4 protein and thereby to a higher citrullinating enzyme activity in the joint. This will result in a higher concentration of citrullinated proteins that could serve as a target for the immune response against citrullinated Ags.

A third line of evidence is found in the strong association between production of ACPA and the presence of RA susceptibility HLA-DRB1 genes (44).

It has long been observed in many different populations that specific HLA-DR gene variants in the MHC region are highly associated with RA. The association has been mapped to the third hypervariable region of DRβ-chains, especially aa 70–74, encoding a conserved amino acid sequence (QKRRA, QRRRA, or RRRA) that forms the fourth anchoring pocket in the HLA groove. This susceptibility epitope, called the shared epitope (SE), is found in multiple RA-associated DR molecules, including DR4, DR1, and DR14 (e.g., DRB*0401, DRB*0404,
of the RA patients do not harbor these Abs. Likewise, the emergence of these Abs does not appear to have an immediate effect, suggesting the requirement for an additional factor involved in disease onset.

Therefore, we propose a “multiple hit model” for RA (Fig. 2) in which environmental and genetic factors have to come together within one individual for induction and progression of the disease and in which ACPA are associated both with an increased risk for developing RA and with progression to erosive disease. Considering the clinical association data and the immunological data published thus far, we have elaborated a two-step model attempting to explain the development of RA.

**Step one: induction of ACPA**

For the induction of a response against citrullinated Ags, activation of both B cells specific for citrullinated Ags and, most likely, Th cells has to occur. Citrullination has been described as a physiological process occurring during apoptosis at multiple sites in the body (22, 47, 48). This process is believed to involve mainly intracellular proteins, which need to be unfolded to become more accessible to degradation by proteases (23) and most probably does not lead to an immune response against citrullinated Ags. Citrullination can, in contrast, occur also during inflammation (28, 29). Considering that citrullinated proteins are attractive targets for the immune system when presented in a proinflammatory environment, this could lead to the induction of effector T cells providing help to B cells specific for citrullinated Ags. Moreover, inflammation could also result in the generation of citrullinated neoepitopes, which, in contrast to the physiologically generated citrullinated proteins, occur extracellularly, and are thus “visible” for B cells specific for citrullinated Ags. Fibrin is the main extracellular citrullinated protein identified thus far in the synovial extract from the inflamed joint and is, as such, a prominent synovial candidate Ag in ACPA-positive RA (30). However, the presence of citrullinated fibrin is not specific for RA, but rather a result of inflammation (28, 29, 49). Nevertheless, despite the fact that inflammation is relatively abundant in everyone’s daily life, only <1% of the population develops ACPA. Therefore, it is conceivable that an accumulation of genetic and environmental factors is necessary for a response to citrullinated Ags to develop.

**FIGURE 2.** Multiple hit model for RA.
For example, it is possible that citrullination of proteins as a result of inflammation or inflicted by environmental factors will initiate an HLA class II-restricted T cell response (Fig. 3; nos. 1 and 2 uptake and presentation) only in a genetically predisposed (e.g., SE-positive) individual. It is, in this respect, intriguing that the environmental factor smoking is associated with an increased risk to develop ACPA-positive RA only in subjects that are SE positive, pointing to a clear gene-environment interaction involved in the development of ACPA-positive disease (50). Other factors, such as an altered negative selection and/or activation of B or T cells could also play a role in the emergence of an immune response against citrullinated Ags. An altered responsiveness could be a consequence of a mutation in the protein tyrosine phosphatase PTPN22, a negative regulator of lymphocyte activation, which predisposes to multiple autoimmune diseases, including RA (51).

The help provided by T cells allows for maturation and class switching by B cells, which results in a further maturation of the ACPA IgG Ab response (Fig. 3; no. 3 autoantibody production). This B cell response specific for citrullinated Ags, induced either in the lymph nodes draining the joints or other sites of inflammation, can become pathogenic once citrullinated Ags are generated in the joint and ACPA are able to enter the joint.

**Immune complexes allow autoantibodies access to the joints**

Several clinical observations indicate that the joint is an organ sensitive to systemic inflammatory reactions. For instance, arthritis is one of the presenting features of immune complex-associated complications of meningococcal disease in children (52). Moreover, in serum sickness, immune complex deposition and the subsequent inflammatory response not only cause skin lesions but often also (transient) arthritis (53).

**FIGURE 3.** Schematic representation of the potential contribution of ACPA to inflammation in the RA joint. A. Citrullinated proteins (C), produced in the joint or elsewhere, are taken up by APCs (1) and, if generated in an inflammatory environment, are presented to T cells (T) by activated dendritic cells in the draining lymph node. This will promote the induction of a T cell response (2). T cells provide help to B cells specific for citrullinated Ags (B) that will subsequently produce high-affinity IgG ACPAs (red Abs) (3). The Abs are allowed to enter the joint when a local inflammation occurs or as a result of immune complex-facilitated vascular leakage (4). Gra, granulocyte; M, macrophage/monocyte; Ir, irrelevant Ag; black Ab, Ab to irrelevant Ag. B. During inflammation or after trauma, monocytes (M), and granulocytes (Gra) migrate into the synovium of the joint. During cell death (5), the membrane integrity of these cells is lost, and PAD enzymes become activated due to extracellular calcium influx, leading to citrullination of synovial proteins (6). In the joint, ACPAs, bound to citrullinated Ags (7), activate complement and attract and trigger granulocytes, monocytes, and mast cells (8). These cells (mainly granulocytes and macrophages) exert their function and die. As a consequence, more PAD becomes activated. The generation of more Ags will drive the inflammation into a vicious circle, contributing to the development of chronic disease.

In many of the small animal models of human RA, such as in the K/BxN and the collagen-induced arthritis mouse model, immune complexes play an important role in autoantibody-mediated autoimmunity (8). In addition, in both models, joint-specific Ag expression is not a requirement for joint-specific disease. Type II collagen, the autoantibody target in collagen-induced arthritis, is not only present in the joint but also in tracheal and bronchial cartilage, the vitreous humor of the eye, and the cartilage of the ear. Nevertheless, anti-type II collagen Abs cause disease in the joints (54). This is even more pronounced in the K/BxN model where autoantibodies to glucose-6-phosphate isomerase (GPI) cause arthritis, despite the fact that GPI is present in virtually every organ (55).

After injection in healthy animals, anti-GPI Abs rapidly accumulate in the joints (56). This process is dependent on the presence of mast cells, neutrophils, complement receptors, and FcRs and is related to the fact that anti-GPI Abs form immune complexes with GPI in serum (57). Similarly, anti-type II collagen Abs do not accumulate in the distal joints, but do so after coinjection of irrelevant, preformed immune complexes. Interestingly, control autoantibodies localize to the joint in a similar manner, but their presence is of limited duration. Only Abs against Ags expressed in the joint, such as GPI and type II collagen persist in the joint, and cause arthritis. These observations are intriguing, as they indicate that 1) the presence of circulating immune complexes mediates Ab access specifically to the joints and 2) the presence of Ag ensures persistence of Ab in the joint. Moreover, they could explain why ACPAs are present sometimes years before clinical signs of disease, as arthritis will not develop until articular Ags will be expressed and Abs will have access to the joints. The latter could occur when circulating (irrelevant) immune complexes such as RF are produced,
by providing an explanation for the presence of both ACPAs and RF Abs in many RA patients (our unpublished data).

**Step two: citrullinated Ags in the target organ**

The joints are not only sensitive to immune complex-mediated influx of Abs, but are also a site where small bleedings occur regularly. Bleedings in the joints in patients with hemophilia are daily events and are believed to be due to the extensive vascularization of the synovium and to the mechanical stress applied to the joints with every movement (reviewed in Ref. 58). Although coagulation will occur in healthy persons, these findings do indicate that the joint is a site where spontaneous bleedings occur regularly. These could result in hypoxia-induced cell death and release of endogenous danger signals, such as uric acid (59) and high-mobility group box 1 (60), which will alarm the immune system and attract at least a few immune cells (e.g., neutrophils). Especially granulocytes, which express PAD4, are known to have a high turnover under inflammatory conditions (Fig. 3; no. 4 cell death) (61). PAD enzymes are normally present in an inactive state, as they require high concentrations of calcium for activation. Although in living cells the intracellular concentration of calcium is ~100 times lower than the threshold concentration for activation of PAD (62), the situation in dying cells is likely to be completely different. During cell death, the integrity of the cell membrane is disrupted, resulting in a influx of extracellular calcium and subsequent PAD activation within the cell (22). Likewise, PAD enzymes may leak out of the cell and become activated (the normal extracellular calcium concentration is above the threshold), leading to citrullination of the extracellular matrix proteins and thereby generating the target Ag for ACPAs.

PAD4 and PAD2 are expressed by granulocytes, monocytes, and macrophages. Recruitment of granulocytes and monocytes to an inflamed joint, followed by their demise will most probably result in the activation of these two enzymes, allowing the citrullination of intra- and extracellular proteins including extracellular fibrin (Fig. 3; no. 5 citrullination), which is generated upon activation of the coagulation system. Recognition of citrullinated proteins by ACPAs leads to formation of immune complexes and activation of complement, with subsequent re-
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Now the immune response has entered a vicious circle, in which inflammation causes even more Ag to be made, with possible perpetuation of the response as the result.

**Concluding remarks**

ACPAs are unique and predictive for RA and citrullinated pro-
teins are found in the inflamed joint. This response fulfills thus the first two criteria of Witebsky postulates of autoimmunity and points toward a role for the response against citrullinated Ags in the pathogenesis of RA. To fulfill all of the Witebsky postulates, induction of disease upon generation of an immune response against citrullinated Ags in experimental animal models is required. Although this would represent an important step forward, it will mean that we are still only beginning to understand RA and the role of the citrullinated Ag-specific immunity (Table I).

**Table I: Outstanding questions**

1. Using a combination of citrullinated peptides or one citrullinated protein, ACPAs are detected in 60–70% of patients with RA (34, 63, 64). Does this imply that a considerable proportion of patients with RA do not produce ACPAs or are current assays simply not able to detect them?
2. Current assays generally detect ACPAs of the IgG isotype. Does this indicate the presence of an underlying T cell response, and does this imply that this T cell response is specific for citrullinated Ags?
3. Is blocking PAD enzymes a way to treat RA? Selectively blocking PAD enzymes involved in RA may be beneficial, as this would prevent production of citrullinated proteins.
4. What is the Ag responsible for the generation of the immune response against citrullinated Ags? Identifying the relevant Ag is important and could be the basis of a therapeutic intervention, e.g., tolerizing vaccines.
5. How, when, and where is the immune response against citrullinated proteins generated? Or, how, when and where is tolerance broken?
6. Does immunity against citrullinated Ags cause arthritis in animals?

Valuable information may come from follow-up studies in healthy individuals harboring ACPAs. Analyses of their B and T cell responses against citrullinated Ags might give important insights into the events resulting in full-blown arthritis. At the same time, these individuals are the ones most likely to benefit from Ag-specific intervention strategies. If ACPAs truly are involved in the pathogenesis of RA, Ag-specific interventions may prevent chronic arthritis and long-term joint destruction without the side effects associated with today’s treatment regimens.

**References**


