The influence of autoantibody status and characteristics on the course of rheumatoid arthritis

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CHAPTER 11

Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, crossreactivity and the 'AMC-Senshu' method

Ann Rheum Dis. 2013 Jan;72(1): 148-50

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Recently, a novel family of autoantibodies in rheumatoid arthritis (RA) patients was described: anti-carbamylated protein (Anti-CarP) antibodies, which target carbamylated (homocitrulline-containing) epitopes.[1, 2] Since citrulline and homocitrulline have a similar structure, we wished to determine to what extent human autoantibodies can differentiate between them. Unlike human antibodies, the anti-modified citrulline (AMC) antibody developed by Dr Senshu[3-7] is able to recognise citrullinated epitopes irrespective of the neighbouring amino acids. Thus, we also aimed to verify whether the AMC assay could distinguish between these two amino acids.

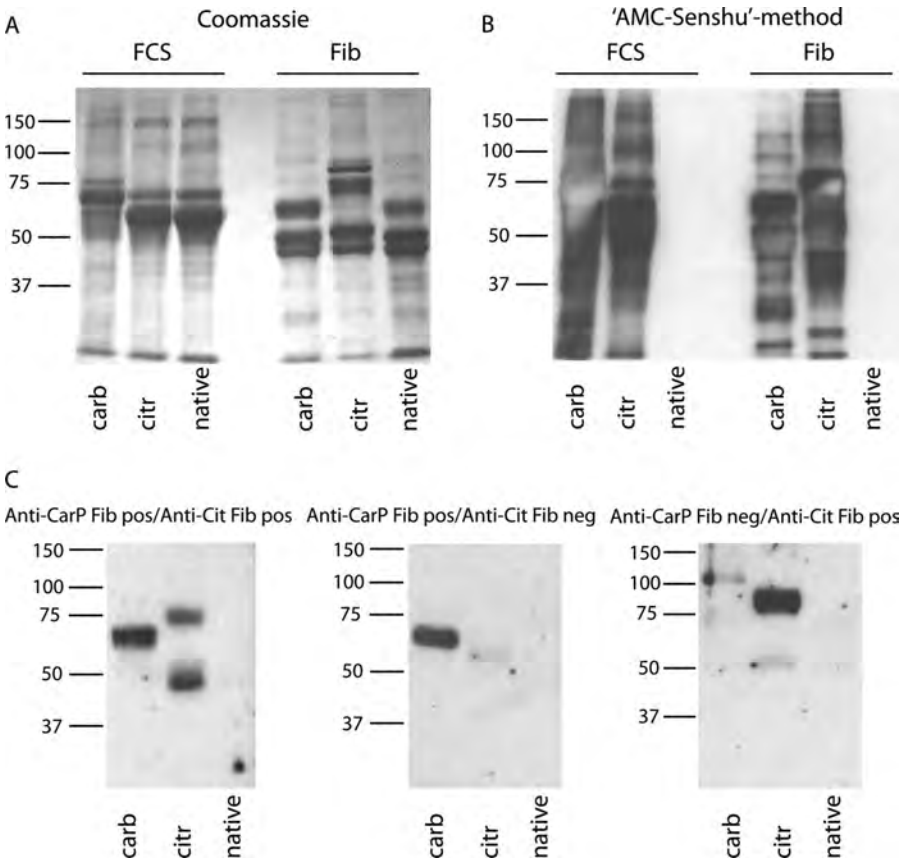

Figure 1 The 'Anti-Modified Citrulline (AMC)-Senshu' method does not discriminate citrullinated and carbamylated antigens, while human autoantibodies do. (A) Coomassie blue staining showed equal loading of foetal calf's serum (FCS), Ci-FCS, Ca-FCS, fibrinogen (Fib), Ci-Fib and Ca-Fib. (B) The 'AMC-Senshu' antibody used according to the protocol of the manufacturer, did not recognise FCS and Fib, but strongly recognised Ci-FCS, Ca-FCS, Ci-Fib and Ca-Fib. (C) Three selected rheumatoid arthritis sera can recognise both Ci-Fib and Ca-Fib, or only one of the modifications specifically.

To address this, we loaded gels with citrullinated, carbamylated and non-modified forms of foetal calf's serum (FCS) and human fibrinogen (Fib). Gels loaded with equal amounts of these protein preparations (figure 1A) were used for western blotting and staining with the 'AMC-Senshu' method. Both the citrullinated and the carbamylated forms of the proteins tested were strongly recognised, whereas, the non-modified form did not reveal any staining (figure 1B). Staining similar western blots with selected human sera[2] revealed that sera-positive for anti-citrullinated protein antibodies (ACPA) and anti-CarP stained both citrullinated (Ci) and carbamylated (Ca) forms of Fib, whereas, single positive sera stained only one form of modified Fib (figure 1C). These data indicate that although the 'AMC-Senshu' method does not discriminate between these two modifications, human sera of RA patients are able to.

In the double positive sera, the antibody response may either be cross-reactive, or harbour two separate reactivities. Four double positive sera were tested in inhibition assays in which the sera were incubated with Fib, Ci-Fib or Ca-Fib at 4°C overnight before detecting binding to Ci/Ca-Fib. Fib did not inhibit sera binding to Ci/Ca-Fib (figure 2A,B). In the sera analysed, binding to Ca-Fib can be inhibited by Ci-Fib to various degrees, whereas, binding to Ci-Fib could be inhibited by Ca-Fib to approximately 30% (figure 2A,B). These data indicate that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

In addition, we performed ACPA depletion studies in which double positive sera were depleted of ACPA. Biotinlylated CCP2 peptide and its arginine control were loaded separately onto HiTrap Streptavidin HP Columns. ACPA/anti-CarP double positive sera were applied to one CCP2 arginine-loaded column connected to one CCP2 citrulline-loaded column. The starting material and flow-through were tested on anti-CarP FCS and CCP2 ELISAs. After ACPA depletion, more than 98% of ACPA in the sera was depleted (figure 2C), while more than half the anti-CarP antibodies remained in the flow-through in five out of seven samples (figure 2C), confirming the data presented in figure 2A,B by showing that part, but not all anti-CarP antibodies are cross-reactive to citrullinated epitopes.

As suggested before, we found the 'AMC-Senshu' method cannot differentiate citrullination and carbamylation.[8, 9] Interestingly, part of the human sera can make this distinction. Anti-CarP antibodies and ACPA are often detected together, and here we show that double positive samples harbour anti-citrullinated epitope-specific antibodies, anti-carbamylated epitopes specific antibodies and cross-reactive antibodies. The finding that the 'AMC-Senshu' method recognises both citrullinated and carbamylated proteins does not argue against the notion that citrullinated proteins are present in synovial fluid and tissues, since a number of studies confirmed the presence of citrullinated proteins by mass spectrometry fingerprinting.[3-5, 7]

However, our study suggests that the extent and nature of citrullination and carbamylation in the joint should be re-evaluated especially in light of our recently described anti-CarP response that is, at least in part, not cross-reactive with citrullinated proteins.

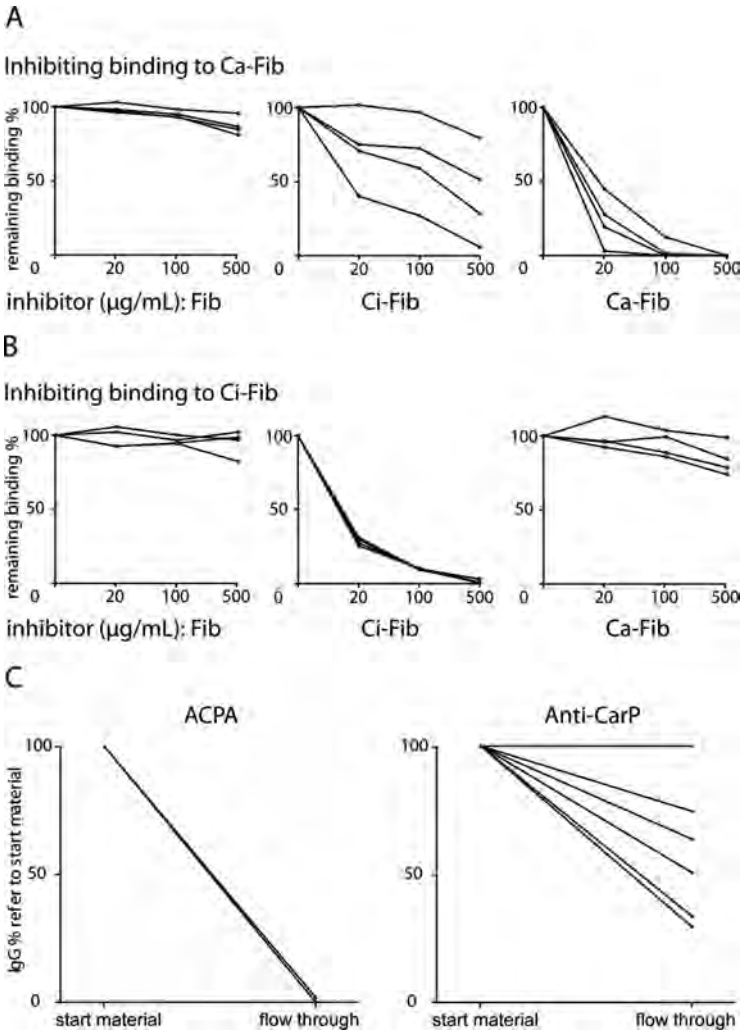

Figure 2 Anti-carbamylated protein (Anti-CarP) antibodies and ACPA represent two families of autoantibodies. (A) Inhibition studies on sera double positive for ACPA and anti-CarP antibodies. Fibrinogen (Fib) does not inhibit sera binding to Ca-Fib. Ci-Fib can partially inhibit sera binding to Ca-Fib, whereas, Ca-Fib can completely inhibit binding to itself. (B) Fib does not inhibit sera binding to Ci-Fib, whereas, Ci-Fib can inhibit more than 97% of binding to itself. Ca-Fib can only inhibit less than 30% of sera binding to Ci-Fib. (C) After ACPA depletion using CCP2 loaded columns, more than 98% of ACPA was depleted from the sera, while more than 50% of anti-CarP antibodies remained in five out of seven samples.

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