How is autoimmunity against citrullinated proteins regulated?
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Citrullinated proteins in rheumatoid arthritis: crucial ... but not sufficient!

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136.
Abstract

Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) and have thus become part of the diagnostic armamentarium in inflammatory arthritis. Circumstantial clinical evidence also suggests that ACPA may participate in important pathophysiological processes in RA. This concept has recently been supported by the specific gene-environment interaction between smoking and HLA-DR4 in ACPA positive but not ACPA negative RA patients and by the enhancement of tissue injury by ACPA in experimental arthritis. In parallel, important progress has been made in the detection and identification of citrullinated proteins as potential targets for ACPA in inflamed synovium as well as other tissues. However, it becomes increasingly clear that well-defined citrullinated epitopes rather than the mere presence of citrullinated proteins as such may be relevant for the induction of ACPA and, eventually, for the pathogenicity of anti-citrulline reactivity. Therefore, defining the clinically relevant citrullinated epitopes and experimentally assessing the requirements for optimal and coordinated immune activation by these epitopes are two major challenges in this field.
From systemic antibodies to synovial targets

One of the major features of rheumatoid arthritis (RA) is the presence of serum autoantibodies in the vast majority of patients. Besides the well-known rheumatoid factor (RF), there has been increasing scientific and clinical interest in the so-called anti-citrullinated protein antibodies (ACPA). These antibodies have been known for more than 40 years as anti-perinuclear factor, anti-keratin antibodies, and anti-filaggrin antibodies (1-3). Their commonalities have been evidenced by the crucial finding that they all target epitopes in which arginine residues have been converted to citrulline by the post-translational action of peptidyl arginine deiminase (PAD) enzymes (4, 5). The high specificity for RA (6-8) and the development of citrullinated substrates which allow easy and reliable detection of these autoantibodies (4, 5, 9, 10) have boosted clinical interest in ACPA as a new diagnostic tool.

Besides this diagnostic application, recent clinical observations have also provided some circumstantial evidence that ACPA may be related to important pathophysiological processes in RA. Firstly, ACPA can be found early in the disease course of RA (11-14), even years before onset of clinical symptoms (15, 16). Secondly, the presence of ACPA is associated with more severe joint destruction (13, 17, 18) and higher disease activity (13, 18, 19), with ACPA positivity at the time of diagnosis being an important predictor for a more aggressive disease course (13, 15, 17). Thirdly, recent studies suggest that ACPA defines a separate etiologic entity within RA (20, 21) and a striking gene-environment interaction between HLA-DR shared epitope and smoking has been described in ACPA positive but not ACPA negative RA patients (22). Despite this evidence, the role of ACPA in pathogenesis of RA is not firmly established. Citrullinated epithelial (pro)filaggrin, the originally described target of ACPA (3, 23, 24), is not expressed in the joint and citrullinated filaggrin-containing epithelial tissues such as skin and buccal mucosa are not involved in RA. These seemingly contradictory findings were put in a new perspective by the demonstration of ACPA of the IgM isotype (6) and local ACPA production in the inflamed RA joint (25), which strongly suggests a local, antigen-driven B cell response. Collectively, these data raised the hypothesis that distinct citrullinated proteins present in the inflamed synovium are involved in the induction and/or perpetuation of ACPA responses, whereas citrullinated (pro)filaggrin is probably a cross-reactive substrate.
Identification of citrullinated proteins in RA synovium

The first step to address this hypothesis was the demonstration of the presence of citrullinated proteins in RA synovium. Immunoblotting experiments provided the first evidence of the presence of several deiminated proteins in synovial tissue extracts (26, 27): focusing on proteins strongly recognized by ACPA, Serre and coworkers demonstrated the abundant presence of deiminated α- and β-chains of fibrin and co-localized extracellular/interstitial fibrin deposits with anti-citrulline immunostaining. A follow-up study showed that the citrullinated fibrin is not specific for RA synovium but is also found in SpA and inflamed OA synovium (26). Other synovial citrullinated proteins have also been detected in these immunoblotting experiments (27). One of these could be the ‘Sa antigen’ originally described in extracts from human placenta, spleen, and RA synovium (28). Later, it was shown that the placental ‘Sa antigen’ corresponds to citrullinated vimentin and that the RA-specific anti-Sa antibodies recognize citrullinated (but not unmodified) vimentin in vitro (29).

Other possible citrullinated synovial proteins include fibronectin, alpha-enolase, EBNA-1, and nuclear proteins. Fibronectin colocalizes with citrulline reactivity on parallel stainings of consecutive sections and on 1D-western blotting after immunoprecipitation of fibronectin (30) but, as fibronectin can crosslink with fibrin (31, 32), these results need to be confirmed by identification of the citrulline containing epitopes. Alpha-enolase, which is recognized in its in vitro citrullinated form by RA sera, can be found in the same regions of the synovium as anti-citrulline staining, but Western blotting of immunoprecipitates from synovial cells failed to confirm in vivo citrullination of alpha-enolase (33). Similarly, it has been demonstrated that ACPA bind specifically to the deiminated EBNA-1 protein encoded by Epstein-Barr virus, but the presence of this deiminated protein in RA synovium remains to be demonstrated (34). Finally, immunohistochemical analysis of RA and control synovium also reveals nuclear staining (26, 27), suggesting that nuclear proteins such as citrullinated histones might also be present (35, 36).

Citrullinated proteins at other sites of inflammation

The common occurrence of protein citrullination in different forms of joint inflammation raises the question of whether this posttranslational modification is more generally associated with inflammation. Immunohistochemical analysis in RA-associated interstitial fibrosis revealed that citrullinated proteins are present in
about half of the RA lung samples whereas lung tissue from normal controls showed almost no staining (37). However, citrullinated proteins were also detected in half of the cases of idiopathic interstitial pneumonia, with no significant difference in the amount or pattern compared to RA associated pneumonia. In contrast to bronchoalveolar lavage cells (22), there was no association between smoking status and pulmonary citrullination in lung tissue. A recent study also demonstrated the presence of citrullinated proteins in other inflamed tissues such as muscle in polymyositis, gut mucosa in Crohn’s disease, and tonsils in chronic tonsillitis (38).

The central nervous system (CNS) is of particular interest since, similar to the joint, it is usually isolated from the peripheral immune system and can form the target of a MHC class II restricted autoimmune attack in multiple sclerosis (MS). In chronic and fulminating forms of MS the citrullination of proteins such as myelin basic protein (MBP) (39, 40) and glial fibrillary acidic protein (GFAP) (41) is increased in the brain and spinal cord. As citrullination results in proteins with more loose secondary structures (42), deimination of myelin proteins can lead to less compact myelin which is more rapidly degraded by the proteinase cathepsin D (43, 44). This may in turn enhance the release of antigenic peptides (45) and thus propagate an autoimmune response as observed in experimental autoimmune encephalitis in mice (46). Interestingly, citrullinated proteins are not only found in pathological conditions but also in the non-diseased CNS. Indeed, 6 of 19 arginines are deiminated in the C8 isoform of MBP (47-49) in the normal developing brain (39) and deiminated GFAP was also evidenced in normal human brain tissue (41). Therefore, the CNS illustrates well that protein deimination occurs also in non-RA inflammation as well as in physiological situations.

Is citrullination enough to trigger ACPA responses?

The widespread presence of citrullinated proteins in a variety of pathological but also physiological (36, 50, 51) conditions clearly indicates that protein deimination as such is not specific for RA synovitis. The more fundamental issue raised here, however, is the striking contrast between the ubiquitous presence of citrullinated proteins and the RA-specificity of ACPA, since ACPA have for instance not been found in MS (Figure 1). Although it is clear from immunoblotting experiments that not all citrullinated proteins are specifically recognized by ACPA (27), even the presence of citrullinated proteins with proven antigenic affinity for ACPA, such as citrullinated (pro)filaggrin in the skin, is not sufficient to initiate the antibody response (52) Moreover, even the presence of such deiminated proteins in a suitable inflammatory milieu is not sufficient to break tolerance, since citrullinated fibrin
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Figure 1: Schematic representation of the discrepancy between the ubiquitous presence of citrullinated proteins in a variety of pathological as well as physiological conditions and the highly rheumatoid arthritis (RA) specific presence of anti-citrullinated protein antibodies (ACPAs). Potential explanations for this discrepancy include: 1) the requirement of an appropriate MHC class II background (HLA-DR4 shared epitope), 2) the amount of citrullinated antigen, and 3) the presence of RA-specific citrullinated epitopes. Such RA-specific citrullinated epitopes have been described in inflamed synovium but their exact biochemical identity is still under investigation.

is present in the inflamed synovium of patients with other chronic inflammatory joint diseases besides RA (26). Thus, citrullination of proteins, even in inflammatory conditions, is not sufficient to trigger the RA-specific ACPA response.

Restriction elements in the ACPA response towards ubiquitous citrullinated proteins?

In other conditions where a highly specific immune response is directed towards an ubiquitous antigen, such as the anti-dsDNA antibodies in systemic lupus erythematosus, experimental models have demonstrated that the balance between tolerance and autoimmunity is controlled by a multitude of complex mechanisms which fall out of the scope of the present review. A similar analysis for ACPA-related autoimmunity is still hampered by the absence of an appropriate experimental model of ACPA induction (53, 54) but human data have pointed to two interesting mechanisms which could be involved in the restriction of the immune response
towards citrullinated proteins. Firstly, it is known from other immune-mediated diseases that not only the presence but also the amount of antigen presented to the immune system determines the immune response by breaking a putative tolerance-threshold (55) (Figure 1). Based on an association between PADI4 polymorphisms and RA in a Japanese population, Suzuki et al. proposed that increased PAD4 mRNA stability in RA could lead to an increase in the expression of the deiminating enzyme, which in turn could augment protein citrullination and contribute to breaking the tolerance threshold (56). However, this genetic association is still controversial (57-59) and the hypothesis is challenged by 1/ the lack of direct evidence for an increased amount of PAD4 protein (52) or mRNA in RA, 2/ the fact that citrullinated proteins such as fibrin are found in equal amounts in RA and control synovium (26), and 3/ the absence of a link between PADI4 haplotypes, synovial citrullinated proteins, and ACPA (52, 59-61).

A second mechanism which may contribute to the restriction of the antibody response is the requirement for a specific MHC background, as suggested by the higher ACPA levels in HLA-shared epitope positive RA patients (62-64) and the prominent gene-environment interaction between shared epitope and smoking in ACPA positive but not ACPA negative RA patients (22) (Figure 1). One caveat here is that some RA patients who lack the HLA-shared epitope still develop high ACPA titers, but this could be explained by the presence of other permissive MHC molecules such as HLA-DRB1*1501 (65). A more important drawback is that patients with spondyloarthritis and psoriatic arthritis, who have citrullinated proteins such as deiminated fibrin in their synovial membrane (26), do not develop ACPA despite the presence of the HLA-DR shared epitope in almost half of these patients (66). Therefore, it is clear that the HLA-DR shared epitope is involved in the ACPA response but that the MHC background alone does not explain the specificity of ACPA for RA.

**Defining autoepitopes**

The link between ACPA and MHC class II strongly suggests that the presentation of specific peptides by HLA-DR to CD4+ T lymphocytes and the resulting T cell activation play an important role in the ACPA response (Figure 2). The in vitro conversion of arginine into citrulline at the peptide side-chain position interacting with the HLA-shared epitope significantly increased peptide-MHC affinity and led to the activation of CD4+ T cells in DR4-IE transgenic mice (67). However, the higher affinity for HLA-DR4 was demonstrated only with one citrullinated epitope of vimentin and could not be reproduced using a wide panel of peptides derived from fibrin (68). Moreover, T cell responses were directed towards both citrullinated
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Whereas further research is needed to resolve the discrepancies with regard to HLA-DR4 binding, these data point towards two important issues: 1) activation of T lymphocytes could be a crucial step in the loss of B cell tolerance towards citrullinated proteins and, 2) whereas citrullination is crucial for B cell/antibody reactivity, it is uncertain whether citrullination is required for the T cell epitope in order to provide adequate T cell help for the humoral autoimmune response (Figure 2). A citrullinated protein recognized by ACPA and containing the correct deiminated B cell epitopes and non-citrullinated fibrin epitopes and were detected in RA as well as in control arthritides (68).

Figure 2: Schematic representation of how citrullinated proteins could be involved in the pathogenesis of rheumatoid arthritis. Citrullinated proteins are recognized by autoreactive B cells, which subsequently can differentiate to plasma cells producing anti-citrullinated protein antibodies (ACPAs). Recent data of an experimental model indicate that these ACPAs can enhance arthritis, but it remains to be proven that ACPAs can also induce disease. The linkage of ACPA levels with the HLA-DR shared epitope and the isotype of the ACPA strongly suggest that the autoreactive B cells require appropriate T cell help. However, this requirement as well as the exact specificity of these T cells (towards citrullinated or non-citrullinated epitopes of the citrullinated protein?) remain to be formally demonstrated. Insights from other autoimmune models suggest that a coordinated activation of both autoreactive B and T lymphocytes may be required to lead to pathology. Whereas circumstantial clinical evidence support this hypothesis, the development of new experimental models for citrulline-related autoimmunity will be crucial to address these issues formally.
but lacking appropriate T cell epitopes (deiminated or not) may fail to induce a strong ACPA response. Indeed, different immunogenic peptides yield quantitatively and qualitatively different autoimmune responses, a phenomenon which appears to be dependent on the antigen-specific interaction between autoreactive T and B cells rather than on the amplitude of the lymphocyte activation as such (69-72). So called autoepitopes appear to have the unique property of promoting self-perpetuating interactions between B and T lymphocytes and reciprocal T-B cell diversification in a variety of autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis and celiac disease (73-77). Thus, the ability to induce a strong and diversified autoimmune response is a specific property of a well-defined autoepitope. In the case of anti-citrulline reactivity in RA, these autoimmune responses may vary widely between different citrullinated epitopes and the presence of such specific autoepitopes rather than citrullination of proteins as such may be crucial to initiate the ACPA response.

Citrullinated B cell epitopes

Although we lack the appropriate experimental models to test these concepts relating to ACPA responses in RA, it is well known that in vivo ACPA are directed against various citrullinated epitopes in which not only the citrulline residue but also the flanking amino acids make up the antibody recognition site (4, 5). This is illustrated by the fact that different citrullinated substrates yield different ACPA sensitivities and specificities for RA when used in the same patient cohorts (63, 78). The anti-citrullinated protein antibodies produced in vitro are also epitope-specific and recognize a distinct subset of citrullinated peptides, as illustrated by the different staining patterns in an immunohistochemical study using different anti-citrulline reagents (79) and by the fact that monoclonal antibodies raised against in vitro citrullinated proteins only rarely cross-react with other citrullinated proteins (DB, unpublished data). As is the case for myelin oligodendrocyte glycoprotein in EAE and MS (80), the citrullinated epitopes may even be conformation dependent and thus loose their reactivity in certain in vitro conditions.

In most of the previously mentioned studies, researchers used the commercially available anti-modified citrulline antibody for the detection of citrullinated proteins in synovial tissue. This antibody detects all citrullinated proteins irrespective of the amino acid context and the structural conformation, because the chemically modified citrulline is voluminous and shields neighbouring amino acids (81). As indicated, however, the challenge at this point is not to identify all synovial citrullinated proteins nor even to identify those citrullinated proteins that bind ACPA with high
affinity and specificity, but rather to identify those selected deiminated autoepitopes that are responsible for the specific induction of ACPA in RA. One way to approach this complex problem is to screen defined citrullinated epitopes for specific disease associations in human RA.

RA-specific citrullinated epitopes as an alternative hypothesis

We have previously demonstrated the presence of distinct intracellular citrullinated proteins in RA synovium using a rabbit polyclonal antibody raised against poly-L-citrulline (82). Several follow-up studies, including a study with a monoclonal mouse antibody, confirmed these findings and ruled out the detection of free citrulline (52, 66, 83, 84). Four observations point to a particular relevance of the detected citrullinated targets for RA: 1) they are highly specific for RA, being present in about half of RA synovia but none of the controls; 2) they colocalize with PAD2, which in contrast to PAD4, appears to be overexpressed in RA synovium and could thus at least partially explain the RA-specificity of the intracellular citrullinated proteins (52); 3) they also colocalize with ACPA reactivity in RA synovium, suggesting that they can be recognized by the autoantibodies (82); and 4) in contrast to other citrullinated proteins (26, 79), the presence of the RA-specific intracellular citrullinated proteins determines the local production of ACPA in a HLA-DR shared epitope restricted manner (52, 60) (Figure 1). Although the biochemical identity of these proteins is still under investigation, these data suggest that, despite the ubiquitous presence of citrullinated proteins in physiological and pathological conditions, the restricted presence of well-defined citrullinated epitopes in RA synovium may contribute to the highly specific induction of ACPA in this disease. These data also suggest that PAD2 rather than PAD4 may be relevant for the deimination of these well-defined epitopes. In contrast with PADI4, functional haplotypes for PADI2 have not yet been investigated.

Implications for the pathophysiology of RA

If well-defined citrullinated autoepitopes rather than merely ubiquitous citrullinated proteins are relevant for the induction of ACPA, the identification of these epitopes could provide crucial insights into the pathophysiology of RA. Indeed, it remains an open question if anti-citrulline autoreactivity in RA is primarily involved
in the pathogenesis, results in secondary enhancement and/or perpetuation of inflammation, or is simply a bystander phenomenon.

Although original reports failed to demonstrate specific anti-citrulline responses in different arthritis models (53, 54), there is now emerging evidence that ACPA may contribute to the enhancement of arthritis. Immunization with deiminated collagen rather than native collagen slightly aggravates collagen-induced arthritis (CIA) in mice (85). More importantly, tolerization by intravenous administration of a citrulline-modified peptide before collagen immunization reduces disease severity compared to a control peptide and ovalbumin, and passive transfer of monoclonal antibodies specific for citrullinated fibrinogen in combination with anti-CII antibodies enhances disease severity compared to anti-CII antibodies alone (86). Although further control experiments with antibodies to non-citrullinated fibrinogen should confirm the specificity of this observation, these data demonstrate for the first time that ACPA can enhance tissue injury in CIA. In contrast, however, antibodies against citrullinated fibrinogen failed to enhance adjuvant-induced arthritis in Lewis rats (87).

Whereas these emerging data show that ACPA could enhance disease severity in arthritis models, there is still no evidence for a primary role of citrullinated proteins and/or ACPA in the induction of the arthritis. Indeed, immunization with citrullinated proteins such as fibrinogen failed to induce arthritis, even in HLA-DR4 transgenic animals, although they were clearly immunogenic (87, 88). Accordingly, transfer of ACPA alone failed to induce arthritis in DBA/1 mice (86). Interpreting these data from the perspective of the recent human findings, it is not surprising that citrullinated proteins as such are not pathogenic. Consistent with the observation of RA-specific citrullinated epitopes in human synovium (52, 82) and of the critical quantitative and qualitative impact of specific epitopes on the immune response in other autoimmune models (69), it is likely that well-defined citrullinated autoepitopes in a background allowing optimal and coordinated immune reactivity will be required to assess the real pathogenic potential of anti-citrulline immunoreactivity (Figure 2).

Conclusions

Increasing circumstantial evidence from clinical research supports the concept that ACPA define a separate pathophysiological entity within RA and that citrullinated proteins may be involved in the induction of this process. Major progress has been made in the detection and identification of citrullinated proteins as potential targets for ACPA in inflamed synovium as well as other tissues. Importantly, however, it becomes clear that well-defined citrullinated epitopes rather than the mere presence
of citrullinated proteins as such may be relevant for the induction of ACPA and, eventually, for the pathogenicity of anti-citrulline reactivity. Therefore, defining the clinically relevant citrullinated epitopes and experimentally assessing the requirements for optimal and coordinated immune activation by these epitopes are two major challenges in this field.

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CHAPTER 7

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