Antibodies to Citrullinated Human Fibrinogen Synthetic Peptides in Diagnosing Rheumatoid Arthritis

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Since aggressive therapy given early in the rheumatoid arthritis (RA) disease course has the greatest therapeutic potential, early diagnostic tests with both high specificity and sensitivity are desirable. Rheumatoid sera were found to contain antibodies against citrullinated peptides, which are considered to be highly specific markers of RA. In the present work several analogues of the α- and β-chains of fibrin peptides containing different degrees of citrullination have been synthesized and analyzed by ELISA using 111 sera from RA patients. In addition, we have also investigated the synergistic effects of different presentation formats of the synthetic constructs. We have designed chimeric and cyclic peptides that bear different peptide sequences within the same molecule. Our results indicate that the synthesis of peptides bearing fibrinogen and filaggrin domains could be a robust method for the design of useful diagnostic strategies in RA.

Introduction

Rheumatoid arthritis (RA[a]) is one of the most common autoimmune diseases of unknown origin, affecting 0.5−1% of the population, that progressively destroys synovial joints and can cause deformity and loss of function as well as systemic complications.

Aggressive therapy given early in the disease course has the greatest therapeutic potential; therefore, to prevent joint destruction, early diagnosis and treatment are required. The classification criteria of the American College of Rheumatology (ACR)[1] are mainly based on clinical parameters; however these criteria are not very suitable for the diagnosis of early RA.[2,3] Although in some patients the diagnosis of RA can be made during the first consultation, in many of them, however, it would be very useful for clinicians to have a serological test that can distinguish RA from other types of rheumatic diseases at an early stage of the disease. In this sense, there are a few RA specific antibodies: anti-perinuclear factor (APF), anti-filaggrin antibodies (AFA), and anti-keratin antibodies (AKA), the epitopes recognized by them being generated by a post-translational modification that is the deimination of the natural amino acid arginine to citrulline by means of the peptidylarginine deiminase.[4]

Antibodies against citrullinated proteins and peptides belong to the most specific serological markers for diagnosing RA. The main advantages of citrullinated peptides over citrullinated proteins for the detection of RA autoantibodies have been reviewed by Nijenhuis et al.[5] In fact, the first commercial assays that have been recognized as a useful disease marker of RA were ELISA cyclic synthetic peptides based tests because they are very specific for RA, are present in more than 70% of the patients, are detectable very early in the disease course, even in preclinical phases, and identify those rheumatoid patients with a poor prognosis and more destructive joint involvement.[6]

In spite of the important progress in the characterization of anticitrullinated protein autoantibodies on filaggrin, it remained clear that citrullinated filaggrin was probably not the in vivo target of the autoantibodies that are present in RA patients but rather an antigen cross-recognized in vitro. In fact, epithelial tissues are not affected during RA, and filaggrin is not expressed in articular tissues. For this reason, new assay methods based on antigens present on the rheumatoid synovia are being developed. In this regard, Koivula et al. have found that synthetic citrullinated peptides related to the carboxy-terminal telopeptides of type I and type II collagens can be used to detect autoantibodies in RA patients.[7] These peptides, characterized by the sequences −YYXA (α1 chain of type I collagen), −FYXA (α2-chain of type I collagen), and −YMXA (α1 chain of type II collagen), where X stands for citrulline, were tested with the chemiluminescence method (Nichols Advantage system) in order to develop a sensitive immunoassay for detecting RA.[8]

Other proteins of the synovial tissue that can be citrullinated are α- and β-chains of fibrin and the Sa antigen (identified as citrullinated vimentin). On the basis of several biochemical and immunological arguments Masson-Bessière et al.[9] identified citrullinated fibrin as the major synovial target of anticitrullinated protein autoantibodies. Recently, Sebbag et al.[10] identified 18 fibrin-derived peptides bearing anticitrullinated protein autoantibodies epitopes, two of them being located in the central globular domain of the protein. These authors reported two

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[a] Abbreviations: RA, rheumatoid arthritis; ELISA, enzyme-linked immunosorbent assay; ACR, American College of Rheumatology; APF, anti-perinuclear factor; AFA, anti-filaggrin antibodies; AKA, anti-keratin antibodies; CCP1, cyclic citrullinated peptide commencial kit 1; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OD, optical density; ODmab, 4-[*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino]benzyl ester; PsA, psoriatic arthritis; ROC, receiver operating characteristic curves; AUC, area under the curve; CCP2, cyclic citrullinated peptide commercial kit 2; HLA, human leukocyte antigen; SE, shared epitope; Fmoc, 9-fluorenylmethoxycarbonyl; Trt, triphenilmethyl; ^{t}Bu, *tert*-butyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Boc, *tert*-butoxycarbonyl; HOBt, 1-hydroxybenzotriazole; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, dimethylformamide; DIPEA, *N*,*N*′-diisopropylethylamine; TFA, trifluoroacetic acid; TIS, triisopropylsilane; HMPA, hydroxymethylphenoxyacetic acid; MS, mass spectrometry; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; DMAP, 4-dimethylaminopyridine; ES-MS, electrospray mass spectrometry; EDT, 1,2-ethanedithiol; BSA, bovine albumin serum; SDS, sodium dodecyl sulfate; PBS, phosphate buffer solution; sulfo-NHS, sulfo-*N*-hydroxysuccinimide; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; DCM, dichloromethane.

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Table 1. Sequences of α-Fibrin and *β*-Fibrin Derived Peptides Synthesized

name	peptide	sequence[a]
	(a) α-Fibrin Derived Peptides	
p1	αfib(185−202)	S R A L A R E V D L K D Y E D Q Q K
p2	[Cit^{190}]αfib(185−202)	S R A L A **X** E V D L K D Y E D Q Q K
p3	[Cit^{186,190}]αfib(185−202)	S **X** A L A **X** E V D L K D Y E D Q Q K
p4	[Cit^{186}]αfib(185−202)	S **X** A L A R E V D L K D Y E D Q Q K
p5	αfib(208−225)	I A K D L L P S R D R Q H L P L I K
p6	[Cit^{218}]αfib(208−225)	I A K D L L P S R D **X** Q H L P L I K
p7	[Cit^{216,218}]αfib(208−225)	I A K D L L P S **X** D **X** Q H L P L I K
p8	[Cit^{216}]αfib(208−225)	I A K D L L P S **X** D R Q H L P L I K
p9	αfib(418−435)	G N V S P G T R R E Y H T E K L V T
p10	[Cit^{426}]αfib(418−435)	G N V S P G T R **X** E Y H T E K L V T
p11	[Cit^{425,426}]αfib(418−435)	G N V S P G T **X** **X** E Y H T E K L V T
p12	[Cit^{425}]αfib(418−435)	G N V S P G T **X** R E Y H T E K L V T
p13	αfib(501−518)	S G I G T L D G F R H R H P D E A A
p14	[Cit^{512}]αfib(501−518)	S G I G T L D G F R H **X** H P D E A A
p15	[Cit^{510}]αfib(501−518)	S G I G T L D G F **X** H R H P D E A A
p16	[Cit^{510,512}]αfib(501−518)	S G I G T L D G F **X** H **X** H P D E A A
p17	αfib(617−631)	H S T K R G H A K S R P V R G
p18	[Cit^{630}]αfib(617−631)	H S T K R G H A K S R P V **X** G
p19	[Cit^{627,630}]αfib(617−631)	H S T K R G H A K S **X** P V **X** G
p20	[Cit^{627}]αfib(617−631)	H S T K R G H A K S **X** P V R G
p21	[Cit^{621}]αfib(617−631)	H S T K **X** G H A K S R P V R G
p22	[Cit^{621,630}]αfib(617−631)	H S T K **X** G H A K S R P V **X** G
p23	[Cit^{621,627}]αfib(617−631)	H S T K **X** G H A K S **X** P V R G
p24	[Cit^{621,627,630}]αfib(617−631)	H S T K **X** G H A K S **X** P V **X** G
	(b) *β*-Fibrin Derived Peptides	
p25	*β*fib(40−57)	F F S A R G H R P L D K K R E E A P
p26	[Cit^{53}]*β*fib(40−57)	F F S A R G H R P L D K K **X** E E A P
p27	[Cit^{47}]*β*fib(40−57)	F F S A R G H **X** P L D K K R E E A P
p28	[Cit^{47,53}]*β*fib(40−57)	F F S A R G H **X** P L D K K **X** E E A P
p29	[Cit^{44,47,53}]*β*fib(40−57)	F F S A **X** G H **X** P L D K K **X** E E A P
p30	[Cit^{44,47}]*β*fib(40−57)	F F S A **X** G H **X** P L D K K R E E A P
p31	[Cit^{44,53}]*β*fib(40−57)	F F S A **X** G H R P L D K K **X** E E A P
p32	[Cit^{44}]*β*fib(40−57)	F F S A **X** G H R P L D K K R E E A P
p33	*β*fib(43−62)	A R G H R P L D K K R E E A P S L R P A
p34	[Cit^{60}] *β*fib(43−62)	A R G H R P L D K K R E E A P S L **X** P A
p35	[Cit^{53}]*β*fib(43−62)	A R G H R P L D K K **X** E E A P S L R P A
p36	[Cit^{53,60}]*β*fib(43−62)	A R G H R P L D K K **X** E E A P S L **X** P A
p37	[Cit^{47,53,60}]*β*fib(43−62)	A R G H **X** P L D K K **X** E E A P S L **X** P A
p38	[Cit^{47,60}]*β*fib(43−62)	A R G H **X** P L D K K R E E A P S L **X** P A
p39	[Cit^{47,53}]*β*fib(43−62)	A R G H **X** P L D K K **X** E E A P S L R P A
p40	[Cit^{47}]*β*fib(43−62)	A R G H **X** P L D K K R E E A P S L R P A
p41	[Cit^{44,47,53,60}]*β*fib(43−62)	A **X** G H **X** P L D K K **X** E E A P S L **X** P A
p42	[Cit^{44,47,53}]*β*fib(43−62)	A **X** G H **X** P L D K K **X** E E A P S L R P A
p43	[Cit^{44,47,60}]*β*fib(43−62)	A **X** G H **X** P L D K K R E E A P S L **X** P A
p44	[Cit^{44,47}]*β*fib(43−62)	A **X** G H **X** P L D K K R E E A P S L R P A
p45	[Cit^{44,53,60}]*β*fib(43−62)	A **X** G H R P L D K K **X** E E A P S L **X** P A
p46	[Cit^{44}]*β*fib(43−62)	A **X** G H R P L D K K R E E A P S L R P A
p47	[Cit^{44,53}]*β*fib(43−62)	A **X** G H R P L D K K **X** E E A P S L R P A
p48	[Cit^{44,60}]*β*fib(43−62)	A **X** G H R P L D K K R E E A P S L **X** P A
p49	*β*fib(72−89)	R R R P A K A A A T Q K K V E R K A
p50	[Cit^{87}]*β*fib(72−89)	R A R P A K A A A T Q K K V E **X** K A
p51	[Cit^{74}]*β*fib(72−89)	R A **X** P A K A A A T Q K K V E R K A
p52	[Cit^{74,87}]*β*fib(72−89)	R A **X** P A K A A A T Q K K V E **X** K A
p53	[Cit^{72,74,87}]*β*fib(72−89)	**X** A **X** P A K A A A T Q K K V E **X** K A
p54	[Cit^{72,74}]*β*fib(72−89)	**X** A **X** P A K A A A T Q K K V E R K A
p55	[Cit^{72,87}]*β*fib(72−89)	**X** A R P A K A A A T Q K K V E **X** K A
p56	[Cit^{72}] *β*fib(72−89)	**X** A R P A K A A A T Q K K V E R K A
p57	*β*fib(151−168)	R K D L W Q K R Q K Q V K D N E N V
p58	[Cit^{158}]*β*fib(151−168)	L K D L W Q K **X** Q K Q V K D N E N V

[a] **X**: citrulline.

citrullinated fibrin-derived peptides, namely, [Cit^{60,72,74}]*β*-fibrin-(60−74) and [Cit^{38,42}]α-fibrin(36−50) peptides, for which they observed that all the anticitrullinated protein autoantibodies-positive sera tested reacted to one and/or the other. Besides, we analyzed in a previous work[11] the reactivity of 133 sera from patients with well-characterized rheumatic diseases, including 66 patients with RA, with several overlapped fibrin citrullinated synthetic peptides. The results of the immunoassays highlighted the usefulness of fibrin-related peptides in RA diagnosis and especially the ability and specificity of the [Cit^{621,627,630}]α-fibrin-(617−631) peptide sequence to recognize the autoantibodies that are present in RA patients. We concluded that fibrin-related peptides may allow the development of improved tests for the diagnosis of RA probably with higher diagnostic performance than those available today, which are based on the detection of in vitro citrullinated whole filaggrin or filaggrin-derived cyclic citrullinated peptides.

In the present work several analogues of α- and *β*-fibrin peptides containing different degrees of deimination have been synthesized. In addition, we have also investigated the synergistic effects of different presentation formats of the epitopes by designing chimeric and cyclic peptides related to α- and

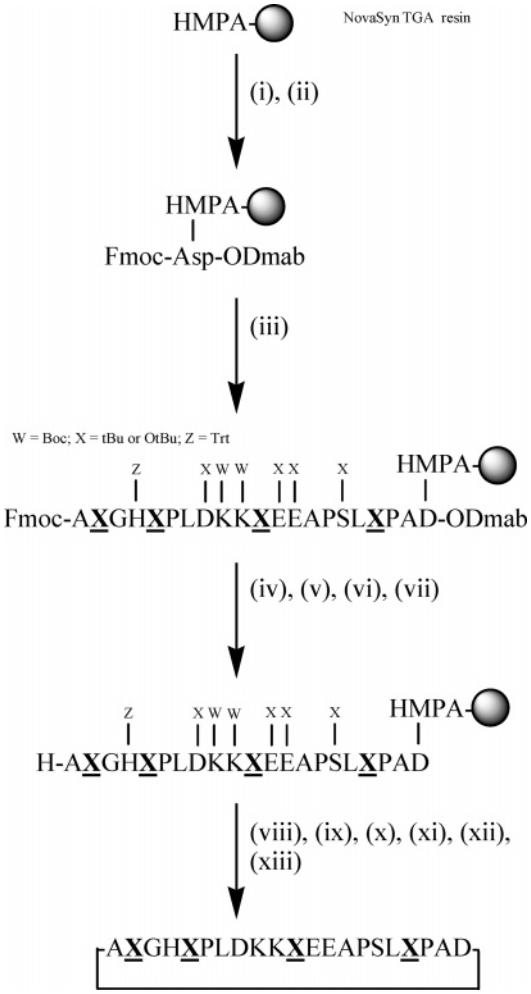

Figure 1. Strategy for solid-phase peptide synthesis of β-fibrin(43−62) head-to-tail cyclic peptide (p38HT). Reagents and conditions are as follows: (i) Fmoc-Asp-ODmab DCM, DIPCDI, 20 min 0 °C; (ii) DMAP, DMF, 3 h; (iii) solid-phase peptide synthesis (SPPS); (iv) 20% piperidine/DMF; (v) 3% hydrazine/DMF; (vi) 20% H_2O/DMF, 16 h; (vii) DMF, DCM, MeOH; (viii) 1% DIPEA/DMF; (ix) 1% HOBt/DMF; (x) PyBOP/DMF, DCM, 72 h; (xi) 1% DIPEA/DMF; (xii) DCM/MeOH; (xiii) TFA/EDT/TIS/H_2O (95:2:1:2), 3 h.

β-fibrin proteins that bear different peptide sequences within the same molecule. Finally, a chimeric peptide bearing the filaggrin cyclic peptide that constitutes the CCP1 test and a fibrin-related sequence has been synthesized and used to determine the presence of specific RA autoantibodies in patients' sera.

Results and Discussion

There is a growing interest in developing specific tests to help in early differentiation of RA and other forms of rheumatic joint and connective tissue diseases, as well as the identification of RA patients with a poorer prognosis and/or early RA patients. Their incorporation into clinical practice would allow identification of those patients who require more aggressive therapy from the moment of diagnosis, leading to more efficient disease control, less joint damage, and better prognosis of disease outcome.

Recent studies have identified antibodies against peptide sequences that have undergone a process of deimination, such as filaggrin (anti-filaggrin antibodies, AFA) in the sera of patients with RA. These AFA are highly specific for RA but have a limited sensitivity. Recently, it has been shown that these antibodies recognize citrullinated peptide sequences of the fibrin chains, which are abundant in rheumatoid synovium. The use

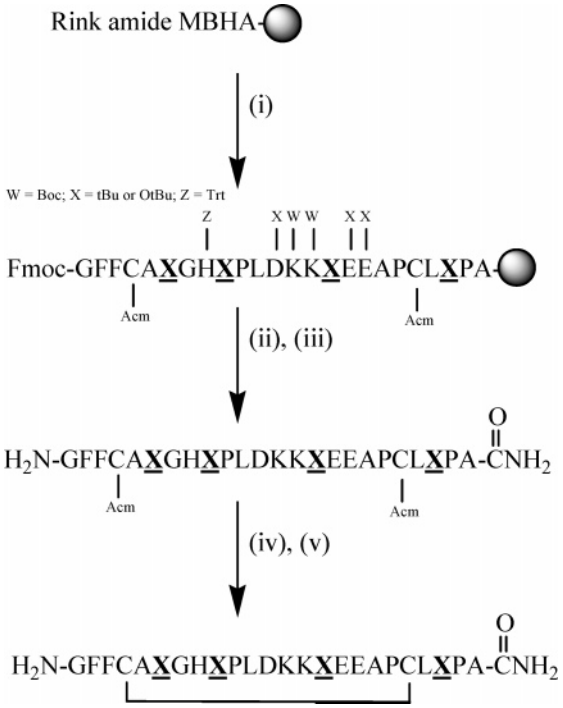

Figure 2. Strategy for solid-phase peptide synthesis of β-fibrin(39−62) disulfide cyclic peptide (p38SS). Reagents and conditions are as follows: (i) solid-phase peptide synthesis (SPPS); (ii) 20% piperidine/DMF; (iii) TFA/EDT/TIS/H_2O (95:2:1:2), 3 h; (iv) I_2/MeOH, 60 min, H_2O, 90 min; (v) DCM.

of fibrin-derived peptides as an antigenic base could improve the sensitivity of ELISA tests in comparison with those commercially available today, which use as an antigenic substrate cyclic citrullinated peptides derived from filaggrin, a protein that, unlike fibrin, is neither expressed at synovial level nor expressed at other joint structures.

In a recent paper we reported the usefulness of fibrin-related peptides in RA diagnosis.[11] In the present work, in order to better comprehend the RA sera reactivities with the fibrin selected peptides, we proceeded to analyze the influence of degree of deimination by synthesizing peptide analogues with different arginine/citrulline relationships. Also, the identification of new epitope-bearing regions in α- and β-chains of fibrin was performed by computerized prediction of antigenicity after analyzing the hydrophilicity, accessibility, and antigenicity profiles of the protein according to Hopp and Woods,[12] Janin,[13] and Welling et al.[14] As previously described,[11] regions with high indices and a large number of residues with high turn probability, which tend to be distributed on the surfaces of proteins and therefore are better recognized by autoantibodies, were identified. Besides, the fibrin sequences that contained the largest number of arginine residues within these regions and therefore the most likely to contain citrulline in the in vivo situation were selected for peptide synthesis.

The selected peptides are shown in Table 1. They were successfully synthesized in the solid phase and characterized by amino acid analysis, analytical high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

These peptides were assayed in a direct binding assay (ELISA test) to analyze their recognition by the autoantibodies present in RA sera. To identify which peptide was the best substrate for the RA autoantibodies, all peptide variants of the selected regions were first assayed with 33 RA sera and 40 control sera. As shown in Figure 4, an increase in sensitivity was seen

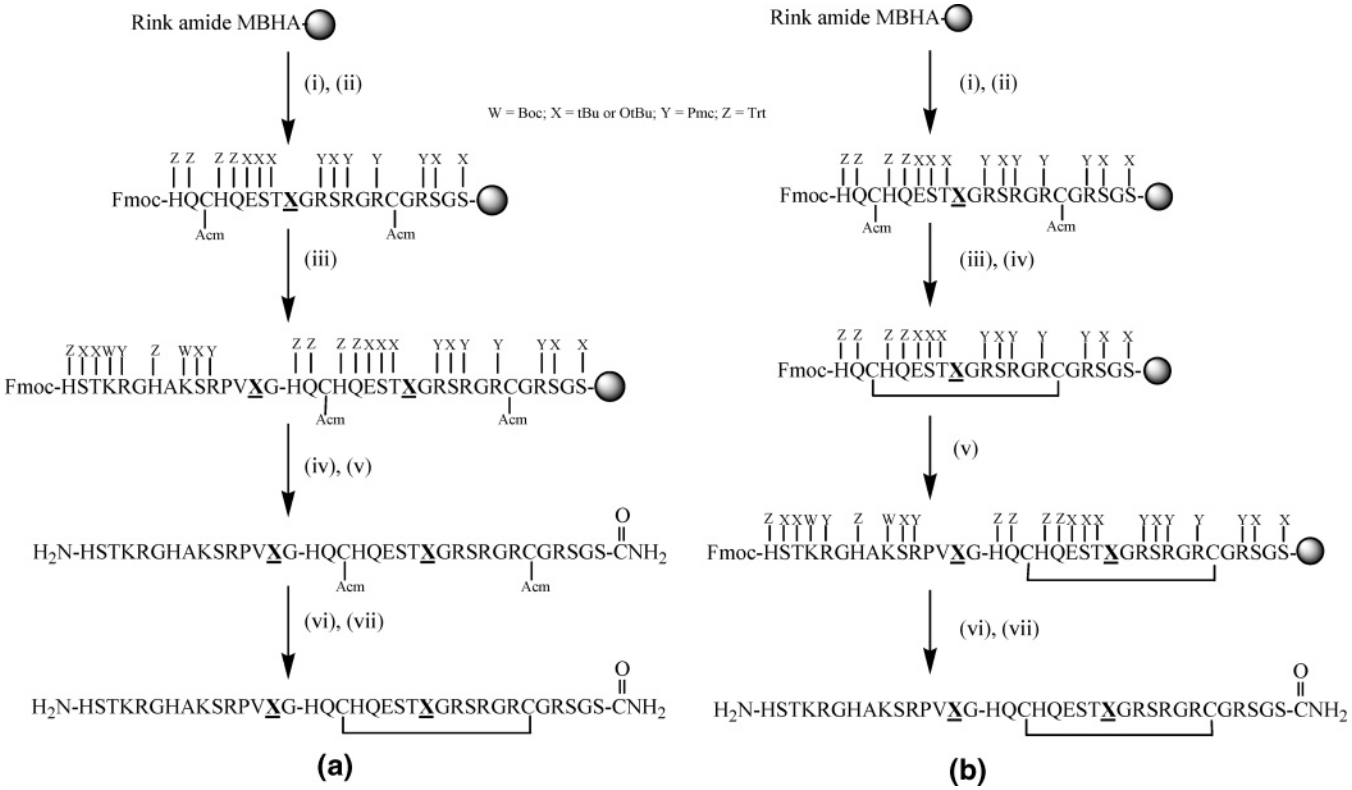

Figure 3. Strategies for the solid-phase peptide synthesis of chimeric α-fibrin−filaggrin synthetic peptide (p18-cfc1cyc). (a) Reagents and conditions are as follows: (i) 20% piperidine/DMF; (ii) solid-phase of cfc1 peptide synthesis (SPPS); (iii) solid-phase of p18 peptide synthesis; (iv) 20% piperidine/DMF; (v) TFA/EDT/TIS/H_2O (95:2:1:2); (vi) I_2/MeOH, 60 min, H_2O, 90 min; (vii) DCM. (b) Reagents and conditions are as follows: (i) 20% piperidine/DMF; (ii) solid-phase of cfc1 peptide synthesis (SPPS); (iii) I_2/MeOH/DCM, 4 h; (iv) DMF/MeOH; (v) solid-phase of p18 peptide synthesis; (vi) 20% piperidine/DMF; (vii) TFA/TIS/H_2O (90:5:5).

Table 2. Cyclic β-Fibrin Peptides Synthesized

name	sequence[a]
p38HT	A R G H **X** P L D K K R E E A P S L **X** P A D
p38SS	G F F C A R G H **X** P L D K K R E E A P C L **X** P A

[a] **X**: citrulline.

Table 3. Cyclic α-Fibrin Peptides Synthesized

name[a]	sequence[b]
p18-sc	H C T K R G H A K C R P V **X** G
p19-sc	H C T K R G H A K C **X** P V **X** G
p22-sc	H C T K **X** G H A K C R P V **X** G
p18-lc	H C T K R G H A K S R P V **X** G I H T C P L
p19-lc	H C T K R G H A K S **X** P V **X** G I H T C P L
p22-lc	H C T K **X** G H A K S R P V **X** G I H T C P L

[a] sc = small cycle; lc = large cycle [b] **X**: citrulline.

predominantly for p18, p19, and p22 peptides from the α-fibrin chain in which arginine was substituted by citrulline in the 630 position. Although p24 also has a Cit in this specific position, this sequence is a completely citrullinated version of the α-fibrin (617−631) region. In agreement with our previous results,[11] a high positive net charge could be an additional factor that contributes to the peptide reactivity. In our hands, by means of an adequate balance of the Arg/Cit residues, the peptides can probably adopt a conformation with enhanced capacity binding to autoantibodies, which is favorable for their diagnostic usefulness.

The rest of peptides belonging to the α-fibrin (617−631) region containing an unmodified arginine at the same position were clearly less reactive, leading to lower absorbance values. This fact agrees well with the results reported by Sebbag et al.[10] about the importance of citrullyl residue 630 in anticitrullinated protein autoantibodies binding. For the peptides of the β-fibrin chain, the region (43−62) rendered the best performance in the ELISA assays, the peptide p38 being the one that gave higher OD_{492} values. Therefore, this fibrin-derived immunoreactive peptide, which is located in the central globular domain of human fibrinogen molecule,[10] could also correspond to a portion of the β-fibrin chain accessible to anticitrullinated protein autoantibodies.

Having in mind that antigenic recognition can be significantly improved by restricting peptide mobility through cyclization[15−18] as well as the results published by Schellekens et al.[19] using a cyclic peptide variant of filaggrin as a diagnostic test with extreme specificity for RA, we pursued the strategy of fibrin peptides cyclization for the engineering of peptides with enhanced affinity to RA autoantibodies. In this regard two different cyclic versions of the β-fibrin (43−62) antigenic site were prepared (Table 2). The head-to-tail peptide (p38HT) correspond to the strict cyclic version of p38 and was obtained in the solid phase by forming an amide bond between the N-terminal end and the carboxyl group of an Asp residue introduced at the C-terminal end of the sequence orthogonally protected by the 4-[*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino]benzyl ester (ODmab) group (Figure 1). The disulfide analogue (p38SS) was formed by substituting two serine residues in this particular β-fibrin region, and the cyclization was performed in solution by iodine/methanol oxidation (Figure 2).

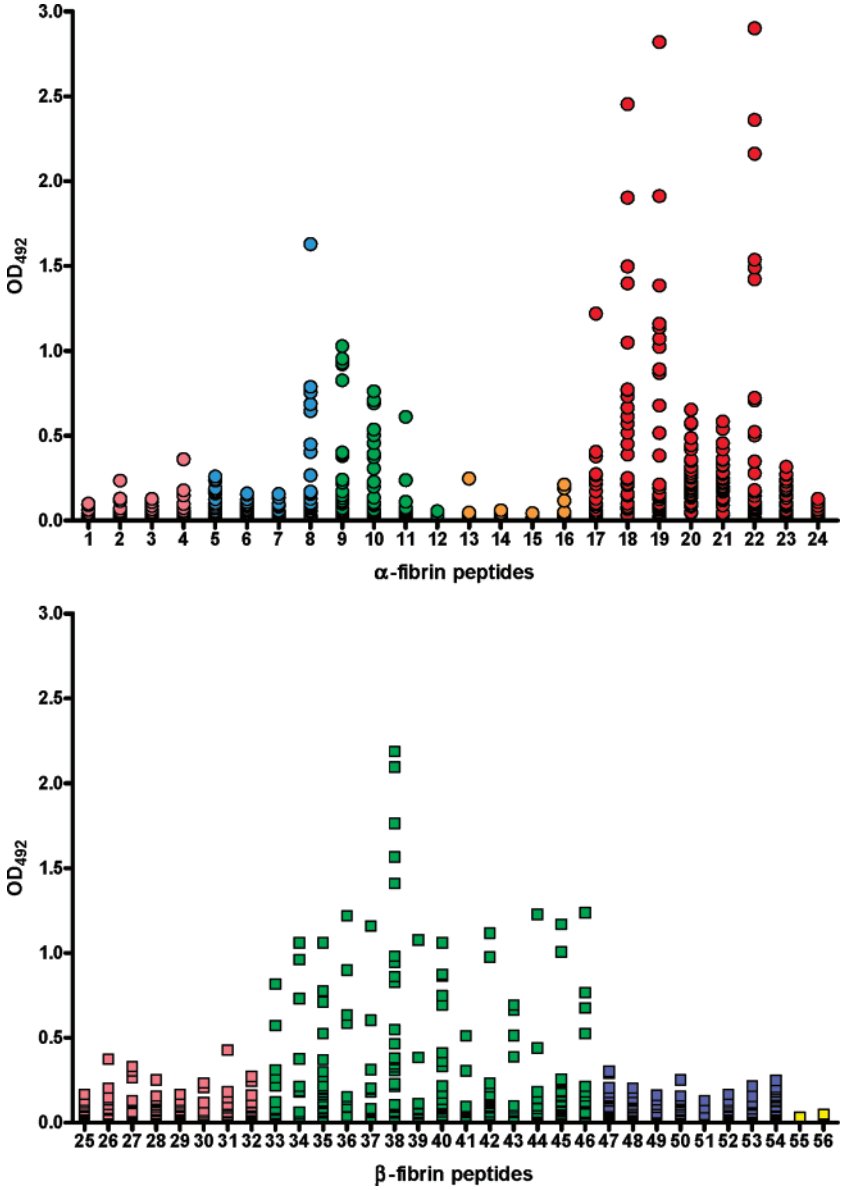

Figure 4. Reactivity of RA sera ($n = 33$) with α- and β-fibrin synthetic peptides.

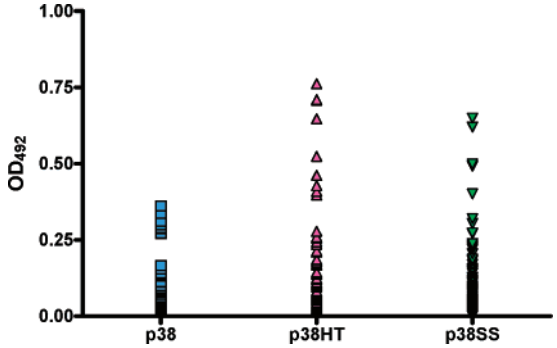

Figure 5. Reactivity of RA sera ($n = 111$) with β-fibrin lineal and cyclopeptides.

The two (head-to-tail and disulfide) cyclic peptides were tested by ELISA against 111 RA sera and 82 control sera. As shown in Figure 5, the ability of the linear peptide to detect autoantibodies was significantly improved by the cyclic peptides ($p < 0.05$); however nonsignificant differences in reactivity were found between the two types of cyclic peptides ($p = 0.162$). These results demonstrated, on one hand, that the constrained peptides could adopt conformations that favor antibody binding; i.e., the peptides may be presented in such a way that they easily adopt the conformational features of the original antigenic site of the native protein. On the other hand, the stabilization of this antigenic site by means of cyclization does not seem to be particularly related to the type of cyclic version of the analyzed β-fibrin peptide.

On the basis of these results and considering that according to Tugyi et al.[20] cyclopeptide with disulfide bridge provided the highest synthetic yield while the amide bond formation was less effective, disulfide versions of the selected α-fibrin peptides were prepared. To analyze the effects of cyclization on the antigenicity of p18, p19, and p22 peptides, two sizes of cycles were obtained (Table 3) and their reactivity was assessed in a cohort of 111 patients with RA and 82 patients with psoriatic arthritis (PsA). Although neither small nor large macrocyclic structures of p18 and p19 rendered an improved reactivity with the sera compared to the linear sequences, the specificities of the ELISA assays performed with the small cyclic compounds (p18-sc and p19-sc) were substantially increased (96.3% and 93.9%, respectively, vs 91.5% for the corresponding linear peptides). When the effect of the size cycle was compared, the ROC curve analysis (Figure 6) showed higher area under the

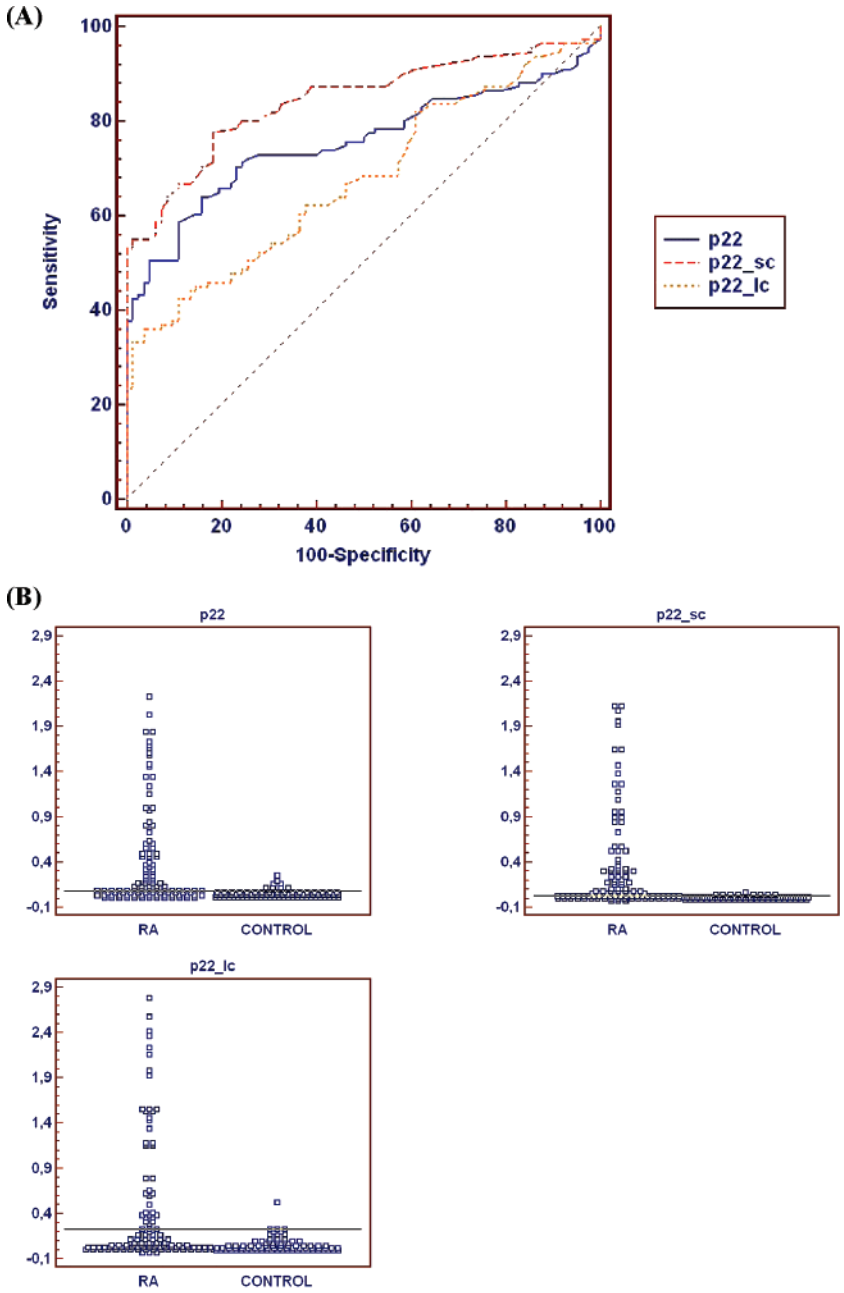

Figure 6. ROC curve analysis and individual results for linear (p22), small cycle peptide (p22-sc), and large cycle peptide (p22-lc) antibody reactivity in the cohort of patients with RA or PsA. (A) Sensitivity and specificity were calculated for all potential cutoff values and plotted ROC curves ($n = 111$ RA patients, $n = 82$ PsA patients). (B) Individual serum antibody reactivity to p22, p22-sc, and p22-lc from patients with RA or PsA (control) was plotted with the optimal cutoff values obtained from ROC curve analysis of each assay. Sensitivities, specificities, positive likelihood ratios, and negative likelihood ratios were 64.0%, 84.1%, 4.03, and 0.43 for p22; 77.5%, 81.7%, 4.24, and 0.28 for p22-sc; and 36,0%, 96.3%, 9.85, and 0.66 for p22-lc.

curve (AUC) values for the three small cycle-containing compounds (0.830, 0.749, and 0.844 for p18-sc, p19-sc, and p22-sc, respectively) compared to the large ones (0.718, 0.690, and 0.675 for p18-lc, p19-lc, and p22-lc, respectively). More pronounced effects of cyclization on antigenicity appear when the ring size in the p22 peptide is reduced. In Figure 6 the relationship between the sensitivity and specificity of the assays performed with p22 and the corresponding cyclic compounds for different cutoff values is presented graphically by means of the ROC curve. As shown, significantly ($p = 0$) higher values of AUC and sensitivity were obtained for p22-sc, thus indicating its usefulness for establishing the diagnosis of RA.

In the search of better antigenic peptides, recent research has also shown that there was an improvement in sensitivity and specificity when sera were tested not only with monomeric but also with multimeric peptides that contain more than one putative epitope.[21−23] Published results[18,24−26] demonstrate that chimeric peptides bearing different peptide sequences within the same molecule are more antigenic than the monomeric peptides and can be used to detect antibodies to more than one epitope simultaneously.

In the present work with the aim of investigating the synergistic effects of different presentation formats of the antigenic fibrin peptides, we designed and synthesized in the solid phase the chimeric peptides shown in Table 4. The results obtained using chimeric and a mixture of single peptides with several RA sera confirmed that the sensitivity of a chimeric peptides-based methodology was higher than that obtained with the

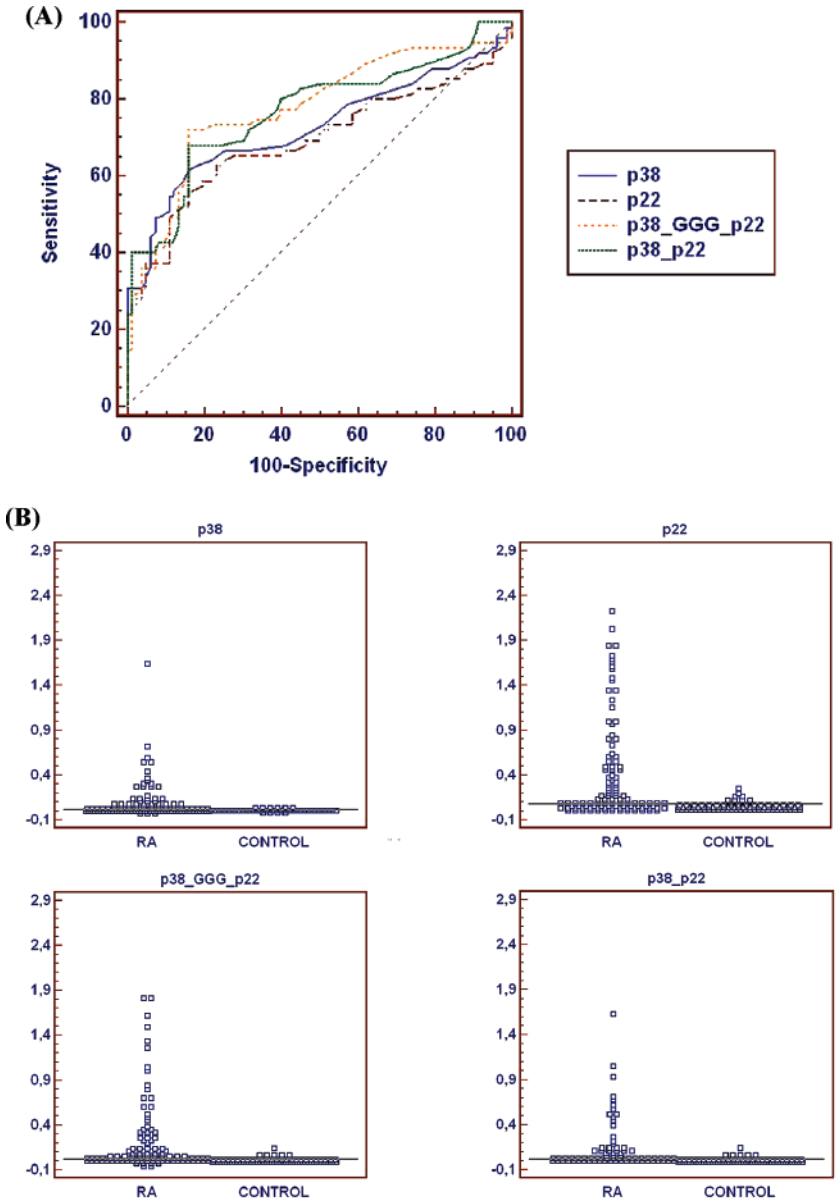

Figure 7. ROC curves analysis and individual results for linear β-fibrin peptide (p38), linear α-fibrin peptide (p22), chimeric peptide (p38-GGG-p22), and mixture of p38 and p22 (p38_p22) antibody reactivity in the cohort of patients with RA or PsA. (A) Sensitivity and specificity were calculated for all potential cutoff values and plotted ROC curves (n = 111 RA patients, n = 82 PsA patients). (B) Individual serum antibody reactivity to p38, p22, and p38-GGG-p22 from patients with RA or PsA (control) was plotted with the optimal cutoff values obtained from ROC curves analysis of each assay. Sensitivities, specificities, positive likelihood ratios, and negative likelihood ratios were 64.9%, 84.1%, 4.09, and 0.42 for p38; 64.0%, 84.1%, 4.03, and 0.43 for p22; 73.0%, 84.1%, 4.60, and 0.32 for p38-GGG-p22; and 68.%, 84.1%, 4.29, and 0.38 for p38+p22.

Table 4. Chimeric β-Fibrin−α-Fibrin and α-Fibrin−Filaggrin Synthetic Peptides

name	sequence[a]
	(a) β-Fibrin−α-Fibrin Peptides
p38-GGG-p18	ARGH**X**PLDKKREEAPSL**X**PA**GGG**HSTKRGHAKSRPV**X**G
p38-GGG-p19	ARGH**X**PLDKKREEAPSL**X**PA**GGG**HSTKRGHAKS**X**PV**X**G
p38-GGG-p22	ARGH**X**PLDKKREEAPSL**X**PA**GGG**HSTK**X**GHAKS**R**PV**X**G
	(b) α-Fibrin−Filaggrin Peptide
p18-cfc1cyc	HSTKRGHAKSRPV**X**GHQCHQEST**X**GRSRGRCGRSGS

[a] **X**: citrulline.

corresponding mixture of the two peptides. However, when analyzing by ELISA the chimeric peptides bearing α- and β-fibrin peptides by means of ROC curves, we could observe moderately higher AUC values for the chimeric peptides compared to the corresponding monomeric or the mixture of single peptides. As an example, in Figure 7 the results obtained for the p38-GGG-p22 chimeric peptide are shown.

More interesting results were obtained when performing the ROC curve analysis of the chimeric peptide that contains the α-fibrin p18 peptide and the cyclic filaggrin peptide, namely, cfc1cyc, which constitutes the CCP1 test.[19] The relationship between the sensitivity and specificity of the two peptides (cfc1cyc and p18-cfc1cyc) for different cutoff values is presented in Figure 8. The sensitivity of the p18-cfc1cyc ELISA was 82%,

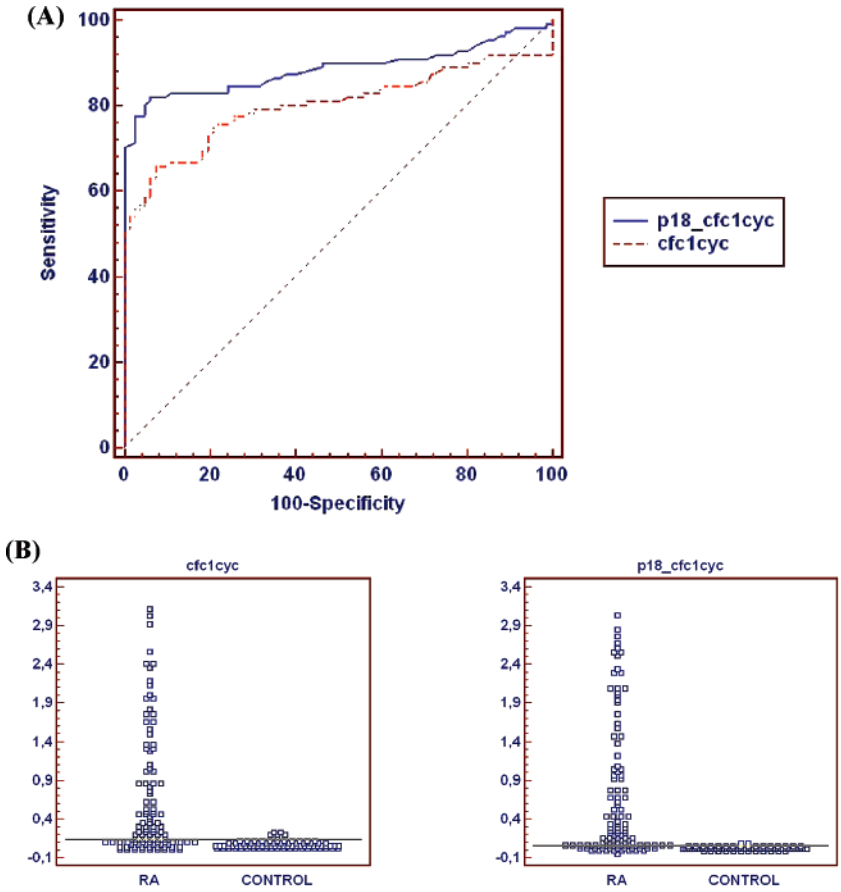

Figure 8. ROC curve analysis and individual results for cyclic filaggrin derived peptide (cfc1cyc) (15) and chimeric peptide (p18-cfc1cyc) reactivity in the cohort of patients with RA or PsA. (A) Sensitivity and specificity were calculated for all potential cutoff values and plotted ROC curves ($n = 111$ RA patients, $n = 82$ PsA patients). (B) Individual serum antibody reactivity to cfc1 and p18-cfc1cyc from patients with RA or PsA (control) was plotted with the optimal cutoff values obtained from ROC curves analysis of each assay. Sensitivities, specificities, positive likelihood ratios, and negative likelihood ratios were 65.8%, 92.7%, 8.99, and 0.37 for cfc1cyc and were 82.0%, 93.9%, 13.45, and 0.19 for p18-cfc1cyc.

which was significantly higher than the 65.8% sensitivity of the cfc1cyc ELISA ($p = 0.002$), the specificity being very high in both cases (93%). This high sensitivity without loss of specificity may be relevant taking into account that the control group consisted of patients affected by PsA, an inflammatory disease with a clinical picture that can simulate RA. On the other hand, the frequency of autoantibodies against the chimeric peptide p18-cfc1cyc (6%) in this control population is in accordance with the results obtained in the sera of patients with PsA in other studies using ELISA commercial tests (CCP2), where a prevalence of 5.6−7.8% has been reported.[27,28]

Finally, in order to improve the ELISA fibrin based tests, we combined peptide cocktails to search for an optimal diagnostic performance. The careful combination of selected specific peptides as antigens in highly sensitive and specific immunoassays has been described for a wide variety of infections.[21,22] Particularly, a combined sensitivity at the same level as the reference reagents without jeopardizing specificity has been obtained by combining peptides from different proteins.[23] On the basis of these observations, we assessed the serologic reactivity of a panel of 23 RA baseline sera, anti-CCP2 test negative, by a standard ELISA procedure using a mixture of synthetic peptides from the most relevant antigenically peptides of α- and β-chains of fibrin and the filaggrin cyclic peptide (Table 5).

These mixtures of peptides were only able to detect 4 of the 23 sera tested. Nevertheless, the chimeric peptide p18-cfc1cyc, composed of the α-fibrin and cyclic filaggrin sequences, was able to detect specific autoantibodies in 8 sera reaching a 34.8% of reactivity. This result demonstrates that the use of chimeric peptides containing several epitopes from different proteins within the same chemical molecule improves the sensitivity of the assay. These results also provide evidence that the use of physical mixtures of synthetic peptides could affect the sensitivity and specificity of the test by the effect of competition for binding to solid phase and for changes in the spatial distribution of antigenic determinants of bound peptides.

This is noteworthy from the results shown in Table 5 of the diagnostic values for RA of fibrin-related peptides. As has been described previously, p18-cfc1cyc was highly specific for RA, was more sensitive than the cfc1cyc (filaggrin-related peptide), and can even detect autoantibodies in CCP2 negative RA patients sera. Up to 43% of these CCP2 negative RA sera reacted to p18-cfc1cyc (α-fibrin−filaggrin peptide) and/or p38 (β-fibrin peptide), clearly indicating that fibrin peptides may allow the development of an improved test for the diagnosis of RA with higher sensitivity than those available today.

We also addressed whether a correlation between the detection of antibody levels by means of p18-cfc1cyc and CCP2 exists. As shown in Figure 9, there was a significant correlation between the fibrin−filaggrin containing peptide and the commercial test levels ($r = 0.86$, $p < 0.001$). These results reinforce the hypothesis that citrullinated fibrinogen is the target of the autoantibodies in RA patients, the synthesis of peptides bearing fibrinogen and filaggrin domains being a robust method for the design of useful diagnostic strategies in RA.

Even more interesting than the diagnostic value of fibrin-related peptides could be the possibility that the autoanti-

Table 5. Reactivity of 23 RA Baseline Sera CCP2 Negative with Synthetic α- and β-Fibrin Peptides

serum sample	p38[a]	p18-cfc1cyc[b]	α-fib+β-fib+cfc1cyc[c]
1	+	+	−
2	−	−	−
3	+	−	−
4	−	−	−
5	−	−	−
6	−	+	−
7	−	−	+
8	−	+	+
9	−	+	+
10	−	+	+
11	−	−	−
12	−	+	−
13	−	−	−
14	−	−	−
15	−	−	−
16	−	−	−
17	−	−	−
18	−	−	−
19	+	+	−
20	−	+	−
21	−	−	−
22	+	−	−
23	−	−	−
reactivity	(4/23) 17%	(8/23) 34.8%	(4/23) 17%

[a] p38 = β-fibrin-related peptide (see sequence in Table 1b). [b] p18-cfc1cyc = chimeric peptide α-fibrin−filaggrin (see sequence in Table 4). [c] α-fib+β-fib+cfc1cyc = p18+cfc1cyc (1:1); p19+cfc1cyc (1:1); p22+cfc1cyc (1:1); p18-sc+cfc1cyc (1:1); p19-sc+cfc1cyc (1:1); p22-sc+cfc1cyc (1:1); p18-lc+cfc1cyc(1:1); p19-lc+cfc1cyc(1:1); p22-lc+cfc1cyc(1:1); p38SS+cfc1cyc (1:1); p38HT+cfc1cyc (1:1); p38SS+p18-lc+cfc1cyc (1:1:1); p38SS+p19-lc+cfc1cyc (1:1:1); p38SS+p22-lc+cfc1cyc (1:1:1); p38HT+p18-lc+cfc1cyc (1:1:1); p38HT+p19-lc+cfc1cyc (1:1:1); p38HT+p22-lc+cfc1cyc (1:1:1).

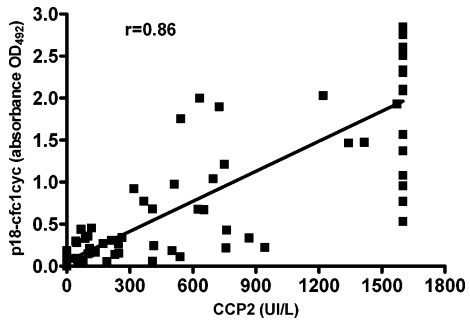

Figure 9. Correlation between anti-p18-cfc1cyc and anti-CCP2 antibody titers.

bodies detected by these peptides may have a prognostic value, in order to detect those patients with recent onset arthritis who are more prone to evolve to a destructive and disabled RA. Several prognostic markers for progressive joint destruction have been described in RA, including female gender, marked inflammatory activity at disease onset, genotype (HLA-DRB1*04 alleles and SE homozygosity), and the presence of rheumatoid factor and antibodies against citrullinated peptide.[29−31] More recently it has been described that the association of HLA class II genes and autoantibodies to cyclic citrullinated peptides influences the severity of rheumatoid arthritis.[32] However, it has also been described the difficulty of using these parameters in normal clinical practice to provide a differentiated therapeutic strategy, because their predictive value for progressive disease is far from 100%. In contrast, the absence of these parameters clearly has a greater clinical relevance in predicting patients who will not suffer disease progression.[33]

The analysis of whether the baseline presence or the titer of anti-p18cfc1cyc antibodies detected in the sera of RA patients is relevant to the identification of a group with a poorer prognosis or contrarily if their absence could be related to nonprogression is currently being investigated.

Conclusion

Our study reinforces the importance of citrullinated fibrin-related peptides in rheumatoid arthritis diagnosis. A chimeric peptide (p18-cfc1cyc) that contains fibrin and filaggrin domains has been designed and synthesized. It is highly specific for rheumatoid arthritis, is more sensitive than filaggrin-related peptides, and can even detect autoantibodies in rheumatoid arthritis patients CCP2 negative. This high sensitivity without loss of specificity may be relevant taking into account that the control group consisted of patients affected by psoriatic arthritis, an inflammatory disease with a clinical picture that can simulate rheumatoid arthritis. In conclusion, it has been demonstrated that p18-cfc1cyc is useful for the development of improved tests for the diagnosis of rheumatoid arthritis with higher sensitivity than those available today.

Experimental Section

Peptide Synthesis. Fifty-eight peptides of the α- and β-fibrin chains containing a different degree of deimination and whose primary sequence is shown in Table 1 were synthesized by semiautomated multiple solid-phase peptide synthesis on a peptide synthesizer (SAM, Multisyntech, Germany) as C-terminal carboxamides on a Tentagel RAM resin (Rapp Polymere GmbH, Germany) (100 mg, 0.28 meq/g) and following a 9-fluorenylmethoxycarbonyl (Fmoc) strategy. Amino acid side chain protection was effected by the following: triphenilmethyl (Trt) for glutamine, asparagine, histidine, and cysteine; *tert*-butyl (^{t}Bu) for aspartic acid, glutamic acid, serine, threonine, and tyrosine; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; and *tert*-butoxycarbonyl (Boc) for lysine and tryptophan.

The coupling reaction was performed in duplicate using 3-fold molar excesses of activated Fmoc-amino acids throughout the synthesis. The amino acids were activated essentially by means of treatment with *N*′,*N*′ diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt). For the second coupling, the carboxyl group was activated by means of addition of a phosphonium salt, benzotriazole-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), in the presence of HOBt and a base such as diisopropylethylamine (DIPEA). The Fmoc deprotection step was also accomplished twice with 20% piperidine in dimethylformamide (DMF) for 10 min. The efficiency of these reactions was evaluated by the ninhydrin colorimetric reaction.

Once the synthesis was complete, the cleavage and deprotection processes of the peptidyl resins were carried out in the semiautomatic synthesizer using the Multisyntech accessories available for this purpose. These reactions took place by means of treatment with 95% trifluoroacetic acid (TFA) in the presence of scavengers, basically 2.5% H_2O and 2.5% triisopropylsilane (TIS) for 4 h.

Peptides were isolated by precipitation with cold diethyl ether, centrifuged, and lyophilized in 10% acetic acid. The peptides were characterized by analytical HPLC on a Kromasil C-18 column (Teknokroma, 5 μm, 25 cm × 0.46 cm) with a linear gradient of 95−50% solvent A in solvent B over 15 min at a flow rate of 1 mL/min using 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) as the eluting system. The peptides were up to 90% pure by analytical HPLC at 215 nm. Their identities were confirmed by MALDI-TOF mass spectrometry.

Peptide Cyclization. To obtain the head-to-tail cyclic peptide p38HT whose sequence is indicated in Table 2, standard protected amino acids were used. p38HT was synthesized manually on Novasyn TGA resin (Novabiochem Ltd., Switzerland) (1 g, resin loading 0.25 mmol/g) as a C-terminal acid by solid-phase synthesis following the procedure outlined in Figure 1. The resin is supplied functionalized with hydroxymethylphenoxyacetic acid (HMPA). It was acylated with the symmetrical anhydride of Fmoc-Asp-ODmab via its side chain. To this end, a preformed symmetrical anhydride

of this orthogonal deprotected amino acid derivative was made fresh prior to use. An amount of 5 equiv of Fmoc-Asp-ODmab (relative to resin loading) was dissolved in dry DCM. Two drops of DMF were needed to aid complete dissolution. An amount of 2.5 equiv of DIPCDI solution in dry DCM was added to the amino acid solution. The mixture was stirred for 1 h at 0 °C, keeping the reaction mixture free of moisture with a calcium chloride drying tube. The DCM was removed by evaporation with N_2 gas. The residue was dissolved in a minimum of DMF with 0.2 equiv of 4-dimethylaminopyridine (DMAP) catalyst, and the solution was added to the resins. The coupling reaction took place for 4 h. The estimation of the level of the first residue attachment was 53%. Peptide synthesis was then continued using a standard Fmoc/^{t}Bu strategy. Three-fold molar excesses of Fmoc-amino acids were used throughout the synthesis. The stepwise addition of each residue was assessed by Kaiser's (ninhydrin) test and by the chloranil test for identification of secondary amines, particularly used when coupling proline residues. After completion of the peptide synthesis, the N-terminal Fmoc group was removed with 20% piperidine in DMF and the removal of Cα-Dmab was carried out with 3% hydrazine monohydrate in DMF followed by treatment with 20% water in DMF for 16 h at room temperature. The regioselectively deprotected peptide resins were washed with 1% DIPEA−DMF followed by 1% HOBt−DMF. The on-resin macrocyclization reactions were carried out with three repeated treatments of 1.5 equiv of PyBOP and 3 equiv of DIPEA dissolved in DMF/DCM (1:1), the activation taking place at room temperature for 72 h with continuous stirring. The resin was washed with 1% DIPEA/DMF and dried with DCM and methanol. The cyclic peptide was concomitantly side chain deprotected and cleaved from the resin by treatment with a mixture of TFA/1,2-ethanedithiol (EDT)/TIS/H_2O (95/2/1/2) for 3.5 h at room temperature.

The cyclic constructs were isolated by precipitation with cold diethyl ether. The residue was dissolved in 10% aqueous acetic acid and lyophilized. The crude peptides were purified by reversed-phase HPLC and identified by electrospray mass spectrometry (ES-MS).

To obtain the disulfide cyclic peptides shown in Tables 2 and 3 (p38SS, p18-sc, p19-sc, p22-sc, p18-lc, p19-lc, and p22-lc), two serine residues within the corresponding sequence were substituted by cysteine residues protected as Cys(Acm). The corresponding linear peptides were synthesized as C-terminal carboxamides as described above and finally cleaved from the resin with TFA/EDT/TIS/H_2O (95/2/1/2) and precipitated from ice-cold diethyl ether. Finally they were dissolved in 10% aqueous acetic acid and lyophilized. Characterization of the resulting peptides was carried out by MALDI-TOF MS and analytical HPLC. For peptide cyclization they were dissolved in acetic acid, and a solution of iodine (10 equiv) in methanol was added for fast building of the disulfide bond. After 60 min of stirring, water was added to accelerate the half-time of S−Acm cleavage. The solution was shaken for another 90 min. After completion of the reaction, water was added and the iodine was extracted with dichloromethane six times. The aqueous phase was diluted 3-fold with water and lyophilized. The crude peptides were characterized by analytical HPLC on a Kromasil C-18 column (Teknokroma, 5 μm, 25 cm × 0.46 cm) with a linear gradient of 95−5% solvent A in solvent B over 30 min at a flow rate of 1 mL/min using 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) as the eluting system and finally were purified by preparative HPLC in a Kromasil-C8 column (Tecknokroma, 5 μm, 25 cm × 2.2 cm) and characterized by ES-MS.

Figure 2 illustrates the synthetic procedure that has been followed for the preparation of the disulfide cyclic peptide p38SS.

Chimeric Peptides. For the synthesis of chimeric linear peptides that contain three flexible glycine residues between the α- and β-fibrin selected peptides (Table 4a), the general procedure on the solid-phase synthesis described above was followed.

For the synthesis of chimeric α-fibrin−filaggrin synthetic peptide (p18-cfc1cyc) whose primary sequence is shown in Table 4b, two different synthetic strategies were assayed. The first one (Figure 3a) consisted of the synthesis of the chimeric linear sequences in the solid phase and their subsequent cyclization in solution by means of the formation of a disulfide bridge. The crude linear peptide was analyzed by ES-MS and analytical HPLC, the purity achieved being about 90%. Consequently, the disulfide bond formation was carried out without purifying this peptide. The cyclization was followed by HPLC and the final product purified by semipreparative HPLC on a Kromasil C-18 column (Tecknokroma, 5 μm, 25 cm × 1 cm) with a linear gradient of 100−75% solvent A in solvent B over 30 min at a flow rate of 4 mL/min using 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) as the eluting system. The purified p18-cfc1cyc was characterized by analytical HPLC and ES-MS. The overall yield from the peptide resin was 30%.

In the second strategy (Figure 3b), the cyclization of the filaggrin peptide sequence was carried out in the solid phase via a disulfide bond formation. The optimal strategy to obtain the desired cyclic product was achieved by using 5 equiv of iodine solved in methanol/dichloromethane (1/1). This solution was given to the preswollen resin and shaken for 4 h. The resin was washed with methanol, dichloromethane, and DMF. This procedure was repeated three times and the cyclization process followed by HPLC and ES-MS. When the oxidation procedure was completed, the synthesis of the α-fibrin sequence was performed on the cyclic peptide attached to the solid matrix.

The cleavage and final deprotection of the chimeric cyclic peptide were finally carried out preserving the previously formed disulfide bond (TFA/TIS/H_2O, 90/5/5). The crude peptide was analyzed by HPLC and ES-MS. Formation of p18-cfc1cyc was more difficult (lower than 10% HPLC purity), and substantial amounts of byproducts were observed.

Although the most elegant method is cyclization of the peptide on the resin (strategy 3b) because chemicals and reagents can be removed easily, in our hands the synthesis of the p18 sequence performed on the cyclic cfc1 peptide attached to the solid matrix was troublesome, thus rendering significantly lower yields of the final desired peptide compared to the ones obtained following strategy 3a.

While on-resin cyclization strategy took advantages of handling, the solution strategy allowed the formation of the cyclic final product with higher purity.

Serum Specimens. Sera were collected from patients of the Rheumatology Service at the Hospital Clinic in Barcelona. A total of 193 sera were collected from patients, of which 111 were diagnosed as RA according to the revised criteria formulated by the American Rheumatology Association in 1987 (now American College of Rheumatology) and previously tested for the presence of anti-CCP2 antibodies by ELISA (Immunoscan RA; Eurodiagnostica, distributed by Diasorin, Madrid, Spain). The remaining 82 sera were taken from unselected patients with PsA and were used as a negative control.

ELISA Assays. Peptide sequences were coupled covalently to ELISA microplates (Costar Corp., Cambridge, MA, DNA-bind *N*-oxysuccinimide surface) as previously described.[11,34]

Briefly, peptides were diluted to 10 μg/mL in 0.05 M carbonate/bicarbonate (pH 9.6) buffer. An amount of 100 μL of peptide solution was added to each well of microplates and incubated overnight at 4 °C. Each plate contained control wells that included all reagents except the serum sample in order to estimate the background reading and control wells that included all reagents except the peptide to evaluate nonspecific reactions of sera. For blank controls, wells were coupled with 2 μg of BSA/well. After incubation, the plates were blocked with 2% BSA in 0.05 M carbonate/bicarbonate (pH 9.6) buffer for 1 h at room temperature. Sera were diluted 200-fold in RIA buffer (1% BSA, 350 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% vol/vol Triton X-100, 0.5 wt %/vol sodium deoxycholate, 0.1% SDS) supplemented with 10% fetal bovine serum. An amount of 100 μL/well was added, and the mixture was incubated for 1.5 h at room temperature. After the mixture was washed six times with PBS/0.05% Tween-20, 100 μL/well of antihuman IgG conjugated to peroxidase diluted 1:1000 in

RIA buffer was added. After incubation for 1 h at room temperature, the plates were washed six times with PBS/0.05% Tween-20 and bound antibodies were detected with *o*-phenylenediamine dihydrochloride (OPD, Sigma Chemical Company) and 0.8 μL/mL 30% hydrogen peroxide. The plates were incubated at room temperature for 30 min. The reaction was stopped with 50 μL of 2 N H_2SO_4 per well, and absorbance values were measured at a wavelength of 492 nm. All sera were tested in duplicate. Control sera were also included to monitor inter- and intraassay variations.

Of the ELISA assays carried out with the physical mixture of fibrin peptides, passive adsorption based on hydrophobic interactions between the molecules and the surface (Maxisorp, 96F Nunc, Roskilde, Denmark) was also carried out according to a method previously described.[26]

Statistical Analysis. Receiver operating characteristic (ROC) curve analysis and regression analysis were conducted using MedCalc, version 7.6 (MedCalc Software, Mariakerte, Belgium).

Correlation analyses were performed using the nonparametric Spearman test.

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Supporting Information Available: Antigenicity prediction algorithm plots of α- and β-fibrin; analytical data and HPLC profiles of the peptides listed in Table 1; HPLC and mass spectrometry characterization of the most antigenic α- and β-fibrin derived peptides; ROC curve analysis for p18, p19, and their cyclic versions; and comparison of reactivity of five RA (CCP2 positive) sera with monomeric and chimeric peptides and the mixture of single peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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