Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage

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Autoimmune responses against posttranslationally modified antigens are a hallmark of several autoimmune diseases. For example, antibodies against citrullinated protein antigens (ACPAs) have shown their relevance for the diagnosis and prognosis of rheumatoid arthritis (RA), and have been implicated in disease pathogenesis. It is conceivable that other autoantibody systems, recognizing other posttranslationally modified proteins, are also present in RA. Here, we describe the presence of an autoantibody system that discriminates between citrulline- and homocitrulline-containing antigens in the sera of RA-patients. IgG antibodies recognizing carbamylated (homocitrulline-containing) antigens were present in sera of over 45% of RA-patients. Likewise, anticarbamylated protein (anti-CarP) IgA antibodies were observed in 43% of RA sera. ACPA and anti-CarP antibodies are distinct autoantibody systems because, in selected double-positive patients, the anti-Carp antibody binding to carbamylated antigens could be inhibited by carbamylated antigens, but not by control or citrullinated antigens. Similarly, ACPA-binding to citrullinated antigens could only be inhibited by citrullinated antigens. In line with this observation, 16% of ACPA-negative RA-patients, as measured by a standard ACPA assay, harbored IgG anti-CarP antibodies, whereas 30% of these patients tested positive for IgA anti-CarP antibodies. The presence of anti-CarP antibodies was predictive for a more severe disease course in ACPA-negative patients as measured by radiological progression. Taken together, these data show the presence of a unique autoantibody system recognizing carbamylated, but not citrullinated, protein antigens. These antibodies are predictive for a more severe clinical course in ACPA-negative RA-patients, indicating that anti-CarP antibodies are a unique and relevant serological marker for ACPA-negative RA.

The identification of anticitrullinated protein antibodies (ACPAs) has contributed significantly to the understanding of rheumatoid arthritis (RA) (1). Significant differences between ACPA-positive and -negative disease have been reported with respect to the contribution of genetic and environmental risk factors, as well as disease progression and remission (2–5). Over the past few years important insight has been gained into the occurrence and etiopathology of ACPA-positive RA. However, less information is available on ACPA-negative RA. This lack of information is partly because of the absence of robust biomarkers characterizing this manifestation of RA.

The posttranslational modification of arginine into citrulline by peptidyl arginine deiminase (PAD) enzymes is essential for the generation of citrullinated antigens that are recognized by ACPA (1). Under physiological circumstances, citrullination is involved in tissues like hair and skin because of its role in terminal epithelial differentiation (6). In the nucleus citrullination plays a role in epigenetic regulation (7) and condensation of chromatin, and has been reported to be involved in translation (6) and the host defense against pathogens (8). Under pathological conditions where cell death may overwhelm the phagocytic capacity of phagocytes, necrotic cells may release PAD into the extracellular space, where higher calcium concentrations now may also allow the citrullination of other proteins located outside the cell (6). These proteins may be targeted by ACPA, possibly leading to inflammation and arthritis.

Citrulline highly resembles homocitrulline (Fig. 1), another posttranslationally modified amino acid (9). Homocitrulline is one methylene group longer, but similar in structure (9). Homocitrulline is generated from lysine residue following a reaction of cyanate, which is present in the body in equilibrium with urea. Under physiological conditions the urea concentration may be too low to allow extensive carbamylation but the conversion process leading to the formation of homocitrulline from lysine in proteins does occur in vivo. In conditions of renal failure, the urea concentration increases and carbamylation of many proteins can be readily detected. However, most carbamylation is believed to take place during inflammation when myeloperoxidase is released from neutrophils (10). This enzyme converts thioycyanate to cyanate, now allowing more carbamylation to occur (11). It has been shown recently that homocitrulline-containing proteins are present in the RA joint and that they may affect T-cell triggering and possibly autoantibody formation in rodents (9, 12). Although highly similar, carbamylation differs from citrullination as, next to their structural difference, lysine is modified instead of arginine. Therefore, homocitrulline will, by definition, be located at other positions in proteins than citrulline. Because of the similarity between citrulline and homocitrulline, we set out to analyze whether antibodies against carbamylated proteins are present in RA and whether these antibodies differ from ACPA with respect to antigen binding and clinical associations.

Results

Anticarbamylated Protein Antibodies and ACPA Are Different Antibody Families. To detect antibodies against carbamylated proteins (anti-CarP antibodies), we developed an ELISA using carbamylated FCS (Ca-FCS) and nonmodified FCS as antigens. Analyzing sera of 40 RA patients and 40 controls, we observed that sera of RA-patients reacted with Ca-FCS compared with sera obtained from healthy subjects with both IgG (Fig. 2 A and B) and IgA (Fig. 2 D and E) reactivity. The enhanced reactivity

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of RA sera to Ca-FCS is further emphasized after subtraction of the reactivity against unmodified FCS (Fig. 2 C and F). Because citrulline and homocitrulline are two rather similar amino acids (Fig. 1), we next wished to determine whether ACPA also recognizes homocitrulline when located at the same position as citrulline in a peptide. For this purpose we performed ELISAs using a citrullinated Fibrinogen (Fib) peptide known to be recognized by ACPA (13). Within this peptide backbone, a citrulline, an arginine, a homocitrulline, or a lysine residue was introduced for further analysis. Analyzing a set of 76 RA sera, we observed that ACPA only recognized the citrullinated peptide, but not the arginine-containing or the homocitrulline-containing peptide (Fig. 3c). These data indicate that ACPA can discriminate between citrulline and homocitrulline presence within the same peptide backbone. Next, we wished to analyze whether there is cross-reactivity between anti-CarP antibodies and ACPA for binding to posttranslationally modified proteins. Therefore, we performed inhibition studies using sera that were reagents to both citrullinated and carbamylationated antigens. We analyzed the binding of anti-CarP antibodies to Ca-FCS-coated plates following preincubation with Ca-FCS, citrullinated FCS (Ci-FCS), native FCS, or by citrullinated peptides used to detect ACPA (cyclic citrullinated peptide-1, Ccpp). Following preincubation, we observed that anti-CarP antibody binding to Ca-FCS can only be inhibited by Ca-FCS but not by Ci-FCS, native FCS, or by peptides used to detect ACPA (Fig. 3b). We also performed the reverse inhibition experiment where we analyzed the binding of ACPA to plates coated with Ci-FCS following the same preincubation procedure. We observed that ACPA binding to Ci-FCS could only be inhibited by Ci-FCS and the citrullinated peptide but not by Ca-FCS, nonmodified FCS, or the arginine form of the peptide (Fig. 3c). Taken together, these data indicate that anti-CarP antibodies and ACPA are not, or only limited, cross-reactive and specifically directed against homocitrulline or citrulline-containing antigens, respectively. Because all observations described above were made using ELISA, we also wished to confirm our findings using a different technique. For this reason we performed a Western blot-analysis using FCS, Ca-FCS, and Ci-FCS on reduced gels, followed by Western blotting. The different blots were incubated with sera of individuals that were either anti-CarP-positive and ACPA-negative or anti-CarP-negative and ACPA-positive. We observed a positive staining of the anti-CarP-positive sample only on Ca-FCS but not on Ci-FCS or FCS (Fig. 3d). In contrast, the anti-CarP-negative, ACPA-positive sample reacted to Ci-FCS, but not to Ca-FCS and FCS (Fig. 3d). To confirm the presence of anti-CarP antibodies we repeated these experiments using a more defined protein, human Fib, as a target antigen. Fib was citrullinated by PAD (Ci-Fib) or carbamylated by cyanate (Ca-Fib). The nonmodified form (Fib), Ca-Fib, and Ca-Fib were used as antigens in ELISA. Similar to the observations for FCS, we observed significant binding of antibodies to the Ci-Fib and the Ca-Fib but not to the Fib-coated wells (Fig. 4a). This finding was largely restricted to the RA sera and not the controls (P < 0.0001). To analyze cross-reactivity we also performed inhibition studies, as described above. ELISA analyses confirmed that ACPA and anti-CarP antibodies are largely noncross-reactive (Fig. 4b). To ensure that reactivity toward carbamylationated proteins is mediated by the antigen-binding part of the antibodies, we generated F(ab′)2. As expected, F(ab′)2, generated from anti-CarP IgG-positive samples but not from negative samples display anti-Car-P reactivity (Fig. 4c and d). As observed using intact antibodies, F(ab′)2-reactivity toward Ca-Fib could also be inhibited specifically by Ca-Fib, whereas F(ab′)2-reactivity toward Ca-Fib could only be inhibited specifically by Ca-Fib (Fig. 4e).

Collectively, these data indicate that anti-CarP antibodies and ACPA recognize different antigens, one recognizing citrullinated proteins (ACP) and the other carbamylationated proteins (anti-CarP). Likewise, these data indicate that antigen-recognition is most likely mediated via the variable domains present in the F(ab′)2 fragments.

**Anti-CarP Antibodies Are Present in RA.** Following the identification of anti-CarP antibodies as an autoantibody family separate from ACPA, we wished to quantify the presence of these anti-CarP antibodies in a large population of RA patients and controls. For this reason, we first generated a standard, comprising of a pool of anti-CarP antibody-positive sera. This standard displayed a specific, dose-dependent binding of both IgG and IgA to Ca-FCS but no binding to unmodified FCS (Fig. 5a and b). For this analysis,

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**Fig. 1.** Illustration of citrullination and carbamylation. Citrullination (A) and carbamylation (B) occur on different amino acids via different mechanisms, but yield similar end-products.

**Fig. 2.** Antibodies against carbamylationated proteins are present in sera of RA patients. The reactivity of IgG (A and B) and IgA (D and E) from sera of healthy controls (NHS) or RA patients (RA) to wells coated with nonmodified FCS (FCS) or carbamylationated FCS (Ca-FCS) is depicted. Data expressed as absorbance at 415 nm. (C and F) Absorbance units of FCS were subtracted from the absorbance units of Ca-FCS, representing the specific anti-carbamylationated protein response. **P < 0.0001** for a t test comparing NHS and RA.
The group of RA patients analyzed in this study showed a limited degree of correlation when analyzing anti-CarP antibodies and ACPA, as measured by the CCP2 assay. Therefore, we analyzed next whether the presence of anti-CarP antibodies are also predictive for a more severe disease course in patients with recent-onset arthritis where X-rays of hands and feet are taken of all RA-patients at yearly intervals to assess radiological damage using the Sharp–van der Heijde method (14). We observed that the presence of anti-CarP IgG strongly associates with a more severe disease progression. Patients positive for anti-CarP IgG had more joint destruction over 7 y than IgG-negative patients without correlation (CI 1.68–2.40, \( P = 8.68 \times 10^{-14} \)) or with correction of ACPA and rheumatoid factor (RF) (\( \beta = 1.41, 95\% \) CI 1.13–1.76, \( P = 0.002 \)) (Fig. S1). Anti-CarP IgA was associated with more joint destruction over 7 y than anti-CarP IgA-negative patients without correction of ACPA and RF (\( \beta = 1.21, 95\% \) CI 1.01–1.45, \( P = 0.041 \)) but not after correction (\( P = 0.855 \)) (Fig. S1). As the analysis described above does not show whether anti-CarP antibodies predict radiological progression in the anti-CCP2-negative, anti-CCP2-positive, or both RA subgroups, we next performed a stratified analysis. Importantly, this analysis revealed that the presence of anti-CarP IgG is associated with a more severe joint damage in the anti-CCP2-negative subgroup (\( \beta = 1.86, 95\% \) CI 1.41–2.66, \( P = 1.8 \times 10^{-5} \)) (Fig. 6). Likewise, a similar trend toward more joint damage over time was observed for anti-CCP2-negative patients who tested positive for IgA anti-CarP antibodies (\( \beta = 1.25, 95\% \) CI 0.98–1.58, \( P = 0.071 \)) (Fig. S1). In contrast, in the anti-CCP2-positive subgroup, which is already characterized by severe joint destruction, no additional increase was observed in individuals who also harbored anti-CarP antibodies (Fig. S1). Taken together, these data indicate that the detection of anti-CarP antibodies at baseline is predictive for a more destructive disease course in anti-CCP2-negative RA as measured by the Sharp–van der Heijde method.

**Discussion**

A family of autoantibodies that recognize carbamylated proteins, anti-CarP antibodies, can be detected in sera of RA patients. Both inhibition studies and cohort studies show that anti-CarP antibodies and ACPA represent two different and independent autoantibody families, one recognizing carbamylated proteins and the other citrullinated proteins. Our data show that anti-CarP antibodies and ACPA are, by and large, noncross-reactive although we do not exclude that some cross-reactivity exists at the population level, as is also indicated in recent data obtained in rabbits after vaccination with carbamylated proteins (12). Interestingly, positivity for anti-CarP antibodies is related to clinical outcome, as individuals positive for anti-CarP IgG, but negative for anti-CCP2 antibodies, have a more destructive disease course compared with anti-CarP IgG-negative RA patients.

It is currently unknown which proteins undergo posttranslational modifications like carbamylation. Carbamylation is mediated by cyanate, which is in equilibrium with urea. Increased urea concentrations, smoking, and inflammation have been reported to shift this equilibrium toward cyanate and, hence, enhanced carbamylation (11). Because currently no in vivo relevant targets for anti-CarP antibodies are known, we used a complex protein mixture as an initial source of carbamylated protein antigens for the detection of anti-CarP antibodies. Western blot

**Fig. 3.** Anti-CarP antibodies and ACPA are two separate autoantibody systems. (A) IgG reactivity of 76 sera from RA patients, toward several forms of a Fibrinogen peptide is depicted. (B and C) Antibody binding to Ca-FCS or Ci-FCS was inhibited using preincubations with fluid-phase inhibitors. (D) FCS, Ca-FCS, and Ci-FCS were separated by SDS-PAGE gels and blotted. The presence of antibodies reactive to proteins on the blots was analyzed by incubating these blots with either anti-CarP-positive ACPA-negative and anti-CarP-negative ACPA-positive sera.

**Materials and Methods**

As the analysis described above does not show whether anti-CarP antibodies are also present in sera of Anti-CCP2-Negative RA Patients. The group of RA patients analyzed in this study consisted of both ACPA-positive and ACPA-negative individuals, as measured by the CCP2 assay. Therefore, we analyzed next the association between anti-CarP antibodies and anti-CCP2 antibodies. The presence of anti-CarP antibodies and anti-CCP2 antibodies showed a limited degree of correlation when analyzing the entire RA population (\( r^2 = 0.27, P < 0.001 \) for anti-CarP IgG or \( r^2 = 0.15, P < 0.001 \) for IgA). However, we also identified substantial numbers of RA patients that are only positive for anti-CCP2 antibodies, as well as a group of patients that is only positive for anti-CarP antibodies (Fig. 5 E and F). We observed that \( \sim16\% \) of the anti-CCP2-negative RA patients displayed anti-CarP IgG antibodies, whereas 30% of the anti-CCP2-negative RA patients tested positive for anti-CarP IgA (Fig. 5 G and H). These data indicate that the presence of anti-CarP antibodies overlaps with the occurrence of anti-CCP2 antibodies, but that this overlap is not absolute, as over 30% of the anti-CCP2-negative patients harbor anti-CarP antibodies. In total, more than 35% of all anti-CCP2-negative patients have either anti-CarP IgG or IgA antibodies.
analyses indicate the recognition by anti-CarP antibodies of at least one dominant protein present in FCS after carbamylation using cyanate (representing high urea concentrations) (Fig. 3D). However, these data are likely not to represent the in vivo situation where carbamylation is a more gradual but constantly occurring process (15). In this respect, it is likely that especially long-lived proteins acquire homocitrulline residues over time, as carbamylation is nearly irreversible and thus will lead to the accumulation of homocitrulline-residues on proteins with a long half-life. Intriguingly, the joint is known for the presence of long-lived proteins, such as collagen and other cartilage-expressed proteins. Therefore, it is conceivable that such matrix-proteins will accumulate homocitrulline residues during life, especially under conditions of inflammation. Indeed, it has been shown that homocitrulline is present in the joint (9), possibly representing the long-lived nature of many joint-derived proteins. Therefore, it is conceivable that such matrix-proteins and whether these can serve as a target for anti-CarP antibodies.

The molecular nature of the antigens recognized by ACPA has been identified more than 15 y ago by describing that citrulline is an essential constituent of antigens recognized by these RA-specific antibodies (16, 17). This finding has made considerable impact, as it has opened up the way to relevant and novel insights into RA-diagnostics and etiopathology (1). For example, ACPA are now part of the new American College of Rheumatology/European League Against Rheumatism criteria for RA (18), and have been implicated in RA-pathogenesis, both in animal models (19–21) and in ex vivo human studies (22–25). Importantly, the description of ACPA has led to the realization that RA constitutes at least two clinical syndromes that share many clinical features, but differ with respect to genetic background, predisposing environmental factors and clinical progression/remission (3, 4, 26–28). Although it is clearly too early to allow any firm conclusions, it is tempting to speculate that anti-CarP antibodies also contribute to disease pathogenesis and display diagnostic value, given the similar nature of the antigens recognized and their presence in ACPA-negative disease.

The presence of anti-CarP antibodies in anti-CCP2–negative disease is highly intriguing, as it could potentially represent a unique biomarker that positively identifies at least part of this manifestation of RA. To gain further insight into this possibility, it is important to establish whether the presence of anti-CarP antibodies is specific for RA or also found in other rheumatic diseases, as well as whether their presence predict the development of (ACPAnegative) RA in patients suffering from early unclassified RA and joint complaints, such as arthralgia.

To establish a cut-off to define a positive sample, we have analyzed the presence of IgG and IgA directed against Ca-FCS and FCS in sera of healthy controls. All samples were tested for reactivity toward Ca-FCS and FCS, and absorbance values were converted into arbitrary units per milliliter using an anti-CarP antibody-positive standard present on the same plate. Because sera from several individual subjects also displayed reactivity toward nonmodified FCS, we subtracted the “FCS-reactivity” from the reactivity toward Ca-FCS using arbitrary units per milliliter as defined by the standard curve. We subsequently calculated the cut-off as the mean plus two times SD and applied the cut-off to the data of the RA patients following a similar strategy. The disadvantage of this method is that a standard is used on Ca-FCS for the determination of arbitrary units per milliliter toward FCS, another antigenic entity. However, this method did allow the calculation of a specific response to the posttranslational modification.

Every method of establishing a cut-off has advantages and limitations. Therefore, we subsequently confirmed our observations using another strategy as well by calculating the cut-off as the mean plus two times SD of the anti-Ca-FCS response in controls. This cut-off was applied to the data of the RA patients as was also used before (29). The association with radiological progression of anti-CarP IgG in ACPA-negative RA remains significant, albeit with a lower level of significance (P = 0.001).
In brief, nonmodiﬁed baxis. Below the SI Materials and Methods IgA and 2.0 7 0.5 H 0.25 1 6 1.0 Materials and Methods 0.25 0.13 5 and SI Materials and Methods 4.0 4.0 2 D F and 3 0.13 axis and the Anti-CarP IgG and IgA antibodies are present in RA sera. (value is derived from the analysis model, as described 2.0 1.0 1.0 31), the detection of anti-CarP antibodies might be beneﬁcial to classify. From a clinical perspective, the detection of anti-CarP antibodies in early arthritis could be highly rewarding because they predict a more severe disease course. Because early aggressive treatment in RA has been shown to prevent future damage (30, 31), the detection of anti-CarP antibodies might be beneﬁcial to identify anti-CCP2–negative patients at risk to develop severe disease. The identiﬁcation of such patients might be important to guide treatment decisions early after onset of symptoms, especially in early arthritis patients that are diﬃcult to classify.

In conclusion, in addition to the autoantibody system that recognizes citrullinated proteins (ACPA), an autoantibody system against carbamylated proteins (anti-CarP) is present in sera of RA patients. Detection of anti-CarP antibodies could offer new possibilities to identify patients at risk for a severe disease course.

**Materials and Methods**

Patient and Control Sera. The sera analyzed were from patients participating in the Leiden EAC cohort. The Leiden EAC is an inception cohort of patients with recent-onset arthritis (symptoms duration <2 y) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 (32). All RA patients fulﬁlled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the classiﬁcation of RA (33) within 1 y of follow up. A total of 571 RA patients were involved in the analyses. Patient samples were compared with 305 healthy control samples also living in the Leiden area. The protocols were approved by the Leiden University Medical Center ethics committee and informed consent was obtained.

Detection of Anti-CarP Antibodies by ELISA. In brief, nonmodiﬁed FCS and modiﬁed FCS were coated on Nunc Maxisorp plates (Thermo Scientiﬁc) over night. Following washing and blocking, the wells were incubated with serum. Bound human IgG or IgA was detected using rabbit anti-human IgG or IgA antibodies (Dako), followed by HRP-labeled goat anti-rabbit IgG antibody (Dako). Bound human IgG or IgA was detected using rabbit anti-human IgG or IgA antibodies (Dako), followed by HRP-labeled goat anti-rabbit IgG antibody (Dako). Following the last washings, HRP enzyme activity was visualized using ABTS (34). A more detailed description of the protein modiﬁcations and ELISAs based on FCS and Fib, including Fab(2), is in SI Materials and Methods. We established the cut-oﬀ for a positive response as the mean plus two times the SD of the speciﬁc anti-CarP reactivity of the healthy controls. The methods for the detection of ACPA and Western blotting are available in SI Materials and Methods.

**Fig. 5.** Anti-CarP IgG and IgA antibodies are present in RA sera. (A and B) Dose-response curves of the anti-CarP antibody-positive standard (IgG and IgA) on Ca-FCS and FCS in ELISA. (C and D) ELISA was performed for the detection of anti-CarP IgG and IgA in sera of healthy controls (NHS) and RA patients. A cut-oﬀ was established using the mean plus two times the SD of the healthy controls, as described in the Materials and Methods. Reactivity is depicted as arbitrary units per milliliter. The number of samples tested and the percentage of positivity is indicated below the graph. (E and F) Pie charts showing the percentage of RA patients positive and negative for anti-CCP2 and anti-CarP antibodies. (G and H) Pie charts showing the percentage of anti-CarP IgG- or IgA-positive patients negative for anti-CCP2.

From a clinical perspective, the detection of anti-CarP antibodies in early arthritis could be highly rewarding because they predict a more severe disease course. Because early aggressive treatment in RA has been shown to prevent future damage (30, 31), the detection of anti-CarP antibodies might be beneﬁcial to identify anti-CCP2–negative patients at risk to develop severe disease. The identiﬁcation of such patients might be important to guide treatment decisions early after onset of symptoms, especially in early arthritis patients that are diﬃcult to classify.

**Inhibition Studies.** To determine whether anti-CarP antibodies and ACPA are cross-reactive antibodies, we performed inhibition studies in which autoantibody-positive serum samples, positive for both ACPA and anti-CarP antibodies, were preincubated with increasing concentrations of either nonmodiﬁed FCS, Ca-FCS, Ci-FCS, or the carbamylate or arginine containing a form of the CCP1 peptide (17). Following preincubation at room temperature, the samples were tested for reactivity against Ca-FCS and Ci-FCS, as described above. Serum and Fab(2) samples positive for both Ci-Fib and Ca-Fib were preincubated with Fib, Ci-Fib, and Ca-Fib at 4 °C overnight and subsequently analyzed on the Fab ELISA (SI Materials and Methods).

**Radiological Progression.** In the EAC cohort, radiographs of the hands and feet, which had been obtained in a longitudinal fashion, were scored according to the Sharp–van der Heijde method (35). Scoring and analysis have been described in detail previously (14). Data were analyzed directly, or using repeated measurement analysis to optimally make use of the longitudinal data obtained for each patient (14). More detailed information is available in SI Materials and Methods.
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