

SUPPLEMENT

Autoimmunity in rheumatoid arthritis: different antigens—common principles

L A Trouw, Tom W J Huizinga, René E M Toes

Department of Rheumatology,
Leiden University Medical
Center, Leiden,
The Netherlands

Correspondence to

Professor Dr René E Toes,
Department of Rheumatology,
C1-R, Leiden University
Medical Center, PO Box 9600,
Leiden 2300 RC,
The Netherlands;
R.E.M.Toes@lumc.nl

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ABSTRACT

Since the identification of antibodies directed against citrullinated protein antibodies (ACPA) as specific serological markers for rheumatoid arthritis (RA) the insight into the pathogenesis of RA has made significant progress. It is now realised that RA does not represent one disease entity, as ACPA-positive and ACPA-negative RA differ in several important aspects. For example, the most prominent genetic risk factors, the human leucocyte antigen shared epitope alleles only predispose to ACPA-positive RA, but not to ACPA-negative disease. Likewise, ACPA-positive RA is characterised by a more severe disease course as well as by a lower chance to enter drug-free remission. More recently, it became apparent that next to ACPA, another autoantibody system is also present in RA patients that is directed against a structurally similar determinant. These antibodies recognise carbamylated proteins and thus are called anticarbamylated protein (anti-CarP) antibodies. During carbamylation lysine residues are post-translationally modified to a homocitrulline, the antigenic identity crucial for recognition by anti-CarP antibodies. In this review we will discuss novel insight into the immune responses directed against citrullinated and carbamylated proteins.

INTRODUCTION

Antibodies represent an important and powerful entity of the immune defence. Unfortunately, next to protective antibodies, some individuals also produce self-reactive antibodies that can contribute to unwanted tissue damage. Such self-reactive antibodies can be directed against an array of molecules that are normally present in the body. In particular, antibodies against 'altered self' have received increasing attention in relation to several autoimmune diseases. 'Altered self' is generated as a result of, for example, modifications of self proteins by chemical or enzymatic modifications after the proteins have been produced. These post-translational modifications may become the target of an antibody response that can also attack otherwise healthy tissue when the relevant post-translational proteins are expressed at certain sites in the body. Although protein modifications also occur during normal cellular physiology, these modifications are not 'visible' to the immune system. In this review we consider 'altered self' as these post-translational changes that do become 'visible' to the immune system in a way that normally does not occur. An immune response is likely to be initiated only against those post-translationally modified proteins that are not presented for negative

selection. For example, physiological phosphorylation or citrullination may therefore not initiate an immune response, whereas when the same modification becomes aberrantly presented to the immune system, it may induce an immune response.

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly affects the joints.¹ A large proportion of RA patients is characterised by the presence of antibodies directed against post-translationally modified proteins, especially proteins that have been citrullinated.² The antibodies against such proteins are called anticitrullinated protein antibodies (ACPA) and recognise proteins only when they contain the modified, 'non-encoded', amino acid citrulline.^{3–4} Citrulline is generated by the enzymatic conversion of the amino acid arginine by peptidyl arginine deiminase (PAD)⁵ (figure 1). The presence of ACPA can be detected by several commercially available laboratory tests designed to detect as many ACPA as possible. The first assay brought to the market uses a cyclic citrullinated peptide (CCP) and therefore the antibodies reacting in this assay are called anti-CCP antibodies. Nonetheless, ACPA and anti-CCP antibodies are directed against the same antigens, as anti-CCP antibodies recognise several citrullinated proteins that are also expressed in the inflamed joint such as citrullinated vimentin and citrullinated fibrinogen.^{6–10}

The presence of ACPA in RA patients is not only associated with a more severe disease course in RA, but is also predictive for the development of RA in patients with undifferentiated arthritis or arthralgia.^{11–13} The pathways leading to the development of ACPA are not known, but interestingly, the most prominent genetic risk factors for RA development, such as the human leucocyte antigen (HLA) shared-epitope (SE) alleles or PTPN22, are specifically predisposed to the development of ACPA-positive RA.¹⁴ Likewise, the most prominent environmental risk factor, smoking, also predisposes to the development of ACPA-positive RA and not to the development of ACPA-negative disease.^{15–16} These findings suggest that these risk factors predispose to the development of the citrulline-specific autoimmune response that putatively underlies the disease pathogenesis of ACPA-positive arthritis.

Next to citrullination several other forms of post-translational modifications have also been described to occur.¹⁷ One of these post-translational modifications is mediated in a process named carbamylation and leads to the formation of homocitrulline.¹⁸ Structurally, homocitrulline greatly resembles

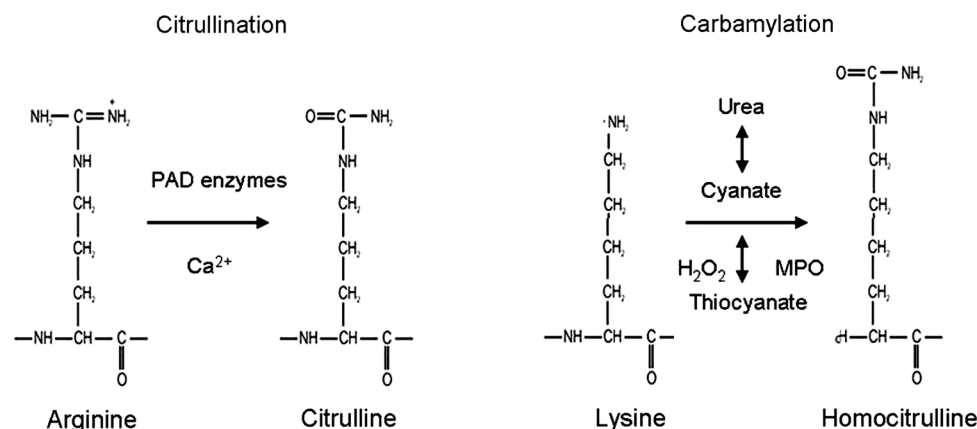


Figure 1 Schematic overview of the post-translational modifications citrullination and carbamylation. The process of citrullination modifies an arginine (R) present in the amino acid sequence of a protein into a citrulline, whereas the process of carbamylation modifies a lysine (K), into a homocitrulline. Citrullinated proteins are recognised by anticitrullinated protein antibodies, whereas carbamylated proteins are recognised by anticarbamylated protein antibodies. The process of citrullination is mediated by enzymatic conversion by peptidyl arginine deiminase (PAD) enzymes, whereas the process of carbamylation is a chemical reaction driven by cyanate. H₂O₂, hydrogen peroxide; MPO, myeloperoxidase.

citrulline but is one methylene group longer. Citrulline is generated when PAD enzymes modify the amino acid arginine. In contrast, the amino acid homocitrulline is generated by a chemical reaction in which cyanate reacts with the amino acid lysine (figure 1). Arginine and lysine are located at different positions in the amino acid sequence of proteins, and therefore these modifications occur at different positions in proteins with different flanking amino acids. Intriguingly, homocitrulline residues can also be recognised by autoantibodies, but these mostly do not crossreact with citrulline.

In this review we will highlight recent advances in the understanding of immune responses against citrulline and homocitrulline-containing proteins in the context of RA.

MATURATION OF THE ACPA RESPONSE

Antibody responses against protein antigens are initially characterised by a wave of antigen-specific IgM followed by an IgG response that has undergone affinity maturation leading to a better clearance of the invading pathogen. Upon subsequent antigen exposure, affinity maturation as well as isotype switching continues to take place further shaping the extent and efficacy of the immune response. Although these general aspects apply to most antibody responses induced by, for example, vaccination, the development and maturation of the ACPA response seems not to adhere to these common principles as became apparent over the past decade.¹⁹

ACPA can recognise a diverse set of proteins when they are citrullinated (figure 2). ACPA are highly crossreactive as ACPA isolated after binding to a particular citrullinated protein, such as citrullinated fibrinogen, will also react to other citrullinated proteins.⁶ Nonetheless, within the ACPA repertoire, different fine specificities are present as became apparent when the reactivity to different citrullinated peptides was analysed.^{4 20} These findings offered hope of the possibility to use the ACPA fine specificities as a biomarker to foretell disease development in the ACPA-positive pre-RA population, or to predict disease progression and/or response to therapy. Although ACPA can be detected many years before the onset of symptoms of RA,^{21 22} emerging data indicate that it is unlikely that predictive algorithms on the basis of the recognition of specific citrullinated epitopes will be helpful, as studies performed in different populations revealed that parallel to a rise in the ACPA titre no

specific reactivity could be identified that associated with the precipitation of disease.^{23–25} Likewise, clustering of these reactivities does not seem to provide additional information regarding the clinical phenotype of RA, for example, with respect to the disease activity score, swollen or tender joint counts^{26 27} or regarding the prediction of disease evolution in the pre-RA stage such as is present in patients with ACPA-positive arthralgia.²⁴ Also, current data do not indicate added value of ACPA fine specificity testing in the prediction of radiological progression in the RA population. For example, it is known that the HLA-SE alleles shape the ACPA fine specificity repertoire as several ACPA reactivities are enhanced in ACPA-positive RA patients who harbour HLA-SE alleles.^{20 28 29} Nonetheless, within the ACPA-positive RA patients, neither the presence of HLA-SE alleles (as a proxy for a different ACPA fine specificity

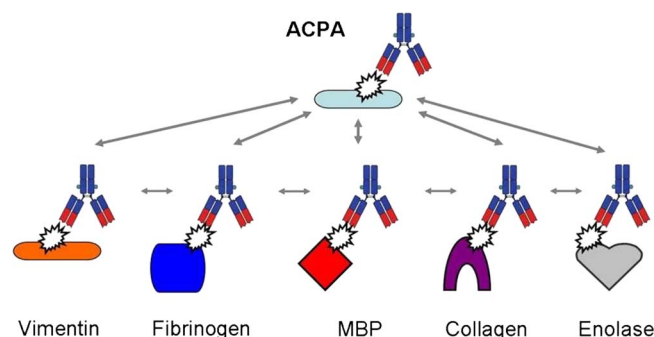


Figure 2 Anticitrullinated protein antibodies (ACPA) are a collection of (partly) crossreactive antibodies recognising citrulline-containing proteins and peptides. The ACPA response, often detected in assays capturing the vast majority of ACPA such as the cyclic citrullinated peptide (CCP) assay, can be divided into several (partly) crossreactive fine specificities. Overall, ACPA captured by a citrullinated protein (e.g., citrullinated fibrinogen) crossreacts with other citrullinated proteins (e.g., citrullinated vimentin). Presumably, these proteins contain multiple citrullinated residues. Reactivity to a specific citrullinated peptide shows a more private recognition pattern, although these reactivities can also be partly crossreactive. At present it is not known whether the reactivity captured by CCP peptides or peptides with sequences derived from citrullinated proteins reflect the ability of these ACPA to recognise citrullinated proteins present in the inflamed joint, although often otherwise speculated. MBP, myelin basic protein.

repertoire) nor the presence of defined ACPA reactivities was predictive of a different rate of joint damage.^{30 31} Similarly, current data suggest that response to therapy is also not influenced by the presence of specific ACPA fine specificities, although more research is needed to substantiate this notion.³² Nonetheless, the studies into the ACPA recognition repertoire has brought important insights into the development of the ACPA response by showing that the ACPA recognition profile broadens before the onset of clinical symptoms, but stabilises after RA has developed, indicating that before disease onset, an active and ongoing immune response is present resulting in a higher level of ACPA antibodies that is associated with a broader recognition of citrullinated antigens.^{2 19}

Next to the fine specificity of the ACPA response, the isotype usage of this reaction has also been analysed extensively. By now all isotypes have been shown to be used in the ACPA response, including IgM, IgA, all IgG subclasses and IgE.^{33 34} Similar to epitope recognition, also for isotype usage the overall view is that parallel to an increase in ACPA levels, an increase in the number of isotypes used by ACPA is observed before the development of full-blown RA. Once RA is established, no changes in isotype usage by ACPA seem to be present.^{34 35}

Nonetheless, the isotype usage of the ACPA response is remarkable as even in patients with over 10 years of ACPA-positive RA IgM ACPA next to IgG ACPA can still be found.^{34 36} Because of the short half-life of IgM (approximately 7 days) and the notion that IgM antibodies directed against protein antigens are mainly produced by new B cells recruited from the naive B-cell repertoire, these observations suggest that the ACPA response is continuously fuelled by new antibody responses directed at ACPA.^{34 36} The observation that the presence of IgM ACPA can go hand in hand with the presence of IgG ACPA is also intriguing given the switch of IgM production to IgG production that is normally observed following repeated antigen exposure. This latter phenomenon is explained by the higher avidity of IgG for antigen upon affinity maturation, and is also used in the clinic through the infusion of anti-rhesus IgG to rhesus-negative women giving birth to a rhesus-positive child to prevent the development of anti-rhesus responses. Therefore, these observations suggest that the avidity maturation of the ACPA response is atypical allowing the recruitment of new B cells into the ACPA response despite the presence of high ACPA IgG titres. Indeed, the ACPA IgG response is overall characterised by a low avidity especially when compared to 'conventional' antibody responses against recall antigens in the same patients.³⁷ The reason for the low avidity of the ACPA response is currently unknown, but may be related to the abundance of autoantigens. Avidity maturation is dependent on competition for a limited source of antigen, and therefore the abundant presence of antigen, such as might be the case for autoantigens like citrullinated proteins, is unlikely to result in fierce competition for a survival signal to the B-cell receptor and thus an overall increase in the avidity of the antibody response. Similar to the observations made for the isotype usage and the number of epitopes recognised, the limited avidity maturation that occurs in the ACPA response also takes its final shape before the onset of symptoms.³⁸ In established RA, the avidity of the ACPA response remains stable, conceivably when antigen presence is most abundant. Whether the absence of proper avidity maturation and the continuous presence of IgM ACPA together with IgG ACPA are related is currently not known. However, it is tempting to speculate that because the IgG antibodies are of low avidity they do not capture antigens sufficiently to prevent these antigens from

binding to and activation of naive IgM ACPA-producing B cells. Therefore, together, evidence is emerging that the biology and evolution of the ACPA response is different from the evolution of 'conventional' antibody responses as only limited avidity maturation is observed despite the presence of abundant isotype switching. Likewise, most events affecting the composition/nature of the ACPA response (avidity, level, isotype usage and fine specificity) take place before the onset of clinical symptoms whereafter it stabilises.

ANTIBODY RESPONSES AGAINST HOMOCITRULLINE-CONTAINING PROTEINS

As outlined above, ACPA recognise proteins that contain citrulline residues that have been generated through the action of PAD. Next to citrulline, other post-translational modifications might also be recognised by autoantibodies. Recently, we became especially interested in carbamylated proteins as these proteins contain a homocitrulline that has been generated through a chemical reaction in which lysine is converted into a homocitrulline through the action of cyanate. Cyanate, necessary for carbamylation, is naturally present in the body and in equilibrium with urea.¹⁸ Under physiological conditions the urea concentration may be too low to allow extensive carbamylation, but nonetheless, carbamylation is a process that occurs throughout the body also under physiological conditions. In particular, proteins with a long half-life are therefore likely to become carbamylated during their 'life time'. Smoking, the most prominent environmental risk factor for RA, is also an environmental factor that enhances carbamylation by increasing the cyanate concentration.³⁹ Likewise, in conditions of renal failure, the urea concentration increases and carbamylation of several proteins can be readily detected.⁴⁰ However, most carbamylation is believed to take place during inflammation when myeloperoxidase is released from neutrophils.^{39 41} This enzyme strongly shifts the equilibrium of thiocyanate towards cyanate, now allowing extensive carbamylation to occur.³⁹ Therefore, it is conceivable that carbamylation is a naturally occurring process in the inflamed synovium of RA patients.

Our interest in carbamylated proteins originated from the hypothesis that ACPA would also recognise carbamylated proteins because of the high resemblance between citrulline and homocitrulline. Much to our surprise, however, the first experiments quickly showed that most ACPA did not recognise peptides in which the citrulline residue was replaced by a homocitrulline residue, indicating a high specificity of ACPA for citrullinated antigens. At the same time, some reactivity towards homocitrulline containing antigens was noted in some patients. For this reason, we developed a novel assay that specifically detects the presence of antibodies directed against carbamylated proteins (anti-CarP).⁴² Using this assay it was revealed that RA patients, next to the well-known ACPA, also harbour anti-CarP antibodies. Interestingly, anti-CarP antibodies were observed both in ACPA-positive and in ACPA-negative individuals and vice versa,⁴² further emphasising that antibodies against one modification do not necessarily crossreact with the other modification. Also inhibition studies confirmed that ACPA and anti-CarP antibodies are largely non-crossreactive antibody systems.⁴³ Importantly, we observed that the presence of anti-CarP antibodies in RA patients was associated with a more severe disease course, as detected by a higher rate of joint damage. This was most prominent in the ACPA-negative subgroup, a group of patients for whom so far no good serological markers are available. The observations

Clinical and epidemiological research

presented above not only indicate the presence of another auto-antibody system in RA patients, but also suggest that the population of RA patients is more heterogeneous than anticipated as, perhaps, next to ACPA-positive disease, anti-CarP-positive RA might also represent an additional disease entity with its own genetic contributions. Clearly, additional studies should be performed to determine whether this notion is, indeed, a reality.

Anti-CarP immune responses are not only restricted to humans, but can also be induced in mice and rabbits by vaccination with carbamylated proteins.^{44 45} As the possible ability of anti-CarP antibodies to contribute to arthritis induction or severity is currently not known, studies in appropriate animal models could be very helpful in answering this outstanding question. Similarly, as also indicated above, additional studies performed with human material derived from different cohorts and biobanks should be performed to determine, for example, whether certain HLA alleles, genetic risk factors or environmental triggers predispose to the formation of anti-CarP antibodies, whether the presence of anti-CarP antibodies predisposes to the development of RA in individuals at risk of developing RA, whether the presence of anti-CarP antibodies influences the risk of achieving (drug-free or drug-induced) remission, whether the presence of anti-CarP antibodies can aid the treatment decisions in ACPA-negative undifferentiated arthritis patients, as well as whether anti-CarP antibodies are specific for RA or can also be found in other rheumatic disorders. These studies should also include investigations into the presence of carbamylated proteins in the joint that could serve as a target for anti-CarP antibodies as well as investigations into the evolution of the anti-CarP response much like the studies performed in the ACPA field.

CONCLUSIONS

The identification of ACPA as a serological marker for RA has had a great impact on the diagnosis and prognosis of RA, as also substantiated by the inclusion of ACPA status into the new criteria for RA established in 2010.⁴⁶ The subgrouping of RA into ACPA-positive and ACPA-negative RA enabled significant progress in the understanding of the pathogenesis of RA. With the identification of anti-CarP antibodies, recognising a highly homologous antigenic entity, similar progress may be achieved to further the understanding of disease pathogenesis, but potentially also to characterise ACPA-negative patients or individuals at risk of developing RA. Particularly for these individuals, new serological markers, either alone or in combination with other serological markers, may provide useful information regarding the future development of disease. However, it is clear that this attractive horizon is not yet in sight as we are only at the beginning of understanding the meaning and value of the anti-CarP response, and are therefore awaiting future studies that hopefully bring fresh encouragement to understand RA pathogenesis.

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