Rheumatoid arthritis

Unlocking the ''PAD'' lock on rheumatoid arthritis

P J Utz, M C Genovese, W H Robinson

Perhaps a panel of antigens containing citrulline may explain RA: many questions remain

Rheumatoid arthritis (RA) affects about 1% of the world population, yet the driving antigen(s) remain unidentified. A number of different antigens have been proposed to have a fundamental role in RA, including antibodies bound by rheumatoid factor (RF), collagen, BiP, Sa, and many others (reviewed by Rantapää-Dahlqvist *et al*[1]). The field has been revolutionised by the discovery that antigens containing arginine residues that have been deiminated to form citrulline are prominent targets of autoantibodies in RA.[2 3] Their detection forms the basis of several assays that are now standard diagnostic tests in rheumatology clinics world wide. The goal of this editorial is to provide a brief history of the discovery of citrullinated antigens in RA, a review of what is known about the enzymes (peptidylarginine deiminases, PADs) involved in the catalysis of a reaction that forms citrulline, and a roadmap of future areas for research.

HISTORY OF CITRULLINATED ANTIGENS

The history of citrullinated antigens is one that is particularly important for students of rheumatology to review, because the initial discovery was largely overlooked and rediscovered on several different occasions over the ensuing four decades. In 1964, in the *Annals of the Rheumatic Diseases*, a search for cell and tissue substrates containing antigens that could be bound by autoantibodies from patients with RA found that granules from differentiating buccal mucosal cells expressed such an autoantigen. The autoantibody system was termed ''antiperinuclear factor''.[4] A similar screen performed 15 years later showed that rat oesophagus was an ideal and more easily studied substrate for detection of these serum autoantibodies, and these were named ''antikeratin antibodies''.[5] This work went largely unnoticed until nearly another 20 years had passed, when two European groups independently demonstrated that the target of both antiperinuclear factor and antikeratin antibodies was a post-translationally modified antigen (filaggrin) containing citrulline residues.[2 3] Other studies have suggested that citrullinated antigens such as filaggrin, vimentin, and fibrin are physiological candidate targets in RA (reviewed by van Boekel *et al*[6]).

SERUM ANTIBODIES AS PREDICTORS OF RA

Three recent papers, including one in this issue of the *Annals*,[7] have added further insights into the role played by PAD enzymes and citrullination in RA pathogenesis. Rantapää-Dahlqvist and colleagues analysed the predictive value of anti-cyclic citrullinated peptide (CCP) antibodies in a cohort of patients with RA who had donated blood years before the development of RA.[1] In addition to anti-CCP antibodies, IgG, IgM, and IgA RF were also measured in 83 people who had donated blood before disease onset. The prevalence both of anti-CCP autoantibodies and IgA RF was 33.7%, with a lower prevalence observed for IgM and IgG RF. For donors who had provided serum $\leqslant 1.5$ years before any symptoms of RA, the sensitivity of the anti-CCP assay was 52%. It is interesting to note that serum autoantibodies were detectable in a few patients as long as 9–22 years (CCP and RF, respectively) before disease onset.

> ''Diagnostic autoantibodies precede the onset of RA by years''

Antibody titres increased over time in almost all people. These studies clearly demonstrate that anti-CCP antibodies precede the onset of RA by over a year. These results further imply that this post-translational modification may lead to the creation of a neoepitope that drives pathogenic autoreactive T and B cells. Although this discovery will not lead to large scale screening of patients outside rheumatology clinics, and is unlikely to lead to therapeutic interventions in patients lacking symptoms, it may represent an important breakthrough in the pathogenesis of RA.

Studies of autoantibodies in other autoimmune diseases demonstrate why the discovery of anti-CCP antibodies in patients before disease onset is of critical importance. Li and colleagues demonstrated in an elegant study that serum derived from family members of patients with pemphigus foliaceus contained antibodies directed against syndesmoglein.[8] Antibodies in non-affected family members recognised the carboxyl (COOH) portion of the molecule, in stark contrast with antibodies from afflicted patients which recognised the amino (NH_2) terminus. Over time, a subset of non-afflicted patients developed disease, and this correlated with the development of antibodies that recognised both the NH_2 and the COOH terminus of syndesmoglein. As with the study of Rantapää-Dahlqvist,[1] this study showed that serum antibodies could serve as useful predictors of disease in people who lack symptoms but are at risk of disease. Similar findings have also been seen in systemic lupus erythematosus,[9] multiple sclerosis,[10] and insulin dependent diabetes mellitus.[11] Taken together, studies in many, but not all, diseases quite clearly demonstrate that diagnostic autoantibodies precede disease manifestations by years. An intriguing possibility is that citrullinated antigen(s) represent an early target of the immune response in RA, and that arthritis only develops when the T and B lymphocyte epitope spreading has reached as yet unidentified dominant epitopes. Work being performed in the laboratory of one of the authors (WHR) employing large scale RA antigen arrays may answer this question.[12–14] Autoantibody recognition may ultimately lead to more accurate diagnostic assays, and perhaps more targeted therapeutic treatments for individual patients.

Although only a handful of antigens containing citrulline residues have been identified, the enzymes that catalyse the deimination of arginine to form citrulline have been the subject of intense interest. The obvious hypothesis being tested is that unidentified antigen(s) present in the synovium are modified by one or more PAD enzymes, generating an immune response that ultimately leads to the clinical manifestations associated with RA. In this issue of the *Annals*, Vossenaar and colleagues address some important aspects of this hypothesis—namely, which PAD enzymes might have a role in RA, and which cells express these enzymes.[7] Four different PAD enzymes have been identified in humans. Of these, PAD2 and PAD4 are thought to be most relevant to RA because both are expressed in haematopoietic cells such

as macrophages, whereas PAD1 and PAD3 are largely found in skin. Vossenaar *et al* report that mRNAs encoding PAD2 and PAD4 can be easily identified by reverse transcriptase-polymerase chain reaction (RT-PCR) from CD14+ peripheral blood mononuclear cells. Upon differentiation into macrophages in vitro, only PAD2 mRNA remained detectable. Analysis at the level of proteins showed that PAD2 was detectable only in macrophages, whereas PAD4 was found in both monocytes and macrophages. This result demonstrates that PAD2 is regulated post-transcriptionally (supported by in vitro studies employing a luciferase gene regulated by the long 3′ untranslated (UTR) region derived from the PAD2 gene), and that PAD4 is likely to be a relatively long lived protein. Similar results were found when studying cells obtained from synovial fluid from patients with RA. No differences were observed when comparing peripheral blood mononuclear cells from normal patients and healthy controls, either at the level of mRNA or protein expression. One of the most elegant aspects of this study was the observation that cells expressing PAD enzymes did not contain citrulline modified antigens unless exogenous stimuli capable of markedly increasing intracellular calcium (a required cofactor for PAD activity) were applied to the cells. In fact, one known autoantigen containing citrulline, vimentin, was detected within 15 minutes of exposure to ionomycin. A unique cadre of citrullinated proteins was observed when comparing lysates prepared from monocytes and macrophages, adding yet another level of complexity to these already intriguing results.

> ''PAD2 and PAD4 are most likely to have a role in RA''

The third recent paper shedding light on citrullination and PAD enzymes took a genetic approach to identify genes that are linked to RA.[15] The genes encoding PAD enzymes are encoded on human chromosome region 1p36, within a previously identified RA susceptibility locus. Single nucleotide polymorphisms in this region were used to identify a haplotype associated with RA in PAD4 but not in any of the other three PADs. Sequencing of this gene in all patients identified two different haploytpes, an RA non-susceptible haplotype (haplotype 1) and an RA susceptible haplotype (haplotype 2). In vitro experiments performed using haplotype 2 mRNA demonstrated significantly increased stability of the transcript, suggesting that enhanced stability of PAD4 mRNA might account for the strong genetic association of haplotype 2 with RA. In light of the findings of Vossenaar *et al* in this issue, it is also possible that the PAD4 protein encoded by haplotype 2 is qualitatively different, rather than simply being quantitatively different, from the protein encoded by haplotype 1. For example, the calcium requirements, ability to interact with other regulatory molecules or subunits, subcellular localisation, or substrate specificity might be uniquely different between these two haplotypes.

QUESTIONS REMAIN

Although the RA ''PAD lock'' seems to have been identified, the door to full understanding of RA remains unopened, but unlocked. At least six questions remain unanswered and should be the focus of future RA research in the coming decade: (*a*) Which of the PAD enzymes are relevant to autoantigen citrullination, and where are they expressed? For example, are there subsets of CD14+ cells, or other relevant cell types, that might express PADs?; (*b*) Are PAD enzyme(s) dysregulated in patients at risk of developing RA? Careful analysis of the biochemical and functional properties of the proteins encoded by PAD4 haplotypes 1 and 2 is a logical place to begin such studies; (*c*) What role might microorganisms play in the citrullination of autoantigens, or PAD activation? Given the potential association of RA with antecedent infections, one might speculate that infectious agents could activate endogenous cellular PAD enzymes, could express biomolecules that are molecular mimics of citrullinated antigens, or could encode their own PADs or PAD-like enzymes within their genomes; (*d*) Can overexpression of PADs, or exposure to citrullinated antigens in a proinflammatory context, break tolerance and induce a disease resembling RA?; (*e*) What are the physiological stimuli that activate PAD enzymes? Clearly, chemicals such as ionomycin and thapsigargin can activate PAD activity leading to production of citrullinated autoantigens such as vimentin. Perhaps other stimuli such as binding of cytokines or chemokines to their respective receptors might activate PADs, or might induce PAD expression in vivo; (*f*) Most importantly, what are the relevant citrullinated antigens that drive RA? The results of Vossenaar *et al* in this issue strongly suggest that other citrullinated antigens will soon be discovered. Perhaps there will be no single antigen to explain RA, but rather a panel of antigens containing citrulline.

It has been an amazing decade for RA research, one that saw the approval of an entirely new class of cytokine inhibiting biological agents, as well as the discovery of a highly sensitive and specific diagnostic assay for RA. As with any important breakthrough, it is often the case that more questions arise than existed before the discovery. Let us hope that the interval between the initial *Annals of the Rheumatic Diseases* paper in 1964 and the repeated ''rediscovery'' of citrullinated antigens over the ensuing 40 years far exceeds the period between the unlocking of the RA ''PAD lock'' and the opening of the door to a firm understanding of RA pathogenesis.

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