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The Use of Chimeric Vimentin Citrullinated Peptides for the Diagnosis of Rheumatoid Arthritis

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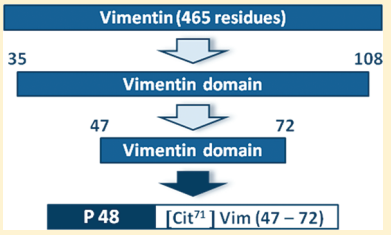
Supporting Information

ABSTRACT: Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes inflammation and, in many cases, destruction of the joints. To prevent progressive and irreversible structural damage, early diagnosis of RA is of paramount importance. The present study addresses the search of new RA citrullinated antigens that could supplement or complement diagnostic/prognostic existing tests. With this aim, the epitope anticitrullinated vimentin antibody response was mapped using synthetic peptides. To improve the sensitivity/specificity balance, a vimentin peptide that was selected, and its cyclic analogue, were combined with fibrin- and filaggrin-related peptides to render chimeric peptides. Our findings highlight the putative application of these chimeric peptides for the design of RA diagnosis systems and imply that more than one serological test is required to classify RA patients based on the presence or absence of ACPAs. Each of the target molecules reported here (fibrin, vimentin, filaggrin) has a specific utility in the identification of a particular subset of RA patients.



INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes inflammation and, in many cases, destruction of the joints. Longstanding and severe cases also develop a systemic disease with extra-articular effects. To prevent progressive and irreversible structural damage, early diagnosis of RA is important because symptoms do not always appear at early stages of the disease.[1]

Anticitrullinated protein/peptide antibodies (ACPAs) are the most specific antibodies present in the sera of RA patients, and they have recently been proposed as a diagnostic criterion for RA.[2] These antibodies may be detected several years before the symptoms appear, and their presence at disease onset may have a high predictive value for the development of erosive joint lesions.[3,4] RA patients can be classified into two major groups: those who have ACPAs and those who do not. The presence of ACPAs at early stages predicts the development of earlier and more widespread erosions and low remission rates.[5,6]

Serological tests have been based on the detection of these antibodies using citrullinated proteins such as filaggrin, fibrin, and vimentin, and several synthetic citrullinated peptide sequences have been applied as antigenic substrates. Schellekens et al. showed that filaggrin-derived peptides detect ACPAs in RA sera, and they reported a cyclic citrullinated peptide derived from filaggrin. This was later used in the first commercial cyclic citrullinated peptide (CCP1) test for the detection of ACPAs in RA patients.[7] The second generation of cyclic citrullinated peptide-based tests (CCP2), whose underlying antigenic substrate has not been published, was launched in 2002. It was found to be both more sensitive and more specific than CCP1,[8] and it was used in most of the published studies in which the presence of ACPAs in RA patients was explored. However, other tests for the detection of ACPAs based on different proteins or peptides have recently been developed.[9−11] Specifically, the chimeric fibrin/filaggrin peptides synthesized by our group are highly sensitive and specific for RA. Moreover, positive early patients, including those who were CCP2-negative, showed greater joint destruction.[11]

Bearing in mind the need for the accurate prediction of patient status, we sought to identify new citrullinated antigens that could supplement or complement existing tests. We examined another citrullinated protein (vimentin), which is also present in the RA synovial tissue[12] and contains 43 putative citrullinated arginine residues, which might be used to confirm or predict the development of RA. The epitope anticitrullinated vimentin antibody response was mapped using synthetic peptides obtained by a solid-phase peptide synthesis (SPPS) strategy and evaluated by an enzyme-linked immunosorbent assay (ELISA). Finally, to improve the balance between sensitivity and specificity, the vimentin peptide that was selected, and its cyclic analogue, were combined with fibrin- and filaggrin-related peptides to render chimeric peptides.

RESULTS AND DISCUSSION

To identify the peptide regions of vimentin that are reactive against ACPAs, we constructed the citrullinated vimentin-based overlapping peptide libraries illustrated in Figure 1.

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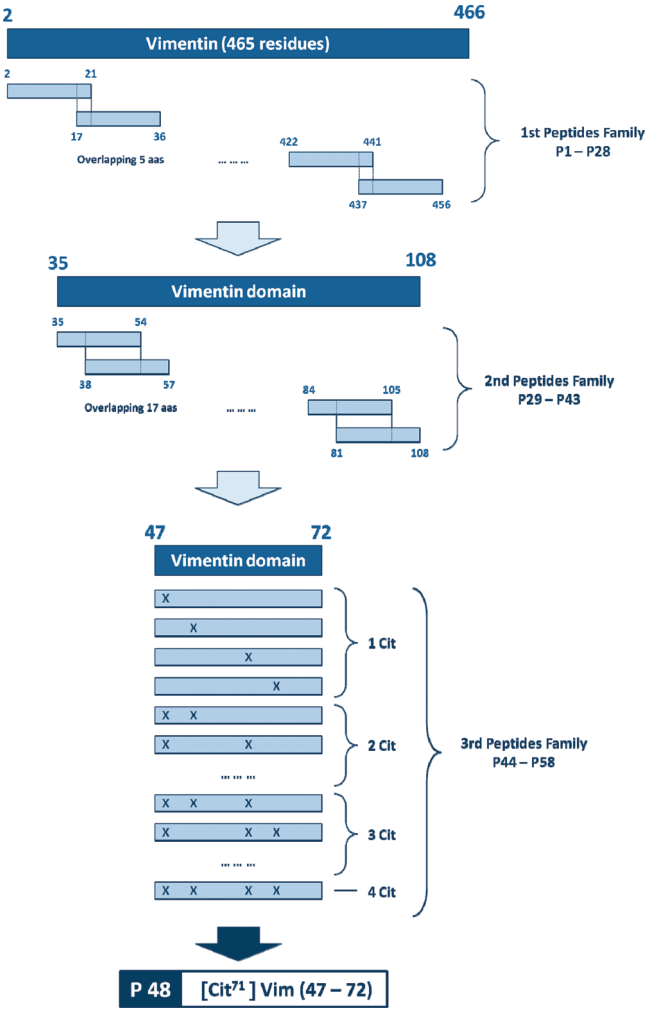

Figure 1. The construction of citrullinated vimentin-based overlapping peptide libraries.

[2]STRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYASSP
GGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQELN
DRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQVDQL
TNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNASLA
RLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTAALRD
VRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTEYRRQ
VQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQNMKEE
MARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLRETNLDS
LPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE[466]

Figure 2. Vimentin (2−466) [initiator Met removed]. GenBank Accession No: P08670.

First, a 28-peptide family covering the full-length citrullinated vimentin (Figure 2; GenBank accession no.: P08670) was designed. In this family, each 20-mer peptide contained at least one citrulline residue in its sequence, and the N-terminal first five amino acids overlapping the five amino acids at the C-terminus of the previous peptide (Table 1). The peptides were synthesized using a semiautomatic multisynthesizer and characterized by HPLC and HPLC-MS (Supporting Information). Synthetic citrullinated peptides were first tested with well characterized samples of serum from four RA patients. According to the median of the absorbance units measured, peptides p4, p7, and p18 showed the strongest reactivity in a direct binding assay

Table 1. Sequences of 20-mer Synthetic Peptides Derived from Vimentin (X: Citrulline)

name	peptide	sequence
p1	[Cit^{4,12,13}]Vim (2−21)	STXSVSSSSYXXMFGGPGTA
p2	[Cit^{23,28,36}]Vim (17−36)	GPGTASXPSSSXSYVTTSTX
p3	[Cit^{36,45,50}]Vim (32−51)	TTSTXTYSLGSALXPSTSXS
p4	[Cit^{50,64}]Vim (47−66)	STSXSLYASSPGGVYATXSS
p5	[Cit^{64,69,71,78}]Vim (62−81)	ATXSSAVXLXSSVPGVXLLQ
p6	[Cit^{78}]Vim (77−96)	VXLLQDSVDFSLADAINTEF
p7	[Cit^{100}]Vim (92−111)	INTEFKNTXTNEKVELQELN
p8	[Cit^{113,122}]Vim (107−126)	LQELNDXFANYIDKVXFLEQ
p9	[Cit^{122}]Vim (122−141)	XFLEQQNKILLAELEQLKGQ
p10	[Cit^{145,155}]Vim (137−156)	QLKGQGKSXLGDLYEEEMXE
p11	[Cit^{155,158,159,170}]Vim (152−171)	EEMXELXXQVDQLTNDKAXV
p12	[Cit^{170,175,184,186}]Vim (167−186)	DKAXVEVEXDNLAEDIMXLX
p13	[Cit^{184,186,196}]Vim (182−201)	IMXLXEKLQEEMLQXEEAEN
p14	[Cit^{207}]Vim (197−216)	EEAENTLQSFXQDVDNASLA
p15	[Cit^{217,222}]Vim (212−231)	NASLAXLDLEXKVESLQEEI
p16	[Cit^{270,273}]Vim (257−276)	DVDVSKPDLTAALXDVXQQY
p17	[Cit^{273}]Vim (272−291)	VXQQYESVAAKNLQEAEEWY
p18	[Cit^{304}]Vim (287−306)	AEEWYKSKFADLSEAANXNN
p19	[Cit^{304,310,320,321}]Vim (302−321)	ANXNNDALXQAKQESTEYXX
p20	[Cit^{320,321}]Vim (317−336)	TEYXXQVQSLTCEVDALKGT
p21	[Cit^{342,345}]Vim (332−351)	ALKGTNESLEXQMXEMEENF
p22	[Cit^{364}]Vim (347−366)	MEENFAVEAANYQDTIGXLQ
p23	[Cit^{364,378,381}]Vim (362−381)	IGXLQDEIQNMKEEMAXHLX
p24	[Cit^{378,381}]Vim (377−396)	AXHLXEYQDLLNVKMALDIE
p25	[Cit^{401,410}]Vim (392−411)	ALDIEIATYXKLLEGEESXI
p26	[Cit^{410,424}]Vim (407−426)	EESXISLPLPNFSSLNLXET
p27	[Cit^{424,440}]Vim (422−441)	NLXETNLDSLPLVDTHSKXT
p28	[Cit^{440,450}]Vim (437−456)	HSKXTFLIKTVETXDGQVIN

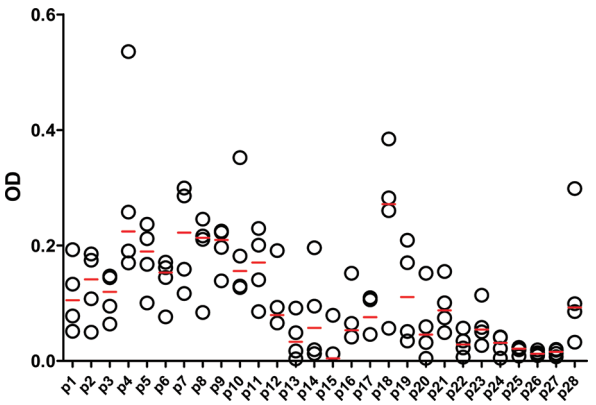

Figure 3. Reactivity of RA sera (n = 4) with the 20-mer synthetic peptides derived from vimentin shown in Table 1. The median of the absorbance values is shown in red.

(Figure 3). These three peptides were then further tested against 139 RA sera. At this point, nonsignificant differences in the reactivity were found between p4 and p7 (p = 0.6566). However, p18 gave significantly lower optical density values (Figure 4). Considering the peptide regions of the protein described above, a second peptide family was synthesized (Table 2) (Supporting Information). This family covers the (35−108) vimentin domain

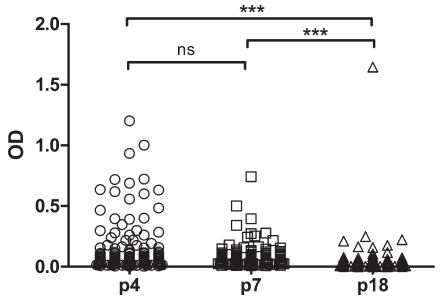

Figure 4. Reactivity of RA sera (n = 139) with p4, p7, and p18 peptides. ***p < 0.001; ns indicated p = 0.6566.

Table 2. Sequences of 20-mer Synthetic Peptides Derived from (35−108) Vimentin Domain (X: citrulline)

name	peptide	sequence
p29	[Cit^{36,45,50}]Vim (35−54)	TXTYSLGSALXPSTSXSLYA
p30	[Cit^{45,50}]Vim (38−57)	YSLGSALXPSTSXSLYASSP
p31	[Cit^{45,50}]Vim (41−60)	GSALXPSTSXSLYASSPGGV
p32	[Cit^{45,50}]Vim (44−63)	LXPSTSXSLYASSPGGVYAT
p33	[Cit^{50,64,69}]Vim (50−69)	XSLYASSPGGVYATXSSAVX
p34	[Cit^{64,69,71}]Vim (53−72)	YASSPGGVYATXSSAVXLXS
p35	[Cit^{64,69,71}]Vim (56−75)	SPGGVYATXSSAVXLXSSVP
p36	[Cit^{64,69,71,78}]Vim (59−78)	GVYATXSSAVXLXSSVPGVX
p37	[Cit^{69,71,78}]Vim (65−84)	SSAVXLXSSVPGVXLLQDSV
p38	[Cit^{69,71,78}]Vim (68−87)	VXLXSSVPGVXLLQDSVDFS
p39	[Cit^{71,78}]Vim (71−90)	XSSVPGVXLLQDSVDFSLAD
p40	[Cit^{78}]Vim (74−93)	VPGVXLLQDSVDFSLADAIN
p41	[Cit^{100}]Vim (83−102)	SVDFSLADAINTEFKNTXTN
p42	[Cit^{100}]Vim (84−105)	FSLADAINTEFKNTXTNEKV
p43	[Cit^{100}]Vim (89−108)	ADAINTEFKNTXTNEKVELQ

Table 3. Sequences of p4-2, p34, and p4-2-34 Synthetic Peptides (X: Citrulline)

name	peptide	sequence
p4−2	[Cit^{64}]Vim (47−66)	STSRSLYASSPGGVYATXSS
p34	[Cit^{64,69,71}]Vim (53−72)	YASSPGGVYATXSSAVXLXS
p4− 2−34	[Cit^{64,69,71}]Vim (47−72)	STSRSLYASSPGGVYATXSSAVXLXS

and is composed of 15 peptides overlapping by 17 aa residues (peptides p29−p43). In addition, two analogues of p4, p4−1 [Cit^{50}]Vim(47−66), and p4−2 [Cit^{64}]Vim(47−66), were synthesized in order to assess the influence of the Arg/Cit ratio on ACPA recognition. The immunoassays performed with the peptides shown in Table 2, and the p4 analogues, using the four well-defined RA sera described above, revealed that peptides p34 and p4−2 had better antigenic activity (Supporting Information). Interestingly, these two peptides overlapped by 14 aa. Therefore, to present these two vimentin-related peptide domains within one peptide sequence, a 26-mer peptide (p4−2−34) was designed and synthesized in solid phase (Table 3). Then, by changing the Arg/Cit ratio in p4−2−34, which would represent the (47−72) vimentin domain, a third peptide family containing 15 26-mer peptide analogues (peptides p44−p58) was synthesized (Supporting Information) to study

Table 4. Sequences of 26-mer Synthetic Peptides Derived from (47−72) Vimentin Domain with Different Arg/Cit Relationship (X: Citrulline)

name	peptide	sequence
p44	[Cit^{50,64,69,71}]Vim (47−72)	STSXSLYASSPGGVYATXSSAVXLXS
p45	[Cit^{50}]Vim (47−72)	STSXSLYASSPGGVYATRSSAVRLRS
p46	[Cit^{64}]Vim (47−72)	STSRSLYASSPGGVYATXSSAVRLRS
p47	[Cit^{69}]Vim (47−72)	STSRSLYASSPGGVYATRSSAVXLRS
p48	[Cit^{71}]Vim (47−72)	STSRSLYASSPGGVYATRSSAVRLXS
p49	[Cit^{69,71}]Vim (47−72)	STSRSLYASSPGGVYATRSSAVXLXS
p50	[Cit^{64,71}]Vim (47−72)	STSRSLYASSPGGVYATXSSAVRLXS
p51	[Cit^{50,71}]Vim (47−72)	STSXSLYASSPGGVYATRSSAVRLXS
p52	[Cit^{64,69}]Vim (47−72)	STSRSLYASSPGGVYATXSSAVXLRS
p53	[Cit^{50,69}]Vim (47−72)	STSXSLYASSPGGVYATRSSAVXLRS
p54	[Cit^{50,64}]Vim (47−72)	STSXSLYASSPGGVYATXSSAVRLRS
p55	[Cit^{64,69,71}]Vim (47−72)	STSRSLYASSPGGVYATXSSAVXLXS
p56	[Cit^{50,64,71}]Vim (47−72)	STSXSLYASSPGGVYATXSSAVRLXS
p57	[Cit^{50,69,71}]Vim (47−72)	STSXSLYASSPGGVYATRSSAVXLXS
p58	[Cit^{50,64,69}]Vim (47−72)	STSXSLYASSPGGVYATXSSAVXLRS

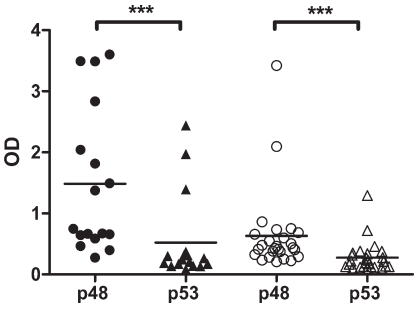

Figure 5. Levels of anti-p48 and anti-p53 antibodies in CFFCPs highly positive RA sera (●, ▲) and CFFCPs negative RA sera (○, △). Bars indicate the median levels of antibody response. ***p < 0.001.

CVFCP: chimeric vimentin/filaggrin citrullinated peptide

H_2N-STSRSLYASSPGGVYATRSSAVRLXS-HQCHQESTXGRSRGRCGRSGS-CNH_2

CFVCP: chimeric fibrin/vimentin citrullinated peptide

H_2N-HSTKRGHAKSRPVXG-STSRSLYASSPGGVYATRSSAVRLXS-CNH_2

CFVcCP: chimeric fibrin/vimentin cyclic citrullinated peptide

H_2N-HSTKRGHAKSRPVXG-STSRSLYACSPGGVYATRSCAVRLXS-CNH_2

Figure 6. Primary structure of chimeric peptides bearing an analogue of (47−72) vimentin domain.

the effect of both the degree and the position of citrullination on the peptide antigenicity (Table 4). Next, using six well-defined RA sera, these 15 peptides were tested in ELISA. The p48 and p53 analogues of the (47−72) vimentin region showed higher optical density values against RA sera than controls

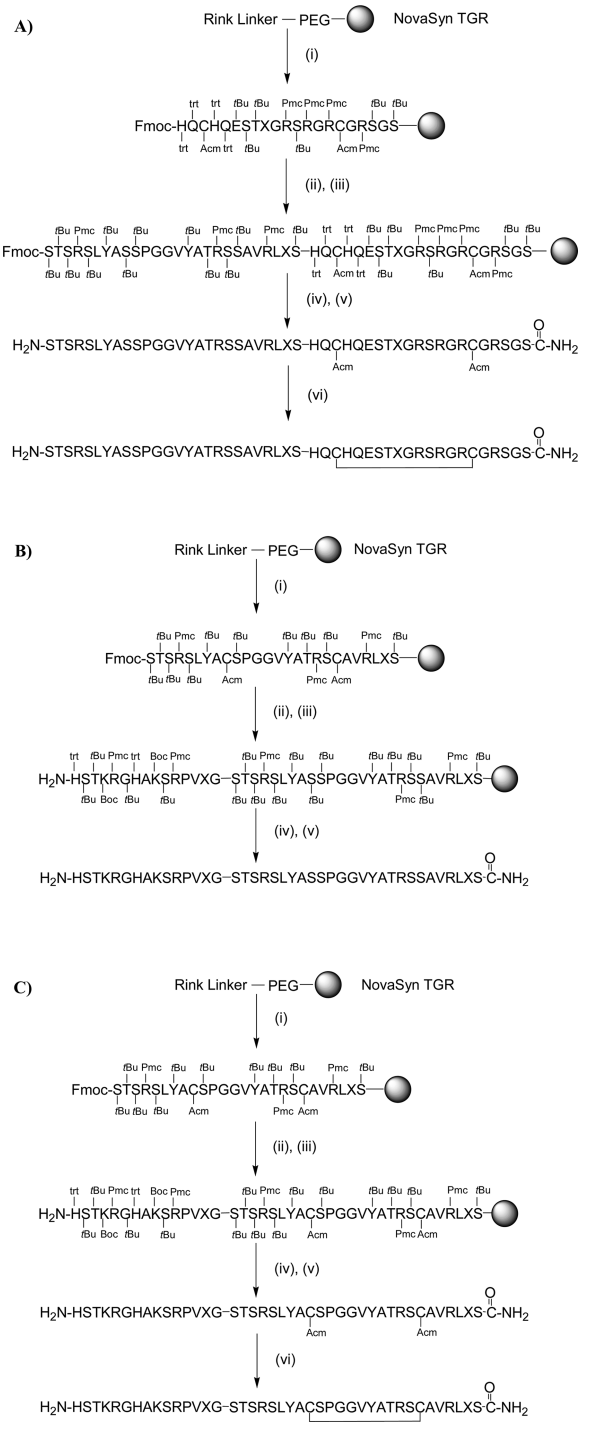

Figure 7. Strategies for the solid-phase peptide synthesis of (A) **CVFCP**, reagents and conditions are as follows: (i) solid-phase synthesis of cfc1-cyc; (ii) 20% piperidine/DMF; (iii) solid-phase synthesis of p48; (iv) 20% piperidine/DMF; (v) TFA/EDT/TIS/H_2O (95:2:1:2); (vi) I_2/AcOH (50:50), 4 h. (B) **CFVCP**, reagents and conditions are as follows: (i) solid-phase synthesis of pv5; (ii) 20% piperidine/DMF; (iii) solid-phase synthesis of α-fibrin p18; (iv) 20% piperidine/DMF; (v) TFA/EDT/TIS/H_2O (95:2:1:2). (C) **CFVcCP**, reagents and conditions are as follows: (i) solid-phase synthesis of p48; (ii) 20% piperidine/DMF; (iii) solid-phase synthesis of p18; (iv) 20% piperidine/DMF; (v) TFA/EDT/TIS/H_2O (95:2:1:2); (vi) I_2/AcOH (50:50), 4 h.

(Supporting Information). We then compared these two peptides by using a large number of well-defined RA sera that had

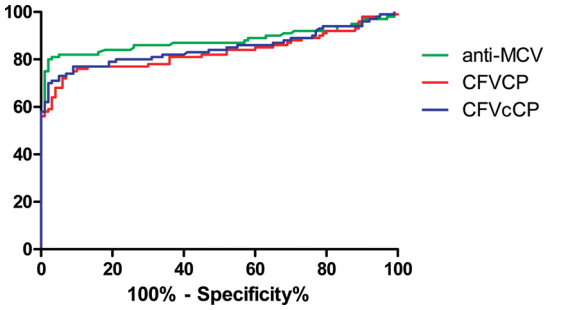

Figure 8. ROC curves analysis for chimeric fibrin/vimentin (CFVCP and CFVcCP) citrullinated synthetic peptides and anti-MCV commercial test in the cohort of patients with RA (n = 100) and blood donors (n = 100). Sensitivity and specificity were calculated for all potential cutoff values (sensitivity of 58%, 62%, and 75% for CFVCP, CFVcCP, and anti-MCV, respectively, at a specificity of 99%; sensitivity of 75%, 77%, and 82% for CFVCP, CFVcCP, and anti-MCV, respectively, at a specificity of 91%).

Table 5. Reactivity of 31 RA CFFCP1 Negative Sera with Chimeric Vimentin Peptides and Anti-MCV Commercial Test vs CCP2

RA serum	CCP2	anti-MCV[a]	CVFCP[b]	CFVcCP[c]
1	+	neg	neg	neg
2	+	neg	neg	+
3	+	+	+	+
4	neg	neg	neg	neg
5	neg	neg	neg	neg
6	neg	neg	neg	neg
7	neg	neg	neg	neg
8	+	neg	neg	neg
9	+	neg	neg	neg
10	neg	+	+	+
11	neg	neg	neg	neg
12	neg	neg	neg	neg
13	neg	neg	neg	neg
14	neg	neg	neg	neg
15	neg	neg	neg	neg
16	neg	neg	+	+
17	neg	neg	neg	+
18	neg	neg	neg	neg
19	neg	neg	neg	neg
20	+	neg	neg	neg
21	neg	neg	neg	neg
22	+	+	neg	neg
23	neg	neg	neg	neg
24	+	+	+	neg
25	+	+	+	neg
26	+	+	+	+
27	neg	neg	neg	neg
28	neg	neg	neg	neg
29	neg	neg	neg	neg
30	neg	neg	neg	neg
31	neg	neg	+	neg

[a] cutoff 91% specificity: 0.285. [b] cutoff 91% specificity: 0.518. [c] cutoff 91% specificity: 0.380.

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been tested against chimeric fibrin/filaggrin peptides CFFCP1, CFFCP2, and CFFCP3.[11] p48 was significantly more reactive than p53 against CFFCP-positive sera ($n = 17$) ($p = 0.0003$). p48 was also significantly more reactive than p53 against CFFCP-negative sera ($n = 26$) ($p < 0.0001$) (Figure 5).

The p48 peptide chosen, [Cit^{71}]Vim(47−72), overlaps partially with other reported vimentin peptides. Hill et al.[13] selected a vimentin-derived peptide sequence (65−77) based on predicted peptide affinity for the DRB1*0401 allele, which induced T-cell activation in DR4-IE tg mice. Verpoort et al.[14] tested a panel of vimentin-derived peptides against several RA sera, and a peptide derived from the region (59−74) showed the highest reactivity. Feitsma et al.[15] reported two vimentin-related peptide sequences, (26−45) (^{28,36}Cit) and 415−433 (^{424}Cit), that induced a T-cell response in HLA-DR4-transgenic mice. The former was located next to the vimentin domain p48 that was chosen in the present study.

On the basis of the increased antigenicity observed with multimeric peptides bearing different epitope peptide sequences within the same molecule, and given our previous results,[16] we designed and synthesized in solid phase a new chimeric peptide. This was composed of the selected vimentin peptide p48 and the cyclic filaggrin peptide constituting the CCP1 test, and it is referred to herein as CVFCP (chimeric vimentin/filaggrin citrullinated peptide) (Figure 6).

To evaluate the effect of replacing the fibrin-derived domain in CFFCP by the selected vimentin peptide (p48), a comparative ELISA assay in a cohort of 100 RA sera and 100 sera from blood donors was performed using CVFCP and CFFCP1 peptides as coating antigens. As shown by the ROC curves obtained (Supporting Information), the sensitivity/specificity balance did not improve because with cut-offs giving 91% specificity for RA versus blood donors, the sensitivity was 76% for CVFCP and 89% for CFFCP1.

To improve the antigenic activity, we further explored the chimeric peptides by designing and synthesizing fibrin-vimentin related peptides. A new chimeric peptide that contains the previously reported α-fibrin p18 peptide[16] and the vimentin peptide p48 was obtained, and it is referred to herein as CFVCP (chimeric fibrin/vimentin citrullinated peptide). Moreover, as constrained peptides could adopt conformations that favor antibody binding,[7,16,17] the vimentin antigenic site was stabilized by cyclization. Thus, a cyclic analogue of CFVCP, named CFVcCP, was also synthesized (Figure 6). To this end, two serine residues in CFVCP were replaced by two cysteine residues and cyclization was performed in solution by iodine/AcOH:H_2O oxidation following peptide cleavage. The strategies for the solid-phase peptide synthesis of CVFCP, CFVCP and CFVcCP are shown in Figure 7. The HPLC analysis and MS analysis are shown in Supporting Information.

Again, the reactivity of the two fibrin/vimentin chimeric peptides (CFVCP and CFVcCP) against 100 RA sera and 100 controls was analyzed by ROC curves and compared to the results of the commercial anti-MCV test (Orgentec Diagnostika GmbH, Germany), which is based on the mutated citrullinated vimentin protein. When the cutoff values were adjusted to the same specificity, CFVcCP was more sensitive than CFVCP (Figure 8). The sensitivity, positive likelihood ratio and negative likelihood ratio were: 75.0%, 8.33, and 0.27 for CFVCP; and 77%, 8.56, and 0.25 for CFVcCP, respectively. These results indicate that these chimeric fibrin/vimentin peptides could be used in the design of new serological tests for RA. Synthetic peptides are simpler and cheaper to prepare than whole protein, and it is easier to control the extent of citrullination.

Moreover, we had previously reported that CFFCP1 detected ACPAs in 34% of CCP2- negative RA patient sera.[16] In the present study, anti-CVFCP/CFVcCP antibodies were found in 29% (9/31) of RA patients without anti-CFFCP1 and also in four patients who tested negative for both anti-CFFCP1 and anti-CCP2 (12.9%) (Table 5). Although the levels of antivimentin peptide antibodies in these four patients were close to the cutoff values (91% of specificity), our results indicate that the chimeric fibrin/vimentin/filaggrin peptides reported herein may help to diagnose RA in patients with negative tests for anti-CCP2.

Besides, antivimentin antibodies correlate with other anticitrullinated antibodies (anti-CCP2 and anti-CFFCP1) and with RF. Those patients who were positive for antivimentin peptide antibodies had higher rates of erosive disease and lower rates of clinical remission (DAS28 < 2.6) (Tables 6 and 7).

Table 6. General Characteristics of Patients with RA

	$n = 99$
gender (female)	74%
mean age (years)	57.3 ± 14.5
mean disease follow-up (years)	7.63 ± 5.2
early onset RA	31%
previous smoking	43%
rheumatoid factor[4]	77%
anti-CCP2	80%
antichimeric fillagrin/fibrin	76%
anti-CVFCP antibodies	76%
anti-CFVcCP antibodies	77%
mean CVFCP antibodies titers	1.60 ± 1.16
mean CFVcCP antibodies titers	0.75 ± 0.42
extra-articular manifestations	28%
erosive disease	60%
mean DAS28 score	3.0
remission (DAS28 < 2,6)	44%
biologic treatment	33%

Table 7. Serological, Clinical and Radiological Differences in RA Patients with and without Antivimentin Antibodies

	CVFCP positive	CVFCP negative	p value	CFVcCP positive	CFVcCP negative	p value
RF positive (%)	83	56	0.009	84	54	0.003
CCP2 positive (%)	93	34	<0.0001	95	33	<0.0001
antichimeric positive (%)	92	22	<0.0001	93	21	<0.0001
erosive disease (%)	69	32	0.002	72	26	<0.0001
remission (%)	37	67	0.028	37	67	0.028

CONCLUSIONS

The epitope anticitrullinated vimentin antibody response in RA patients has been mapped using synthetic peptides. A selected vimentin peptide p48, [Cit^{71}] Vim (47−72), and its cyclic analogue, were combined with fibrin- and filaggrin-related peptides to render chimeric peptides.

Our findings highlight the putative application of these chimeric peptides for the design of RA diagnosis systems and imply that more than one serological test is required to classify patients based on the presence or absence of ACPAs. Each of the target molecules reported here (fibrin, vimentin, filaggrin) has a specific utility in the identification of a particular subset of RA patients. On the other hand, recent evidence indicates possible epitope spreading for ACPA recognition due to ongoing inflammation in RA. Consequently, the characteristics of ACPAs in established disease, with more citrullinated peptides acting as autoantigens, may differ from those observed in healthy individuals.[18]

Our results support the notion that the development of multiple tests combining all these target peptides in a single analysis (i.e., microarrays) would permit a more detailed analysis of the autoantibody reactivity found in the sera of RA patients.

EXPERIMENTAL SECTION

Sixty peptides derived from vimentin containing a different degree of deimination and whose primary sequence is shown in Tables 1−4 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) (12 μmol, 0.24 meq/g) and following a 9-fluorenyl-methoxycarbonyl (Fmoc) strategy. Amino acid side chain protection was effected by the following: triphenilmethyl (Trt) for glutamine, asparagine, and histidine; *tert*-butyl (tBu) for aspartic acid, glutamic acid, serine, threonine, and tyrosine; 2,2,5,7,8-pentamethyl- chroman-6-sulfonyl (Pmc) for arginine and *tert*-butoxycarbonyl (Boc) for lysine and tryptophan.

The coupling reaction was performed using 3-fold molar excesses of activated Fmoc-amino acids throughout the synthesis. The amino acids were activated essentially by means of treatment with 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA). The Fmoc-deprotection step was accomplished also twice with 20% piperidine in dimethylformamide (DMF) for 10 min. The efficiency of the reactions was followed by the ninhydrin colorimetric test. 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% H_2O, 2% ethane-1,2-dithiol (EDT), and 1% triisopropylsilane (TIS) for 3 h. The cleavage was repeated one more time. 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% A in B over 15 min at a flow rate of 1 mL/min using 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B) as eluting system. The peptides were 95% pure by analytical HPLC at 215 nm. Their identity was confirmed by ES-MS.

Chimeric Peptides. The three chimeric peptides shown in Figure 6 were synthesized manually in polypropylene syringes, each fitted with a polyethylene porous disk. Fully protected amino acids (3 equiv) and HATU (3 equiv) were added sequentially to the resin in DMF (3 mL) followed by DIEA (6 equiv). The mixture was allowed to react with intermittent manual stirring for 30 min. The solvent was removed by filtration, and the resin was washed with DMF (5 × 30 s). The extent of coupling was checked by the Kaiser colorimetric assay or De Clercq test. The Fmoc group was removed by treating the resin with 20% piperidine in DMF (3−4 mL/g resin, 2 × 10 min). The peptide elongation continued by coupling the second amino acid and the following amino acids with the same procedure. The peptides were cleaved from the resin by means of treatment with 95% TFA in the presence of scavengers, basically 2% H_2O, 2% EDT, and 1% TIS for 3 h. To synthesize the cyclic chimeric peptide, two serine residues in the linear peptide were substituted by two cysteines. After peptide cleavage, the peptide was dissolved in AcOH/H_2O (1:1, 3 mg/mL) under N_2, then HCl (1 M, 0.1 mL/mg) followed by I_2 (20 equiv/Acm). After 4 h, I_2 was quenched by adding 1 M ascorbic acid dropwise until the mixture became colorless and concentrated by evaporation under reduced pressure to approximately one-third of the original volume. The final product was purified by semipreparative HPLC on a Kromasil C-18 column (Tecknokroma, 5 μm, 25 cm × 1 cm) with a linear gradient of 100−75% A in B over 30 min at a flow rate of 4 mL/min using 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B) as eluting system. The purified chimeric peptides (purity higher that 95%) were characterized by analytical UPLC and ES-MS.

Serum Specimens. Patients who fulfilled the 1987 American College of Rheumatology (ACR) criteria for the classification of RA were enrolled in this study. All were outpatients attending the Rheumatology Unit of the Hospital Clinic of Barcelona.

Serum samples used as negative controls were obtained form blood donors at the same hospital.

Sera were previously tested in duplicate about the presence of anti-CCP2 and anti-MCV antibodies by ELISA (Immunoscan RA; Eurodiagnostica, distributed by Diasorin, Madrid, Spain, and Orgentec Diagnostika GmbH, Germany).

The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona.

ELISA Assays. Peptide sequences were coupled covalently to ELISA microplates (Costar Corp., DNA-bind *N*-oxysuccinimide surface, Cambridge, MA) as previously described.[16]

Briefly, peptides were diluted to 10 μg/mL in 0.05 M carbonate/bicarbonate (pH 9.6) buffer. Then 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 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 50-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 Na-deoxycholate, 0.1% SDS) supplemented with 10% fetal bovine serum 100 μL/well were added and incubated for 1.5 h at room temperature. After washing 6 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 2N 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 intra-assay variations.

Statistical Analysis. Receiver operating characteristic (ROC) curve analysis and regression analysis was conducted using GraphPad Prism 5 program.

ASSOCIATED CONTENT

Supporting Information. HPLC and HPLC-MS characterization of peptides p1−p58. Ultrapressure liquid chromatography

(UPLC) chromatograms and ES-MS spectra of CVFCP, CFVCP, and CFVcCP chimeric peptides. Reactivity of RA sera with synthetic peptides derived from the (35−108) vimentin domain shown in Table 2. Reactivity of RA sera with synthetic peptides derived from the (47−72) vimentin domain shown in Table 4. ROC curves analysis for chimeric fibrin/filaggrin (CFFCP1) and vimentin/filaggrin (CVFCP) citrullinated peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

ACPA, anticitrullinated protein/peptide antibody; ACR, American College of Rheumatology; Boc, *tert*-butoxycarbonyl; BSA, bovine albumin serum; CCP1, cyclic citrullinated peptide commencial kit 1; CCP2, cyclic citrullinated peptide commercial kit 2; CFFCP1, chimeric fibrin/filaggrin citrullinated peptide 1; CFFCP2, chimeric fibrin/filaggrin citrullinated peptide 2; CFFCP3, chimeric fibrin/filaggrin citrullinated peptide 3; CFVCP, chimeric fibrin/vimentin citrullinated peptide; CFVcCP, chimeric fibrin/vimentin cyclic citrullinated peptide; CVFCP, chimeric vimentin/filaggrin citrullinated peptide; DAS, disease activity score; DIEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; ELISA, enzyme-linked immunosorbent assay; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HLA, human leukocyte antigen; HPLC, high performance liquid chromatography; OD, optical density; OPD, *o*-phenylenediamine dihydrochloride; PBS, phosphate buffer solution; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; RA, rheumatoid arthritis; RF, rheumatoid factor; ROC, receiver operating characteristic curves; SAM, semiautomatic synthesizer; SDS, sodium dodecyl sulfate; SPPS, solid-phase peptide synthesis; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, triphenilmethyl; Vim, vimentin

REFERENCES

(1) Landewe, R. B. M. The benefits of early trea tment in rheumatoid arthritis: confounding by indication, and the issue of timing. *Arthritis Rheum.* **2003**, *48*, 1–5.

(2) Aletaha, D.; Neogi, T.; Silman, A. J.; Funovits, J.; Felson, D. T.; Bingham, C. O.; Birnbaum, N. S.; Burmester, G. R.; Bykerk, V. P.; Cohen, M. D.; Combe, B.; Costenbader, K. H.; Dougados, M.; Emery, P.; Ferraccioli, G.; Hazes, J. M. W.; Hobbs, K.; Huizinga, T. W. J.; Kavanaugh, A.; Kay, J.; Kvien, T. K.; Laing, T.; Mease, P.; Menard, H. A.; Moreland, L. W.; Naden, R. L.; Pincus, T.; Smolen, J. S.; Stanislawska-Biernat, E.; Symmons, D.; Tak, P. P.; Upchurch, K. S.; Vencovsky, J.; Wolfe, F.; Hawker, G. Rheumatoid Arthritis Classification Criteria An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum.* **2010**, *62*, 2569–2581.

(3) Meyer, O.; Labarre, C.; Dougados, M.; Goupille, P.; Cantagrel, A.; Dubois, A.; Nicaise-Roland, P.; Sibilia, J.; Combe, B. Anticitrullinated protein/pepticle antibody assays in early rheumatoid arthritis for predicting five year radiographic damage. *Ann. Rheum. Dis.* **2003**, *62*, 120–126.

(4) Forslind, K.; Ahlmen, M.; Eberhardt, K.; Hafstrom, I.; Svensson, B.; Grp, B. S. Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). *Ann. Rheum. Dis.* **2004**, *63*, 1090–1095.

(5) van der Helm-van Mil, A. H. M.; Verpoort, K. N.; Breedveld, F. C.; Toes, R. E. M.; Huizinga, T. W. J. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res. Ther.* **2005**, *7*, R949–R958.

(6) Machold, K. P.; Stamm, T. A.; Nell, V. P. K.; Pflugbeil, S.; Aletaha, D.; Steiner, G.; Uffmann, M.; Smolen, J. S. Very recent onset rheumatoid arthritis: clinical and serological patient characteristics associated with radiographic progression over the first years of disease. *Rheumatology* **2007**, *46*, 342–349.

(7) Schellekens, G. A.; Visser, H.; de Jong, B. A. W.; Van den Hoogen, F. H. J.; Hazes, J. M. W.; Breedveld, F. C.; van Venrooij, W. J. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* **2000**, *43*, 155–163.

(8) van Venrooij, W. J.; Hazes, J. M.; Visser, H. Anticitrullinated protein/peptide antibody and its role in the diagnosis and prognosis of early rheumatoid arthritis. *Neth. J. Med.* **2002**, *60*, 383–388.

(9) Pruijn, G. J. M.; Wiik, A.; van Venrooij, W. J. The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis. *Arthritis Res. Ther.* **2010**, *12*, 1–8.

(10) Lundberg, K.; Kinloch, A.; Fisher, B. A.; Wegner, N.; Wait, R.; Charles, P.; Mikuls, T. R.; Venables, P. J. Antibodies to Citrullinated alpha-Enolase Peptide 1 Are Specific for Rheumatoid Arthritis and Cross-React With Bacterial Enolase. *Arthritis Rheum.* **2008**, *58*, 3009–3019.

(11) Sanmarti, R.; Graell, E.; Perez, M. L.; Ercilla, G.; Vinas, O.; Gomez-Puerta, J. A.; Gratacos, J.; Balsa, A.; Gomara, M. J.; Larrosa, M.; Canete, J. D.; Haro, I. Diagnostic and prognostic value of antibodies against chimeric fibrin/filaggrin citrullinated synthetic peptides in rheumatoid arthritis. *Arthritis Res. Ther.* **2009**, *11*, R135.

(12) Vossenaar, E. R.; Despres, N.; Lapointe, E.; van der Heijden, A.; Lora, M.; Senshu, T.; van Venrooij, W. J.; Menard, H. A. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res. Ther.* **2004**, *6*, R142–R150.

(13) Hill, J. A.; Southwood, S.; Sette, A.; Jevnikar, A. M.; Bell, D. A.; Cairns, E. Cutting edge: The conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J. Immunol.* **2003**, *171*, 538–541.

(14) Verpoort, K. N.; Cheung, K.; Ioan-Facsinay, A.; van der Helm-van Mil, A. H. M.; de Vries-Bouwstra, J. K.; Allaart, C. F.; Drijfhout, J. W.; de Vries, R. R. P.; Breedveld, F. C.; Huizinga, T. W. J.; Pruijn, G. J. M.; Toes, R. E. M. Fine specificity of the anti-citrullinated protein antibody response is influenced by the shared epitope alleles. *Arthritis Rheum.* **2007**, *56*, 3949–3952.

(15) Feitsma, A. L.; van der Voort, E. I. H.; Franken, K.; el Bannoudi, H.; Elferink, B. G.; Drijfhout, J. W.; Huizinga, T. W. J.; de Vries, R. R. P.; Toes, R. E. M.; Ioan-Facsinay, A. Identification of Citrullinated Vimentin Peptides as T Cell Epitopes in HLA-DR4-Positive Patients With Rheumatoid Arthritis. *Arthritis Rheum.* **2010**, *62*, 117–125.

(16) Perez, M. L.; Gomara, M. J.; Ercilla, G.; Sanmarti, R.; Haro, I. Antibodies to citrullinated human fibrinogen synthetic peptides in diagnosing rheumatoid arthritis. *J. Med. Chem.* **2007**, *50*, 3573–3584.

(17) Perez, T.; Ercilla, G.; Chan, W. C.; Haro, I. Antigenicity of chimeric and cyclic synthetic peptides based on nonstructural proteins of GBV-C/HGV. *J. Pept. Sci.* **2006**, *12*, 267–278.

(18) Scott, D. L.; Wolfe, F.; Huizinga, T. W. J. Rheumatoid arthritis. *Lancet* **2010**, *376*, 1094–1108.