Serum Autoantibodies that Bind Citrullinated Fibrinogen Are Frequently Found in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Autoantibodies that bind citrullinated antigens are a sensitive and specific marker for rheumatoid arthritis (RA). While synthetic cyclic citrullinated peptides (CCP) are typically used to identify these antibodies, little is known about antibody reactivity to the predominant citrullinated protein found in the inflamed synovium, citrullinated fibrinogen (CitFib). We assessed the prevalence of anti-CitFib antibodies in patients with various rheumatic diseases.

Methods. In total, 65 patients with established RA and 63 patients with other rheumatic diseases were tested for serum IgM rheumatoid factor (RF), IgG anti-CCP^2, and IgG anti-CitFib antibodies. This cohort was used to determine optimal positive cutoff values for antibody reactivity to CitFib through receiver operating characteristic curve analysis. The specificity of these assays was confirmed with sera from 49 patients with psoriatic arthritis.

Results. Antibodies to both citrullinated antigens were identified in the majority of RA patients tested. The overall sensitivity and specificity of the assays were: CCP 82%, 96%, CitFib 75%, 98%, and IgM RF 80%, 64%, respectively. All but one patient that was positive for CitFib was also positive for CCP^2, and close to half the RF-negative RA patients were positive for CitFib and CCP^2.

Conclusion. These results suggest that autoimmunity to CitFib is common in patients with RA and may play a role in disease pathogenesis. (First Release Aug 15 2006; J Rheumatol 2006;33:2115–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
CYCLIC CITRULLINATED PEPTIDE
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The diagnosis and establishment of the prognosis of rheumatoid arthritis has been enhanced with the ability to detect autoantibodies that bind to citrullinated proteins and peptides. While many substrates have been used to detect these antibodies, most are derived from citrullinated filaggrin, a protein found in the cornified layer of the skin. These anticitrulline antibodies include antiperinuclear factor, antikeratin antibodies, antifilaggrin antibodies, and the first-generation cyclic citrullinated peptide (CCP) (reviewed by Vossenaar, *et al*[1]). Further advances have been made in order to increase the sensitivity for detecting anticitrulline antibodies through the use of dedicated peptide libraries, leading to development of the second-generation CCP assay[2]. Although these antigens are useful for *in vitro* diagnostic assays, they are unlikely to play a role in disease pathogenesis, since they are not found in synovial tissue.

Research into the features of the synovial-derived antigen that might bind these autoantibodies identified citrullinated fibrinogen (CitFib) through biochemical and immunohistological analysis[3-5]. We describe the frequency of serum autoantibodies that bind CitFib in patients with RA and other rheumatic diseases and compare these results with those for reactivity to CCP^2 and IgM rheumatoid factor (RF).

MATERIALS AND METHODS

Patients. Sera were obtained from 128 patients attending the St. Joseph's Health Centre Rheumatology Clinic, London. Of these, 65 fulfilled the 1987 American College of Rheumatology classification criteria for RA, while the remaining patients had a diagnosis of other rheumatic diseases (ORD) as determined by chart review (confirmed by DAB and JA; Table 1). Seventy-seven percent of patients with RA were female with a mean age of 58 (range 32–87) years, while 67% of the patients with ORD were female, with a mean age of 55 (range 16–87) years. Sera from 49 patients with psoriatic arthritis

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Table 1. Antibody responses in patients with RA and other rheumatic diseases including 49 patients with psoriatic arthritis.

	Number	ΔCitFib	CCP^2	RF
RA	65	49	53	52
RF-positive	52	44	47	52
RF-negative	13	5	6	0
Other rheumatic diseases	112	2	5	30
Ankylosing spondylitis	3	0	1	0
Fibromyalgia	2	0	0	2
Gout	1	0	0	0
Hepatitis C virus associated arthralgia	2	0	0	2
Osteoarthritis	4	0	0	2
Overlap syndrome	1	0	0	0
Polymyalgia rheumatica	6	0	0	2
Psoriasis	3	0	0	2
Psoriatic arthritis	49	1	1	7
Sarcoidosis	1	0	0	0
Scleroderma	1	0	0	0
Sjögren's syndrome	4	0	0	3
Systemic lupus erythematosus	5	0	1	3
Undifferentiated arthritis	24	0	1	3
UCTD/MCTD	3	1	1	3
Vasculitis	3	0	0	1
Sensitivity, %		75	82	80
Specificity, %		98	96	73
Positive predictive value		96	91	63
Negative predictive value		87	90	86

UCTD/MCTD: undifferentiated connective tissue disease/mixed connective tissue disease.

(PsA) recruited at the Centre for Prognosis Studies in the Rheumatic Diseases, Toronto Western Hospital, Toronto, were also tested.

The Ethics Committees of the University of Western Ontario and University of Toronto approved the use of these sera for this study.

Autoantibody detection. Serum IgM RF concentrations were determined by nephelometry, with a value > 20 IU/ml considered positive. The second-generation CCP (CCP^2) ELISA (Inova, San Diego, CA, USA) was performed according to the manufacturer's instructions, with a positive value considered > 25 RU/ml. The Toronto cohort of patients with PsA was tested for CCP^2 antibodies using an ELISA kit from Euroimmun (Luebeck, Germany). Autoantibodies to CitFib were determined by ELISA using both CitFib and unmodified fibrinogen as solid-phase antigens. Briefly, plasminogen-depleted human fibrinogen (Calbiochem, San Diego, CA, USA) was citrullinated *in vitro* by incubating 7 units of peptidylarginine deiminase (Sigma, Mississauga, ON, Canada) with 1 mg of fibrinogen in 0.1 M Tris-HCL (pH 7.4), 10 mM $CaCl_2$ for 3 h at 50°C. CitFib was then washed extensively using a 100 kDa Macrosep centrifugation filter (VWR, Mississauga, ON, Canada) with dH_2O followed by phosphate buffered saline (PBS). Deimination was confirmed by mobility shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Western blot using RA serum. ELISA plates (Nunc Maxisorp, VWR) were coated overnight at 4°C with 10 μg/ml (100 μl/well) of antigen (CitFib or Fib) diluted in carbonate buffer, pH 9, then washed (PBS, 0.05% Tween) and blocked for 1 h at room temperature [PBS, 0.1% bovine serum albumin (BSA)]. Serum samples were diluted 1:100 in PBS, 0.1% BSA, 0.05% Tween and incubated in duplicate for each antigen for 2 h at room temperature. After washing, wells were incubated with biotin-conjugated goat anti-human IgG (1:10,000; Sigma) and streptavidin horseradish peroxidase polymer (1:4000; Sigma) diluted in PBS, 0.1% BSA, 0.05% Tween for 30 min at room temperature. After washing again, the wells were incubated 10 min with TMB substrate (Sigma), then the reaction was stopped with 2 M H_2SO_4 and the absorbance was determined at OD 450. Values given represent the average result for each sample tested in duplicate. All samples were tested at the same time, and a CitFib-positive standard was used to monitor interassay variation, which was < 10% between plates. Nine healthy donors were also tested for reactivity to Fib and CitFib. Values for the difference in antibody reactivity between CitFib and Fib (ΔCitFib) were calculated by subtracting the average OD + 2 SD for reactivity to Fib from the average OD for reactivity to CitFib. Each serum sample was tested for reactivity to CitFib and Fib on the same plate.

Statistical analysis. Receiver operating characteristic (ROC) curve analysis, Mann-Whitney U test, and regression analysis was conducted using MedCalc version 7.6 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Detecting antibodies to citrullinated fibrinogen. Antibody reactivity to both citrullinated and unmodified fibrinogen was assessed in a cohort of 65 patients with RA and 63 patients with ORD and were subsequently subjected to ROC curve analysis. Reactivity was measured to unmodified fibrinogen alone and citrullinated fibrinogen alone as well as the difference between the 2 (ΔCitFib). These values were then plotted to identify the most effective measure to discriminate between the 2 populations and to establish positive cutoff values (Figure 1). As expected, serum antibody reactivity to unmodified fibrinogen did not differ between RA patients and those with ORD (median OD = 0.322 and 0.322, respectively; $p > 0.75$), with ROC area under the curve (AUC) of 0.510. Moreover, a significant difference in reactivity to Fib was not found in patients with RA or ORD compared to healthy donors (data not shown). When CitFib alone was assessed, RA patients had significantly higher reactivity than those with ORD (median OD = 0.631 vs 0.288; $p < 0.0001$) and the AUC from the ROC curve was 0.864, suggesting good discrimination between the 2 populations using this measure. Again, a significant difference in reactivity to CitFib was not found between patients with ORD and healthy donors (data not shown). The positive cutoff value (giving the highest positive likelihood ratio and lowest negative likelihood ratio) for CitFib resulted in a sensitivity of 82% and a specificity of 83%. In order to compensate for any nonspecific reactivity in these serum samples we also performed analysis with values taken from the difference in reactivity between CitFib and Fib (ΔCitFib). While there was no significant difference between AUC values for CitFib and ΔCitFib in ROC analysis (0.864 and 0.850, respectively; $p > 0.65$), the positive cutoff value for ΔCitFib (OD > 0) resulted in a sensitivity of 75% and an improved specificity of 98%. Comparatively, when the specificity for reactivity to CitFib is set at 98%, a pronounced reduction in sensitivity to 58% occurred. These results show that there is no increased reactivity to unmodified fibrinogen in RA patients, and identify ΔCitFib as the most accurate means to differentiate these 2 disease populations.

Comparing antibody reactivity to ΔCitFib, CCP^2, and IgM-RF. To determine how frequently autoantibodies to CitFib arise in patients with RA and ORD in relation to other diag-

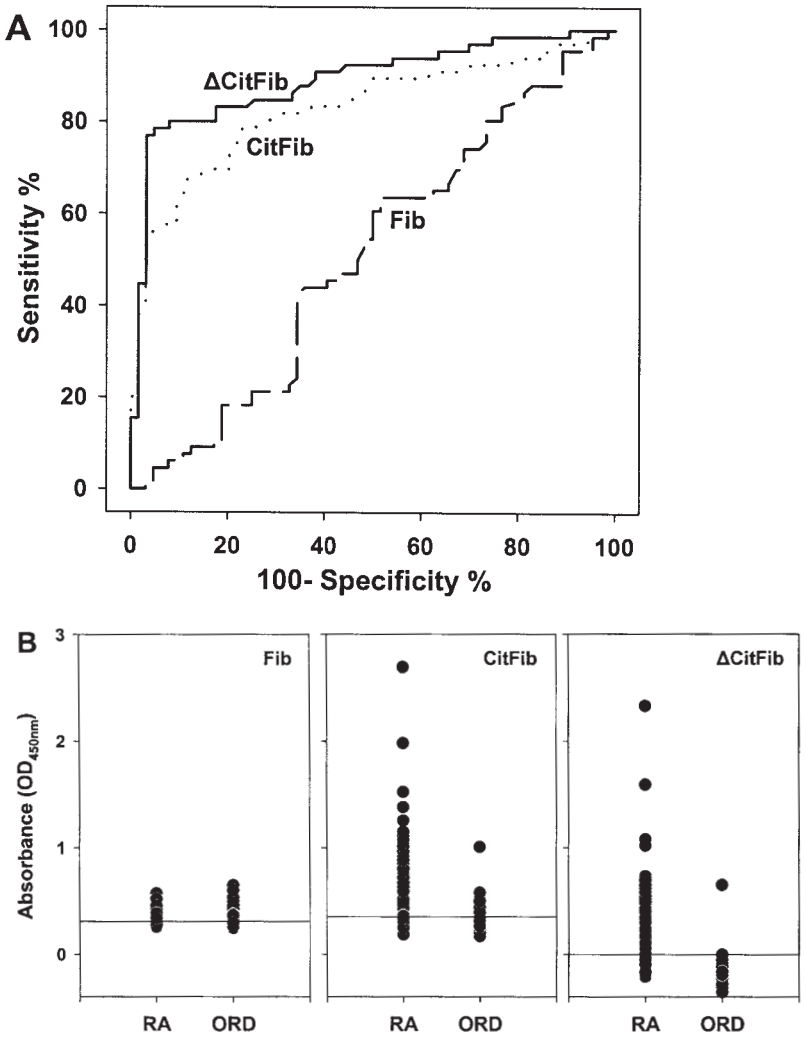

Figure 1. ROC curve analysis and individual results for Fib, CitFib, and ΔCitFib antibody reactivity in the cohort of patients with RA or other rheumatic diseases (ORD). A. Sensitivity and specificity was calculated for all potential cutoff values and plotted as ROC curves (65 patients with RA, 63 patients with ORD). B. Individual serum antibody reactivity to Fib, CitFib, and ΔCitFib from patients with RA or ORD plotted with the optimal cutoff values obtained from ROC curve analysis for each assay. Sensitivities, specificities, positive likelihood ratios, and negative likelihood ratios were Fib 64%, 48%, 1.23, 0.75; CitFib 82%, 83%, 4.67, 0.22; ΔCitFib 75%, 98%, 47.49, 0.25, respectively.

nostic measures, we tested for serum reactivity to CCP[2] and IgM-RF. We also included in the analysis an additional 49 patients with PsA to confirm the specificity of reactivity for ΔCitFib using the cutoff value described above. Predetermined cutoff values were used for CCP[2] and IgM-RF as described in Materials and Methods. A summary of these results is presented in Table 1, which shows that the majority of RA patients had CCP[2]-reactive antibodies or IgM-RF, resulting in a sensitivity of 82% and 80%, respectively. These values were similar to those obtained for ΔCitFib. There was, however, a large difference in the specificity of these assays, as both ΔCitFib and CCP[2] were found rarely in patients with ORD (2/112 and 5/112, respectively), while IgM-RF was detected quite frequently (30/112). Almost all patients positive for ΔCitFib were also positive for CCP[2] (49/50), while CCP[2] reactivity was evident in additional patients with both RA (5 patients) and ORD (3 patients).

We also addressed whether a correlation between antibody levels was evident in the serum samples by regression analysis (65 patients with RA and 63 patients with ORD). As expected, there was a significant correlation between ΔCitFib and CCP[2] levels ($r = 0.695$, $p < 0.01$; Figure 2A), while IgM-RF levels were independent of antibody reactivity to either citrullinated antigen (Figure 2B, 2C).

DISCUSSION

We analyzed the presence of serum autoantibodies to citrullinated fibrinogen in the context of reactivity to both CCP[2] and IgM-RF in patients with RA and other rheumatic diseases. Anti-CitFib antibodies have a high specificity for the diagno-

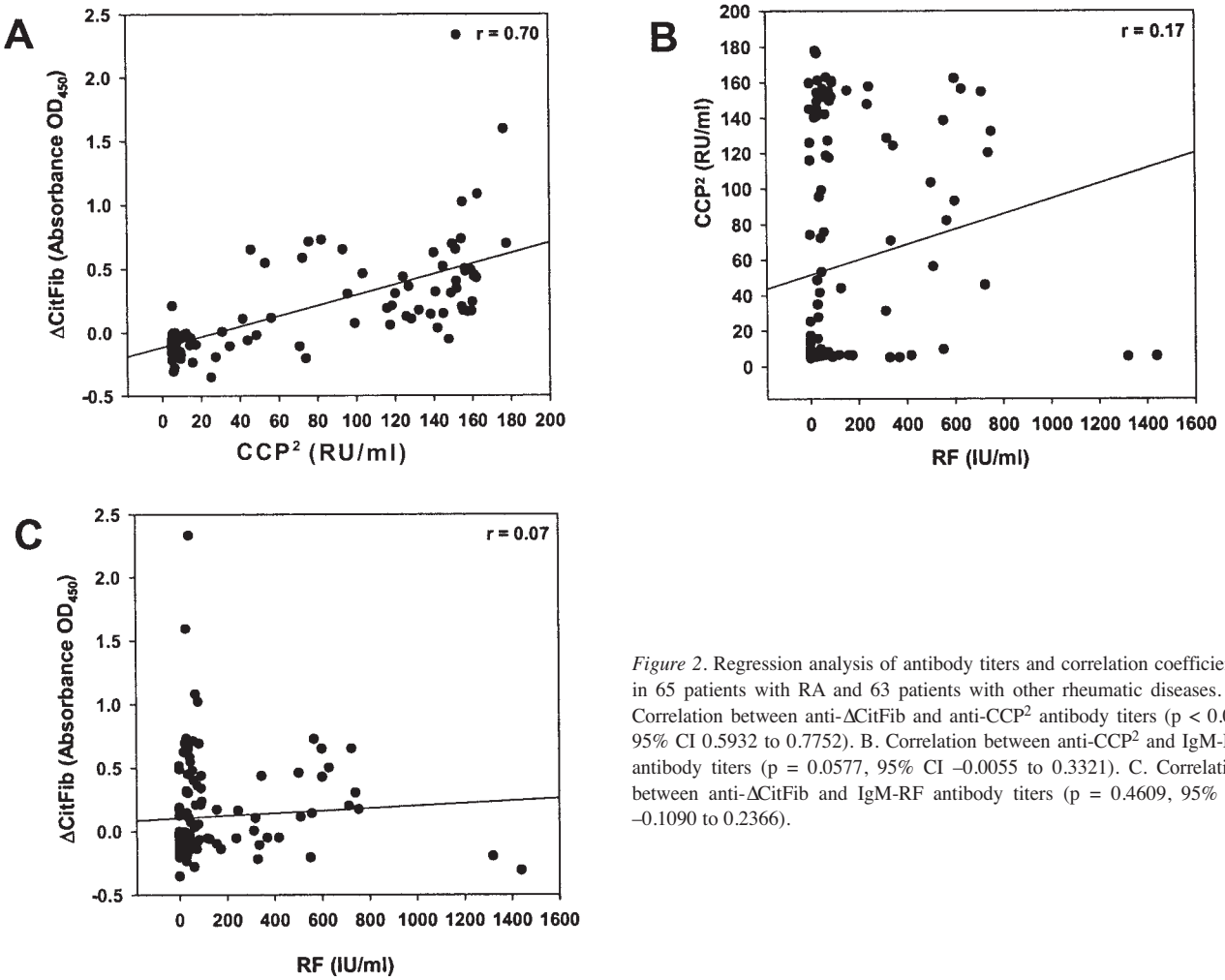

Figure 2. Regression analysis of antibody titers and correlation coefficients in 65 patients with RA and 63 patients with other rheumatic diseases. A. Correlation between anti-ΔCitFib and anti-CCP^2 antibody titers ($p < 0.01$, 95% CI 0.5932 to 0.7752). B. Correlation between anti-CCP^2 and IgM-RF antibody titers ($p = 0.0577$, 95% CI –0.0055 to 0.3321). C. Correlation between anti-ΔCitFib and IgM-RF antibody titers ($p = 0.4609$, 95% CI –0.1090 to 0.2366).

sis of RA and largely overlap with reactivity to CCP^2. Indeed, the concordance of reactivity to these citrullinated antigens was quite high, with only 9/177 serum samples showing differential binding, 8 samples positive for CCP^2 only (5 RA, 3 ORD), and one positive for ΔCitFib only (RA). The additional reactivity to CCP^2 was likely citrulline-specific, since nonspecific binding of these 8 sera to unmodified fibrinogen was not evident [average OD for Fib = 0.326 (range 0.263–0.362) vs overall average of 0.341]. Whether these patients have autoantibodies that bind citrullinated vimentin (another protein found in the inflamed synovium) remains to be determined[6,7]. IgM-RF was found in a number of patients with ORD and had a poor specificity in this cohort. This is not entirely surprising, considering that patients with diseases such as systemic lupus erythematosus, Sjögren's syndrome, and hepatitis C virus-associated arthralgia frequently possess high titers of serum IgM-RF (as seen in this cohort)[8,9]. In accord with previous findings, antibody reactivity to citrullinated antigens could be found in a number of RA patients that were IgM-RF-negative, further supporting the utility of these assays in the diagnosis of RA. Importantly, anticitrulline antibody was rarely detected in patients with PsA, despite its often similar clinical presentation, including the presence of positive rheumatoid factor. This highlights an important difference in these disorders.

Our study demonstrates that antibodies identified by a nonphysiologic antigen used for diagnostic purposes only (CCP) often indicates, in a high proportion of patients with RA, the existence of an immune response to an antigen, citrullinated fibrinogen, that may play a pathogenic role in this disease. Thus, citrullinated fibrinogen has been identified in RA synovial tissue by others, and we have been able to induce arthritis experimentally with citrullinated fibrinogen immunization in DR4-IE tg mice that express the HLA shared-epitope[10].

A characteristic of these RA-specific antibodies is that citrulline is specifically targeted in the context of certain proteins or peptides, while no reactivity is seen to the unmodified forms of these antigens. In accord with this, reactivity to unmodified fibrinogen in this cohort was not different between patients with RA and those with other rheumatic diseases, nor was a difference seen when patients were grouped according to positive antibody reactivity to ΔCitFib or CCP^2 (data not shown). This circumstance is rather unusual in light of data obtained from patients with other autoimmune dis-

eases, where posttranslational modifications may initially trigger autoantibody production, but then antibody specificity often spreads intramolecularly to target other unmodified regions of the autoantigen, a phenomenon known as epitope spreading[11-13]. Why, then, doesn't intramolecular epitope spreading occur in RA patients that have anticitrullinated fibrinogen antibodies?

Based on our finding and those of others, we propose a model that may account for this citrulline-specific response. In order to activate IgG-secreting B cells, CD4 T cell help must be provided, a mechanism that can be initiated by MHC class II molecules that contain the RA shared epitope. We showed previously that the conversion of arginine to citrulline at the peptide side-chain position interacting with the shared epitope significantly increases peptide-MHC affinity and leads to the activation CD4 T cells[14]. While this citrulline-specific T cell activation is sufficient to provide help to B cells for the production of IgG antibodies, it does not impart intramolecular epitope restriction in antibody production, a phenomenon that has been observed experimentally by ourselves and documented by others[15]. Therefore, in order for the antibody response to be confined to citrulline-containing regions of the antigen there must be additional mechanisms of tolerance in play. This is likely mediated by mechanisms of B cell tolerance such as deletion, receptor editing, or inactivation, since these processes occur during exposure to systemically expressed self-antigens found at high concentration[16-20], such as fibrinogen. B cells, then, should be tolerized to unmodified fibrinogen, since it is found at mg/ml quantities in the plasma, suggesting that only those cells with Ig receptors with the potential to interact with citrulline-containing regions of fibrinogen would be amenable to CD4 T cell help. Tolerance then appears to be present at both the T and B cell level and is likely required for the citrulline-specific antibody response to fibrinogen seen in patients with RA.

Our studies provide direct evidence that citrullinated fibrinogen, the predominant citrullinated antigen found in diseased synovium, is a target of autoantibodies in the majority of patients with RA, and that reactivity to this antigen largely overlaps that with CCP[2]. Due to the specificity of this antibody response, further study of its perpetuation will likely yield insight regarding the etiology of this complex disease.

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