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Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the “AMC-Senshu” method

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Abstract

Objectives

Anti-citrullinated protein antibodies (ACPA) play an important role in the diagnosis and prognosis of rheumatoid arthritis (RA). The anti-modified citrulline (AMC) (‘Senshu’) method is the most frequently used method to detect citrullinated proteins. Recently, we identified antibodies against carbamylated proteins (anti-CarP antibodies) and studied whether the ‘AMC-Senshu’ method and human antibodies could discriminate citrullinated and carbamylated proteins.

Methods

We analyzed the reactivity of the ‘AMC-Senshu’ method and human antibodies on western blots targeting citrullinated, carbamylated or non-modified fetal calf serum (FCS) and fibrinogen (Fib). The cross-reactivity of ACPA and anti-CarP antibodies in double positive sera were also examined via the inhibition assays and ACPA depletion columns.

Results

The ‘AMC-Senshu’ method strongly stained both citrullinated and carbamylated FCS and Fib but not the non-modified counterparts. There are sera which stained both citrullinated and carbamylated forms of Fib and sera stained only one form of modified Fib. In the inhibition assays, sera binding to Ca-Fib can be inhibited by Ci-Fib to various degrees whereas binding to Ci-Fib could only be inhibited by Ca-Fib to approximately 30%. After ACPA depletion, more than half of anti-CarP antibodies remained in the flow through in 5 out of 7 samples, confirming that also in double positive individuals two separate antibody families exist.

Conclusions

The ‘AMC-Senshu’ method can not differentiate citrullinated and carbamylated epitopes. However, human antibodies can partially differentiate between them. In light of the recently identified anti-CarP antibodies, the extent and nature of citrullination and carbamylation in the joint should be re-evaluated.
Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease mainly affecting synovial joints. The disease process is characterized by joint damage and bone destruction and may lead to severe disability and increased mortality. Around 20 years ago, anti-citrullinated protein antibodies (ACPA) were discovered in RA patients and are now established as an important diagnostic and prognostic marker. [1] Although its role in the pathogenesis of RA is not yet fully elucidated, evidence on a possible pathologic role is accumulating. [2-7] Identifying citrullinated antigens recognized by ACPA is not only essential for a better understanding of RA but may also open a new window for early intervention. The assay used most frequently to identify citrullinated proteins is the anti-modified citrulline (AMC) assay. This assay is based on chemically adding ureido group adducts to citrulline residues followed by detection with a specific antibody developed by Dr. Senshu. [8] This assay has brought insight into the understanding of RA since it was used to show the presence of citrullinated proteins in the target tissues. [9-17] An advantage of the “AMC-Senshu” antibody is that it recognizes citrullinated epitopes irrespective of the neighboring amino acids. Recently, we and others described a novel family of autoantibodies in RA patients, anti-carbamylated protein (Anti-CarP) antibodies, which target carbamylated epitopes instead of citrullinated epitopes. [18,19] Since citrulline and homocitrulline are very similar, we wished to verify whether this method could distinguish between citrulline and homocitrulline as well as to determine to which extent human autoantibodies can differentiate between these two antigenic entities. To address this question, we studied whether the “AMC-Senshu” antibody and selected human sera can differentiate citrullinated and carbamylated proteins.

Methods

Generation of antigens

In brief, citrullinated fetal calf serum (Ci-FCS) and citrullinated fibrinogen (Ci-Fib) as well as carbamylated FCS (Ca-FCS) and carbamylated fibrinogen (Ca-Fib) were generated via incubation with either peptidylarginine deiminase 4 (Sigma) or potassium cyanate (Sigma) and confirmed by mass-spectrometry as described before. [18]

Autoantibody assays and inhibition assays

ELISA for the detection of anti-CarP Fib and anti-CarP FCS was performed as previously described. [18] Anti-CCP2 reactivity was detected using commercial CCP2 assays (Eurodiagnostica). [18] For the inhibition assays, four sera were incubated with Fib, Ci-Fib or Ca-Fib at 4°C overnight before detecting binding to Ci/Ca-Fib.
Coomassie blue staining and western blot (WB)

FCS, Ci-FCS, Ca-FCS, Fib, Ci-Fib and Ca-Fib were loaded with equal amounts onto 10% SDS-polyacrylamide gels and stained by SimplyBlue™ SafeStain staining (Life technologies) following the protocol from the manufacturer. The same gel was prepared and transferred onto Hybond-C Extra membranes (Amersham).

Selected human sera and the “AMC-Senshu” antibody were used to stain the blots. Ci-Fib positive/Ca-Fib negative, Ca-Fib positive/Ci-Fib negative and double positive sera were selected from previously performed anti-CarP Fib ELISA’s. The protocol using these 3 sera to stain the Fib, Ci-Fib and Ca-Fib blots is the same as previously described.[18] Staining using the “AMC-Senshu” antibody (Millipore) followed the protocol in the kit. In brief, before applying the AMC antibody and its detection antibody, the blot was incubated with 2,3-butanedione monoxime and antipyrine in strong acidic environment to form ureido group adducts.

ACPA depletion/purification column

Biotinylated CCP2 peptides or the arginine control were loaded onto 1ml HiTrap Streptavidin HP Columns (GE healthcare). One column containing the CCP2 arginine peptide was placed on top of two columns containing CCP2 peptides. After washing, ACPA/anti-CarP double positive sera were applied to the columns. Following washing the column was eluted by pH 2.5, 0.1 M glycine-HCl. The eluted antibodies were neutralized by 1M Tris, pH 8. The starting material, flow through and elution were tested on anti-CarP FCS and CCP2 ELISA.

Results

The “AMC-Senshu” method does not discriminate between citrullinated and carbamylated proteins

One gel was used to visualize equal loading of citrullinated, carbamylated or non-modified FCS or Fib (Figure 1A). The other equally loaded gel was used for Western-blotting and the resulting blot was used for the “AMC-Senshu” staining. Development of this blot revealed that both the citrullinated and the carbamylated forms of both FCS and Fib were strongly stained whereas the non-modified proteins did not reveal any staining (Figure 1B). These data indicate that the “AMC-Senshu” method identifies both citrulline and homocitrulline containing proteins and that it does not discriminate between citrullination and carbamylation.
Figure. 1 The “AMC-Senshu” method does not discriminate citrullinated and carbamylated antigens while human autoantibodies do. (A) Coomassie blue staining showed equal loading of FCS, Ci-FCS, Ca-FCS, Fib, Ci-Fib and Ca-Fib. (B) The “AMC-Senshu” antibody used according to the protocol of the manufacturer did not recognize FCS and Fib, but strongly recognized Ci-FCS, Ca-FCS, Ci-Fib and Ca-Fib. (C) Three selected RA sera can recognize both Ci-Fib and Ca-Fib or only one of the modifications specifically.

Human autoantibodies can discriminate between citrullinated and carbamylated antigens

To analyze whether human antibodies can actually discriminate between citrullinated and carbamylated proteins we applied sera of selected RA patients to stain blots containing Ci-Fib, Ca-Fib and Fib. Staining similar western blots with selected human sera revealed that sera positive for 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 (Fig 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 distinguish.

In the sera analyzed in the inhibition assays, 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% (Fig 2A, 2B). These data indicate that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

**Figure 2** Anti-CarP antibodies and ACPA represent two families of autoantibodies

(A) Inhibition studies on sera double positive for ACPA and anti-CarP antibodies. 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 were depleted from the sera while more than 50% of anti-CarP antibodies remained in 5 out of 7 samples.
We have shown previously that anti-CarP antibodies can be found in a subgroup of ACPA negative individuals but that the majority of anti-CarP positive individuals are also ACPA positive. We have shown above that in double positive individuals two separate reactivities exist by performing pre-incubation experiments. We have now verified this aspect further by studying whether in double positive individuals anti-CarP reactivity would remain after removal of ACPA using CCP2 columns. After ACPA depletion, more than 98% of ACPA in the sera were depleted (Figure 2C) while more than half of the anti-CarP antibodies remained in the flow through in 5 out of 7 samples (Figure 2C). Together, these data confirm that two separate autoantibody systems exist directed against citrullinated or carbamylated antigens. Nonetheless, in double positive individuals, there appears to be a cross-reactive portion as well as two mono-specific portions.

**Discussion**

To answer the question whether the “AMC-Senshu” method and human autoantibodies can distinguish citrullination from carbamylation, we performed western blots using the “AMC-Senshu” system and human sera. We found the “AMC-Senshu” method can not differentiate citrullination and carbamylation. Our finding is in line with a previous report suggesting that the AMC-Senshu method also detects homocitrulline.[20,21]

Importantly, our experiments showed that certain human sera can recognize either citrullinated or carbamylated proteins but not both. Since anti-CarP antibodies and ACPA are often found together we analyzed whether in double positive individuals two separate reactivities co-exist or that this only reflects cross-reactivity. By depletion of ACPA, we showed that double positive samples harbor anti-citrullinated epitope-specific antibodies, anti-carbamylated epitopes specific antibodies as well as cross-reactive antibodies.

In this study, we found that the “AMC-Senshu” method can recognize both citrullinated and carbamylated proteins. This finding does not argue against the notion that citrullinated proteins are present in the synovial fluid and tissues. Especially since in a number of studies, citrullinated proteins were first detected by the “AMC-Senshu” method and then further confirmed by mass-spectrometry fingerprinting.[9-12] However, our findings highlight that in these studies next to citrullinated proteins also carbamylated proteins may have been detected. In light of the recently identified anti-CarP antibodies, the extent and nature of citrullination and carbamylation in the joint should be re-evaluated.
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References


