

The Anti-mutated Citrullinated Vimentin Response Classifies Patients with Rheumatoid Arthritis into Broad and Narrow Responders

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ABSTRACT. Objective. Autoantibodies against citrullinated peptide antigens (ACPA) are routinely determined to diagnose rheumatoid arthritis (RA) and are predictive of a more severe course of the disease. We here set out to address an involvement of ACPA in the pathogenesis of RA and investigated the recognition pattern of antibodies against 2 citrullinated antigens in more detail.

Methods. The sera of 77 patients fulfilling the American College of Rheumatology criteria for RA were analyzed for subclass titers of anti-mutated citrullinated vimentin (MCV) and anticyclic citrullinated peptide (CCP) antibodies by combining subclass specific detection antibodies with commercially available CCP and MCV ELISA plates. Cross-reactivities between anti-MCV and anti-CCP antibodies were detected using a sequential ELISA system.

Results. IgG1, IgG3, and IgG4 titers among anti-MCV and anti-CCP antibodies correlated significantly. Cross-reactivity of MCV-specific antibodies against CCP could be detected in 8 of 16 patients' sera; however, cross-binding of MCV-specific IgG4 was weaker compared to total IgG.

Conclusion. The inherent capacity of IgG4 to exchange F(ab) arms provides insight into the anti-MCV antibody diversity and suggests a classification of ACPA positive patients into broad and narrow responders. (J Rheumatol First Release Nov 1 2009; doi:10.3899/jrheum.081263)

Key Indexing Terms:

RHEUMATOID ARTHRITIS

ANTI-CYCLIC CITRULLINATED ANTIBODIES

ANTI-MUTATED CITRULLINATED VIMENTIN

AUTOANTIBODIES

Rheumatoid arthritis (RA) is the most common rheumatic disease affecting about 1% of the population worldwide. In order to prevent debilitation and physical disability characteristic of the late stages of disease, an early and aggressive therapy has proven to be crucial¹. The detection of autoantibodies such as rheumatoid factor (RF) has for decades facilitated the diagnosis of RA, and IgM-RF exhibit a sensitivity of 60-70% and a specificity of 80-90%². More recently, autoantibodies against citrullinated peptide antigens (ACPA) have been described, which can be detected years before the onset of RA and which reach a specificity of more than 95%³.

ACPA are specific for peptides containing citrulline, a posttranslational modification of arginine. Notably, citrullination *per se* is not a specific feature of RA but a normal process during gene regulation, apoptosis, and the terminal differentiation of epithelia⁴. Moreover, citrullinated myelin basic protein has been detected in multiple sclerosis⁵. To date several target proteins of ACPA have been described, among them fibrinogen, filaggrin, vimentin, type II collagen, and α -enolase. Derivatives are used in present diagnostic assays, e.g., the cyclic citrullinated peptide (CCP) including 2 artificial filaggrin peptides⁶ and the mutated citrullinated vimentin (MCV) detectable in the joints of patients with RA and thus representing a real autoantigen⁷.

Although well established for diagnosis, very little is known about the contribution of ACPA to the pathogenesis of RA. They can be detected up to 9 years before the onset of the disease⁸ and even though new B cells producing ACPA develop constantly, isotype switches emerge some time between undifferentiated and established RA⁹. It is therefore reasonable to assume that the specific isotypes of ACPA provide clues as to the underlying immunological processes. Along those lines, we recently demonstrated elevated IgG4 titers of anti-CCP and anti-MCV antibodies in patients with RA¹⁰.

We here show strong correlations of anti-CCP and anti-

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MCV titers among 3 of the 4 IgG subclasses. Hence we developed an ELISA system to investigate cross-reactivity between anti-MCV and anti-CCP antibodies and found a substantial cross-binding in a subgroup of ACPA positive patients.

MATERIALS AND METHODS

Patients. Our cohort comprises 77 patients with RA from 2 different clinics and a private rheumatology practice in Rostock and Berlin. All patients fulfilled the American College of Rheumatology (ACR) revised criteria for the classification of RA¹¹. The patients were selected for being positive for ACPA and all but one were positive for RF. The patients were between 22 and 86 years of age and had a disease duration ranging from recent onset to 38 years. Therapy was heterogeneous and consisted of disease-modifying antirheumatic drugs plus cortisol, adalimumab, etanercept, infliximab, or rituximab, respectively. All sera were obtained prior to the treatment with biological agents. The study was approved by the local ethics committees and informed consent was given by all patients prior to serum sampling.

IgG subclass specific ELISA. An IgG subclass specific ELISA was established and described elsewhere¹⁰. In brief, commercially available ELISA plates coated with CCP (2th generation; EUROIMMUN) or MCV (Orgentec) were combined with peroxidase conjugated human IgG subclass specific antibodies for detection. To quantify CCP and MCV specific antibodies, the sera were diluted 1:400 to analyze IgG1 and 1:20 for IgG2, IgG3, and IgG4. The control sera delivered with the kit systems were used for normalization of the data. Optical densities (OD) were calculated as OD₄₅₀ (specific wave length) minus OD₆₅₀ (reference wave length) as determined by an automated plate reader (Milenia Kinetic Analyser, Diagnostic Products Corp., Los Angeles, CA, USA). All assays were run in duplicates.

Elution ELISA. To test for the cross-reactivity of antibodies directed against MCV and CCP, we established a sequential ELISA. To that end we determined serum dilutions that allow for a 2-fold excess of bound versus unbound antibodies in the ELISA system in order to guarantee comparable equilibria. The dilutions varied depending on the ELISA system and were between 1:200 and 1:1,600 for the detection of anti-MCV and between 1:500 and 1:12,800 for anti-CCP antibodies. The first ELISA was performed in duplicates and one sample served to determine the initial OD of anti-CCP and anti-MCV IgG, respectively. The second sample served to isolate the specific antibody population, either CCP or MCV specific. In detail, bound antibodies were eluted with 200 μ l 0.1M glycine-HCl 0.05M NaCl pH 2.5 and immediately neutralized with 4.5 μ l 1M Tris-HCl pH 9.0 per 100 μ l elution buffer. The eluate was divided in halves and incubated again on 2 ELISA plates. The first one was coated with CCP, the second one with MCV. Detection of bound antibodies was performed with an anti-human IgG antibody (EUROIMMUN, according to manufacturer's instructions) or an anti-human IgG4 antibody (Serotec, Kidlington, UK, 1:25,000). Data were normalized to an expected maximum OD of the second ELISA which corresponded to half of the OD of the initial ELISA. All assays were performed in duplicate.

Statistics. Student's t-test was applied to compare the titers of antibodies against CCP and MCV. To assess the correlation between both titers *p* values and correlation coefficients were calculated by the Pearson method using R.

RESULTS

CCP and MCV autoantibody levels were significantly correlated. We quantified the titers of anti-CCP and anti-MCV antibodies in the sera of 77 patients with RA and compared the levels of each IgG subclass using the OD as indirect indicator. Our results show that the anti-CCP titers exceeded

ed the anti-MCV titers and this proportion held true for IgG1 ($p < 0.001$), IgG2 ($p < 0.01$), and IgG4 ($p < 0.001$), but not for IgG3 (data not shown). Moreover, Figure 1 shows highly significant correlations between anti-CCP and anti-MCV levels for IgG4 ($R = 0.8$), IgG3 ($R = 0.63$) and IgG1 ($R = 0.47$). For IgG2 subclass, only a trend was seen ($R = 0.2$, $p = 0.08$), which may be explained by the low sera levels of this subclass among antibodies recognizing CCP and MCV. The observed correlation between anti-MCV and anti-CCP antibodies can be explained by mutual regulation or cross-reactivity, and we tested for the latter.

Antibodies against MCV exhibit cross-reactivity in 8 out of 16 patient sera. To investigate potential cross-reactivity, we developed a sequential ELISA. The first step includes the isolation of either MCV or CCP specific antibody populations from the sera of 16 patients with RA. This was done by eluting the bound antibodies from the respective ELISA plates. In a second step, the eluted antibodies were allowed to bind to the second ELISA plate, coated with either MCV or CCP (Figure 2A and D). Developing the first plate yielded the percentages of non-eluted antibodies, which were 18% in case of the MCV- and 19% in case of the CCP-specific antibodies and thus comparable. Developing the second ELISA plates yielded the percentages of both antibody re-binding to the same antigen and cross-binding antibodies. Re-binding to the same antigen was observed for a mean of 26% of the MCV- and 55% of the CCP-specific antibodies (Figure 2B and E).

The mean percentage of CCP specific antibodies cross-binding to MCV was 11.5% and only marginally detectable with patients 4 and 5 posing the exception and presenting substantial cross-reactivity (Figure 2E). Interestingly, the cross-binding activities of the MCV specific antibody populations from patients 1-8 again were hardly detectable, presenting a mean percentage of 10%. In contrast, the MCV specific antibodies from patients 9-16 cross-bound even better to CCP than to MCV itself and showed a mean of 44% binding (Figure 2B). Thus, the anti-MCV response subdivided ACPA positive patients with RA into a group with broad and a group with narrow specificities. In order to characterize both groups further, we compared clinical and genetic measures; however, we found no differences with respect to age ($p = 0.26$), disease duration ($p = 0.65$), erythrocyte sedimentation rate ($p = 0.86$), C-reactive protein ($p = 0.92$), Disease Activity Score 28 ($p = 0.86$), HLA-DRB1 haplotypes, or shared epitope status (Table 1). Only the IgG1 titer against CCP showed a significant difference between both groups ($p = 0.02$) with a mean OD of 0.91 for the narrow responders and 1.58 for the broad responders.

Due to its capacity to exchange F(ab) arms, IgG4 is particularly suitable to investigate a broad ACPA specificity. To that end we repeated the sequential ELISA using the IgG4 fraction only. Interestingly, while anti-CCP specific ACPA again only marginally reacted with MCV, anti-MCV specif-

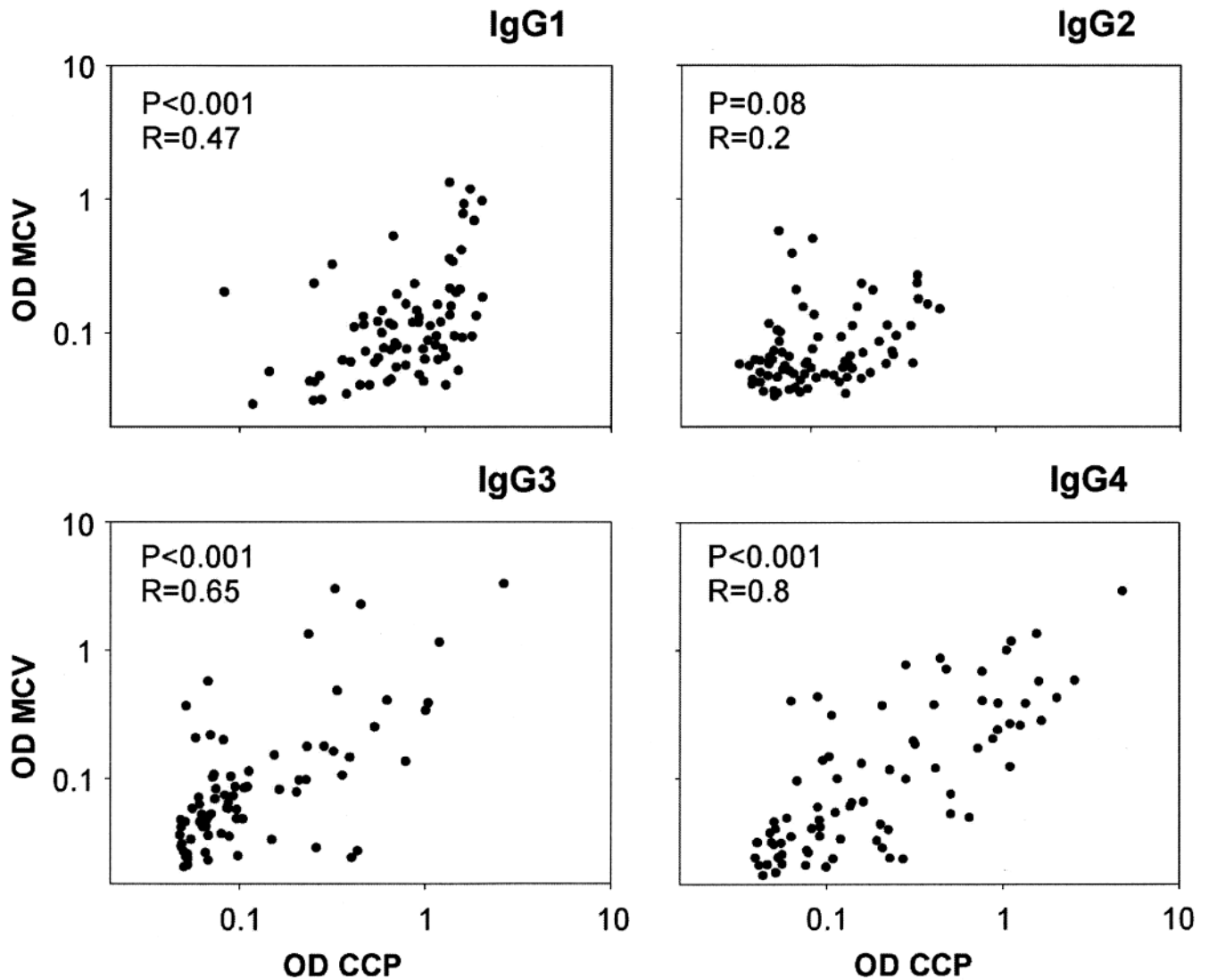


Figure 1. Titers of autoantibodies directed against cyclic citrullinated peptide (CCP) and mutated citrullinated vimentin (MCV) correlate with each other. Scatter plots show anti-CCP and anti-MCV levels of each IgG subclass. Each dot represents one of 77 patients; p values and Pearson correlation coefficients (R) are given.

ic IgG4 substantially bound to CCP in all 3 samples tested (Figure 2C and F) confirming a broader specificity for anti-MCV specific ACPA.

DISCUSSION

We confirmed the previously observed correlation between titers of MCV- and CCP-specific antibodies in patients with RA^{7,12} and extended our findings to the IgG1, IgG3, and IgG4 subclasses. We cannot rule out that mutual regulation during the plasma cell development led to correlating antibody titers; however, for a subgroup of ACPA positive patients we showed cross-reactivity of MCV-specific antibodies against CCP.

While the CCP2 test uses 2 citrullinated peptides select-

ed for maximum specificity and sensitivity in RA diagnosis⁶, MCV as a natural antigen contains many different citrullination sites: In detail, vimentin contains 43 arginine residues with 10 citrullination sites experimentally confirmed⁷. The narrow spacing between 17 of these arginines predicts epitopes with more than one potential citrullination site and, in the case of partial citrullination, allows for an even higher diversity of epitopes. It is therefore reasonable to assume that the MCV antigens at large bind a variety of autoantibodies with different specificities. In fact, the increased sensitivity of anti-MCV- compared to anti-CCP-antibodies in RA diagnosis supports the notion that MCV bears more epitopes than CCP¹².

Our results support this concept even further. While only

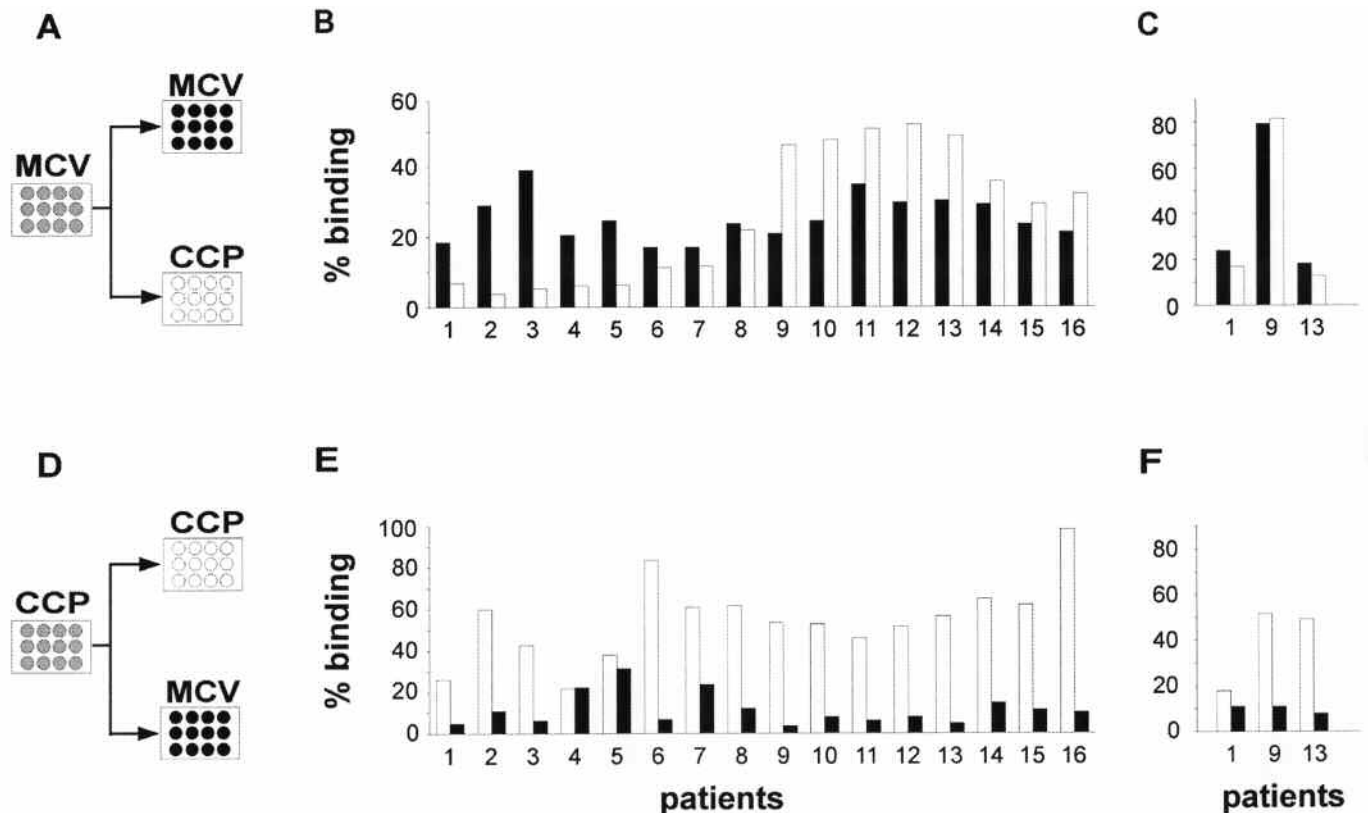


Figure 2. Diversity of anti-MCV response subdivides patients with rheumatoid arthritis (RA) positive for autoantibodies against citrullinated peptide antigens (ACPA) into 2 groups. Mutated citrullinated vimentin (MCV)- (A) and cyclic citrullinated peptide (CCP)-specific antibodies (D) were isolated from the sera of 16 patients with RA using ELISA plates. The eluates were split in 2 and each half applied to a second plate, coated with either MCV or CCP. 100% binding to the second plate corresponds to half of the optical density measured for the primary plate (B and E). C and F are replicates of B and E using the IgG4 positive fraction only.

Table 1. Genetic and clinical measures from RA patients with a broader response spectrum (9–16) and a narrower spectrum (1–8).

Patient Number	Age, yrs	Duration of RA, yrs	Sex	Therapy	ESR, mm	CRP, mg/l	DAS28	HLA-DRB1 Haplotypes	Shared Epitope Status ¹³
1	53	14	m	SASP, MTX	7	13.2	3.07	4/11	S2/S3D
2	67	15	f	E, P	9	13.9	1.5	1/7	S3P/X
3	22	3	m	P	80	104	5.64	4/4	S2/S2
4	65	38	f	A	62	27.9	6.6	3/7	X/X
5	18	3	m	Rx	56	3	6.01	1/4	S3P/S3P
6	71	9	f	A	100	44.6	ND	4/4	S2/X
7	55	4	f	E	100	17.1	6.33	4/13	S2/S1
8	62	3	m	A	64	13.5	6.6	1/4	S3P/S2
9	66	4	m	MTX	48	20.4	3.38	4/6	S2/ND
10	72	27	f	A	54	6.6	ND	1/7	S3P/X
11	76	12	f	A	56	39	5.02	4/4	S2/S2
12	44	18	f	A	38	3	6	3/4	X/S2
13	77	34	f	Rx	88	25.6	3.83	4/4	S2/S2
14	51	3	m	C	66	38.4	6.3	4/7	S2/X
15	62	10	m	I	ND	83.4	7.14	4/4	S2/S3P
16	47	3	f	C	50	8.9	ND	3/4	X/S3P

A: adalimumab, E: etanercept, C: cortisol, I: infliximab, MTX: methotrexate, P: prednisolone, Rx: rituximab, SASP: sulfasalazine; RA: rheumatoid arthritis; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS28: Disease Activity Score 28-joint count.

2 of the CCP isolates bound significantly to MCV, autoantibodies specific for MCV also recognized CCP in 8 out of 16 RA patient sera. Most strikingly, the binding of anti-MCV antibodies to CCP was even stronger than to MCV itself. Two scenarios are conceivable to explain our results. In the first scenario, all antibodies react with both CCP- and MCV-epitope groups, and the sum of all affinities against CCP exceeds the sum of all affinities against MCV. In the second scenario, part of the antibodies react with MCV only, and part react with both epitope groups, again with an elevated affinity of MCV specific autoantibodies for CCP. Our results support the latter scenario as the striking cross-reactivity of anti-MCV specific total IgG to CCP in 8 out of 16 patients was reduced to a comparable binding when bivalent IgG4 antibodies were used (Figure 2B and C).

Our results suggest a subclassification of ACPA positive patients into a group with a narrow and a group with a broad recognition spectrum of anti-MCV specific antibodies. So far we found no differences between both groups with respect to clinical measures and shared epitope status. However, it remains to be investigated in larger cohorts whether the anti-MCV specific recognition spectrum is predictive of a certain course of disease or responsiveness to therapies targeting B cells.

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