Selected cyclic citrullinated peptides derived from the sequence of mutated and citrullinated vimentin (MCV) are targeted by different antibodies subclasses in patients with rheumatoid arthritis in Russian patients

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Abstract

Objective

Antibodies against citrullinated antigens (ACPA) represent one rheumatoid arthritis (RA) classification criteria. Recently, mutated and citrullinated vimentin (MCV), containing approx. 45 potentially citrullinated sites, was characterised as another modified autoantigenic RA target. Therefore, we wanted to screen, select and validate predominant MCV autoantigenic epitopes (called here MCE) as possible new diagnostic targets.

Methods

MCV-derived peptides with citrullinated sites were screened in healthy controls and patients. Based on this, twelve selected MCE were used for validation of ACPA isotypes (IgA/IgG/IgM) with ELISA in early RA (ERA, <12 months) and established RA (>12 months) Russian patients. Sensitivity of MCE reactivity was compared to commercially available ELISAs for anti-CCP IgG, anti-MCV IgG, and anti-RF IgA/IgM/IgG.

Results

Anti-MCE IgG/IgA//IgM antibodies were observed in 64.1%, 23.1%, and 15.4% ERA, and 63.9%, 26.7%, and 13.1% established RA patients, respectively. Anti-MCV IgG was present in 64.1% ERA and 55.0% RA patients. Furthermore, anti-CCP IgG and RF IgG/IgA/IgM were detectable in up to 76.9%, 71.8%, 71.8%, and 38.5% ERA, and 80.1%, 72.3%, 67.5%, and 43.0% RA patients. Anti-CCP IgG single positivity was observed in 7.7% ERA and 6.3% RA patients. Only one RA patient was anti-MCE single positive.

Conclusion

MCV autoantigenic epitopes were emulated by cyclic citrullinated MCV-derived peptides and recognised by all autoantibody-Ig subclasses in RA. Tested MCE were recognized more frequently by IgG as the original MCV antigen. High antibody prevalence against CCP epitopes suggests a strong CCP-linkage to RA pathogenesis in the investigated Russian cohort.

Key words

rheumatoid arthritis, citrullinated peptides, ACPA, modified vimentin

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Introduction

In rheumatoid arthritis (RA), as a chronic autoimmune disease with erosive arthritis and systemic involvement, certain post-translational protein modifications can lead to the formation of autoantigens (1). The so far best described modifications include citrullination and mutation of targeted proteins such as vimentin, fibrinogen, collagen type 2, and alpha-enolase (2-6). Recently, proteins with homocitrullin residues (a carbamylated lysine residue) have also been described as autoantigens in patients with RA (7). In this context, rabbits immunised with homocitrullinated albumin generated antibodies strongly cross react with commercial cyclic citrullinated peptide (CCP) but weakly with mutated citrullinated vimentin (MCV). It has been shown that protein citrullination could be influenced by environmental factors such as smoking and that it is closely linked to a genetic background of the shared epitope (8). Thus, multiple coincidental and cross-linked factors are involved in the pathogenesis of RA leading to the specific autoimmune response observed.

Early diagnosis of RA is essential but often hindered by a slow onset of unspecific symptoms such as fatigue, loss of appetite, weakness, morning stiffness, arthralgias, and mono- or oligoarthritis, which can be also seen in many other diseases. Therefore, to improve early diagnosis of RA, new classification criteria have recently been developed (9, 10). Considering the relative weight of the different available diagnostic parameters, specific laboratory markers, namely seropositivity for rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA), strongly contribute to the early identification of patients with RA (11).

For detection of citrullin-specific antibodies (abs), anti-CCP assays with different performances are used as a standard worldwide (12). In this context, the employed citrullinated peptides are artificial and arranged in a cyclic structure. Furthermore, the sequence of CCP used for the first generation test is derived from filaggrine, and it has not been shown that this antigen is expressed in the synovial tissue (13). Nevertheless, so far the CCPs have been the most specific autoantigenic structures available for the serological diagnosis of RA (14, 15).

In the past years, additional proteins (*e.g.* fibrinogen, vimentin and alpha-enolase) have been identified as modified autoantigens in the pathophysiology of RA. Nowadays these different humoral autoimmune responses, including abs to CCP, are summarised as ACPA. These abs provide superior specificity compared to RF, and also contribute to the prediction of course and outcome of disease (16). The sensitivity of ACPA in the diagnosis of RA is 70-80%, with a very high specificity of more than 90% (15). Importantly, the sensitivity of anti-CCP IgG for patients with early RA (ERA) is also high, with approximately 60% (17, 18).

Recently, MCV was isolated from synovial fluid of patients with RA and characterised as a new promising autoantigen (6). Anti-MCV abs as well as few other ACPAs, including abs to third generation CCP, are now used for diagnostic purposes in the clinic (19). In this study, we have identified and selected antigenic epitopes of MCV (MCE) to generate cyclic peptides thereof. These selected antigens were used for characterisation of autoantibody immunoglobulin (Ig) subclass reactivities in comparison to standard anti-MCV, anti-CCP, and RF assays in RA patients.

Methods

Patients and controls

For standardisation and validation of the self-developed ELISA test for evaluation of reactivity against selected MCV peptides, samples from healthy controls (n=437, mean age 38 years ± standard deviation (SD) = 13.5 years) were tested. Furthermore, samples from individuals with different diseases (*e.g.* rheumatoid arthritis, n=147; arthralgia, n=10; Sjögren's syndrome, n=10; systemic lupus erythematosus (SLE), n=23; polymyalgia, n=1; psoriasis arthritis, n=28; reactive arthritis, n=5; spondylarthritis, n=4; spondylitis ankylosans (Morbus Bechterew), n=1) were collected and included for analysis as disease controls. In order to examine if anti-MCE are detected in infectious diseases, additional individuals with

hepatitis C virus infection (anti-HCV positive, n=31) were tested by ELISA for anti-MCE (Orgentec) and anti-CCP (Medipan). For further validation and evaluation of autoantibody reactivity against cyclic epitopes of MCV, we investigated serum samples from a cohort of Russian patients with RA (n=230, mean age ± SD= 52.06±12.07 years, female:male=4:1) fulfilling the 2010 revised criteria of the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) (9, 10). Patients were classified according to the duration of disease as ERA (<12 months; n=39, mean age ± SD= 47.33±12.22 years, female:male=3:1) or established RA (>12 months; n=191 mean age ± SD=53.03±11.84 years, female:male=5:1). Clinical characterisation of patients was performed with a disease activity score using 28 joint counts (DAS28 ESR), radiographic stage according to Steinbrocker, and functional class according to the ACR revised criteria for classification of functional status in RA (Table I) (20-22). None of the patients had active tuberculosis, active hepatitis or severe co-morbidities. Patients with RA were recruited randomly from the State Educational Institution of Higher Professional Education "Voronezh State Medical Academy N.N. Burdenko of Roszdrav" or the Hospital Therapy Department of the State Institution of Healthcare "Voronezh Regional Clinical Hospital #1" after providing written informed consent. The study was approved by the local ethics committees (protocol no. 3, 2010).

Table I. Clinical characteristics of Russian patients with RA.

Clinical characteristics	ERA (n=39)	Established RA n=191)
Activity of disease (DAS 28 ESR)		
remission (DAS28<2.6)	0	1 (0.5%)
low (2.6<DAS28<3.2)	5 (12.8%)	22 (11.5%)
moderate (3.2<DAS28≤5.1)	32 (82.1%)	140 (73.3%)
high (DAS28>5.1)	2 (5.1%)	28 (14.7%)
X-ray / Steinbroker's stage		
Stage I	8 (20.5%)	20 (10.5%)
Stage II	28 (71%)	110 (57.9%)
Stage III	2 (5.1%)	51 (26.7%)
Stage IV	0	10 (5.2%)
RA Functional class		
Class I	5 (12.8%)	12 (6.3%)
Class II	34 (87.2%)	127 (66.5%)
Class III	0	49 (25.7%)
Class IV	0	3 (1.6%)

Summary of clinical characteristics of Russian patients with early RA (ERA) and established RA according to DAS28 ESR, radiographic stage by Steinbroker's method, and functional class by ACR revised criteria for classification of functional status in RA. Given are absolute patients' numbers and percentages (in brackets) from each group. (From one ERA patient, there were no data available regarding x-ray status classification)

Detection of anti-CCP, RF and anti-MCV antibodies using ELISA

To characterise the serological status of the patients, two different commercially available ACPA assays (using the CCP and MCV as autoantigenic structures) and RF Ig subclass tests were performed. Anti-CCP IgG antibodies were determined according to the manufacturer's instructions using the ELISA Medizym anti-CCP kit (Medipan GmbH, Dahlewitz/Berlin, Germany). Anti-MCV IgG and RF IgG, IgM, and IgA were determined according to manufacturer's instructions using the corresponding commercially available ELISA kits (Orgentec Diagnostika GmbH, Mainz, Germany). Measurement of RF IgM was confirmed by using a second ELISA kit (Medipan).

Synthesis of MCV-derived peptides

The published sequence of MCV (6) was used as a template to synthesise overlapping 17mer peptides, in accordance with Fmoc-chemistry (Perbio Science Deutschland GmbH, Bonn, Germany). Each peptide has an overlap of 12 amino acid residues to the corresponding neighbourhood peptide. The synthesis through the MCV amino acid sequence resulted in 91 peptides with the general formula "Biotin-SGSG-PEPTIDE-Amide". Peptides were compared as non-citrullinated and citrullinated variants. The N-terminal extension of the peptides (Biotin-SGSG) was designed for a defined flexibility and to incorporate an affinity tag. Crude fractions after peptide synthesis were purified using high-performance liquid chromatography. Quality and purity of the peptide was assessed by mass spectrometry and analytical high-performance liquid chromatography in according to the manufacturer procedure (Perbio Science).

ELISA for detection of MCV-derived peptides (Epitope ELISA)

Microtiterplates (Maxisorb, Nunc, Roskilde, Denmark) were precoated with 1 µg/ml streptavidin (Perbio Science) in phosphate-buffered saline (PBS, pH=7.6). After an incubation time of 2 hours at 25°C, the plates were blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature. Precoated plates were used for binding of biotinylated peptides/antigens. Biotinylated 17mer peptides were diluted at a concentration of 0.5 µg/ml in PBS and incubated over night at 4°C (100 µl/ well). In addition to the 91 peptides, biotinylated recombinant MCV, protein A and rheumatoid factor (Fc part of human IgG) were simultaneously used as internal control antigens. Coating and blocking was done as described for the peptides. Non-bound peptides/ antigens were removed by washing the cavities with 200µl/well of 0.1% Tween 20 in PBS.

The assay was performed in accordance with the general protocol for the Orgentec ELISA system. In brief, serum samples were diluted 1:101 in sample buffer (PBS, containing bovine serum albumin and Tween), added to the wells and then incubated for 30 min (100 µl/ well). After three washing steps with 300 µl/well, 100 µl/well horseradish peroxidise-conjugated anti-human IgG (Dianova, Hamburg, Germany) was added and incubated for 30 min. Visualisation was done by incubation with 100 µl/well 3,3',5,5'-tetra-methyl benzidine

substrate (Organon-Teknika, Boxtel, The Netherlands) for 15 min, and the reaction was terminated by adding 50 µl stop solution (0.5 mol/l sulfuric acid) to each well. Finally, absorbance at 450 nm was determined using an ELISA reader (Rainbow Reader, Tecan, Germany). All steps were carried out at room temperature. Background optical density (OD) values were obtained by adding each serum to a well without protein. A positive serum was defined by an OD value more than twice background OD (23). Each serum sample was tested in duplicate. To further define the assay characteristics, 21 normal human sera were assayed in accordance with the instructions for use. In all subjects with RA, anti-MCV and RF abs were also determined with commercially available ELISA test systems according to manufacturer's procedure (Orgentec).

Reactivity against cyclic MCV-derived peptides in ELISA

MCV-derived peptides showing a good reaction in the epitope ELISA screening were selected and synthetised as biotinylated large scale peptide variants (MCE). Single MCE peptides were coupled to microtiterplates (Maxisorb, Nunc, Denmark) via streptavidin and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. For blank controls, wells were coupled with 2 µg/ml biotinylated BSA/well. Antibodies against MCE cyclic epitopes of IgG, IgM, and IgA subclasses were detected by ELISA (Orgentec). Sera were diluted 100-fold in 1x PBS with 1% BSA/0.05% Tween 20 and incubated for 30 min at room temperature. After washing three times with PBS/0.05% Tween-20, 100 µl of anti-human IgG conjugated to peroxidase (Dianova, Germany) diluted 1:15.000 was added to the wells. After incubation (15 min at room temperature), plates were washed again three times, and bound antibodies were detected with 3,3-5,5- tetramethyl-benzidine (Organon-Teknika) as a substrate. After 15 minutes, the reaction was stopped by adding 100 µl 2 M sulfuric acid/ well. Plates were read at a wavelength of 450/620 nm in a BioTek microwell photometer (Synergy HT, BioTek Instruments Inc, USA). The assay was standardised by using positive and negative controls. Furthermore, a standard serum was used for calculation of antibody titers (U/ml) by 4-Parameter-Fit with lin-log coordinates for OD. Normal range (<20 U/ml) was established by testing blood donors (n=437, with mean + 3-fold SD for IgG ab 7.3+3.8 U/ml, for IgA ab 5.4+2.4 U/ml and for IgM ab 8.7+3.6 U/ml).

To assess the imprecision of the anti-MCE peptide ELISA, anti-MCE positive sera (one low value sample [L], one medium value sample [M] and one high value sample [H]) were assayed in five independent runs on one day (inter-assay) or in a single run (intra-assay). For within-run imprecision, L, M and H samples were measured in six replicates on one solid phase. The imprecision data were calculated using analysis of variance. The intra-assay coefficient of variation (CV) was 3.2, 3.1, and 2.3% for the mean concentrations of 70.1, 142.3, and 827.3 U/ml, respectively, whereas inter-assay CV was 4.6, 3.4, and 3.3% for mean concentrations of 62.1, 186.3, and 712.6 U/ml, respectively.

Statistical analysis

Sensitivity was calculated exclusively employing the RA disease control group. Specificity was calculated against both the rheumatic disease control and healthy control groups. Validation of the MCE test and comparison with CCP and MCV tests were made with the Russian RA group. Means, standard deviations, *p*-values and correlation analyses were calculated using GraphPad Prism software (version 4.00). *p*-values of <0.05 were considered to be statistically significant.

Results

Epitope mapping of the MCV antigen

To evaluate the effect of arginine citrullination on the antigenicity of MCV and to localise relevant epitopes on linear polypeptides, a panel of anti-MCV IgG positive sera (n=142) was tested for reactivity with linear 17mer peptides covering the complete amino acid sequence of MCV, based on the MCV sequence as reported elsewhere (6). All tested anti-MCV IgG positive sera showed increased binding to MCV peptides containing citrulline in contrast to those containing unmodified arginine residues. In fact, strong ab reactivities were only observed against peptides with citrulline containing residues.

Reactivity against MCV peptide variants

Subsequently, sera from patients with RA, disease controls, and healthy volunteers reactive with MCV peptides were assayed by ELISA on selected biotinylated peptide variants. Of note, none of the patients from the disease control groups were positive for the test (cut-off 20 U/ml), and in the healthy control group, only one donor (0.22%) was positive (26.3 U/ml), while in the RA group 123 patients (83.7%) were tested positive. Peptides missing five COOH-terminal residues were still reactive with the same number of sera as the original peptide, although in most cases the OD value was lower. Interestingly, reactivity of these sera was enhanced by cyclisation of the same peptide via S-S-bridge from the introduced N- and C-terminal cystein residues (data not shown). This can be explained by an influence of antigen conformation on antibody binding. A total of 12 predominant epitopes were defined by an antibody reactivity of OD >0.5. Characterisation of fine specificity of the ACPAs against MCV resulted mainly in identification of epitopes corresponding to earlier described dominant vimentin peptide sequences for HLA binding and stimulation of T cell proliferation (24-26). Furthermore, there was a positive correlation between the level of ACPAs and the number of recognised epitopes. No single peptide was sufficient to cover the reactivity corresponding to the whole recombinant protein, but rather a mixture of at least six MCV related peptides was required for this. These six peptides, which are presented in Table II, were used for further analyses.

Autoantibody reactivities against MCE and other established antigens in patients with RA

Sera from patients with early and established RA were used for further evaluation of selected MCE by ELISA. In-

creased IgG, IgA and, IgM to MCE were observed in 25 (64.1%), 9 (23.1%), and 6 (15.4%) patients with ERA, as well as in 122 (63.9%), 51 (26.7%), and 25 (13.1%) patients with established RA, respectively (Table III). In comparison, anti-MCV IgG were positive in 25 (64.1%) patients with ERA, and in 105 (55.0%) patients with established RA. With respect to the other autoantibody reactivities, anti-CCP IgG (Medipan) and RF IgG, IgA, and IgM (Orgentec) were elevated in 30 (76.9%), 28 (71.8%), 28 (71.8%), and 15 (38.5%) of patients with ERA, and in 153 (80.1%), 138 (72.3%), 129 (67.5%), and 82 (43.0%) of patients with established RA, respectively. Subsequently, the observed low prevalence of RF IgM measured with the first ELISA assay was confirmed by using a second commercially available ELISA kit (Medipan), revealing positive results in only 10 (25.6%) of patients with ERA, and in 37 (19.4%) of patients with established RA. These results are also shown in Table III. However, for further interpretation, only the results for RF obtained by the Orgentec ELISA kits were considered. Interestingly, 43.6% of the patients with ERA and 43.9% of patients with established RA expressed anti-CCP ab titres of more than 1000 U/ml. Of note, the antibody titres for anti-MCE IgG and for anti-MCV IgG were comparable in individual patients.

A parallel seropositivity for all investigated autoantibodies (anti-CCP IgG, anti-MCV IgG, and anti-MCE IgG) was detectable in 43.6% of samples from patients with ERA, and in 41.9% of patients with established RA. In comparison, seronegative results for all autoantibodies (anti-CCP IgG, anti-MCV IgG, and anti-MCE IgG) was present in 12.8% of samples with ERA and 16.8% with established RA. Single positive results for anti-CCP IgG without detectable anti-MCV IgG and anti-MCE IgG were observed in 7.7% of patients with ERA, and in 6.3% of patients with established RA.

The combination of double positive anti-CCP IgG and anti-MCV IgG, but negative anti-MCE IgG was detectable in 10.3% of patients with ERA, and in 10.5% of patients with established RA. The combination of double positive anti-CCP IgG and anti-MCE IgG, but negative anti-MCV IgG was found in 15.4% of patients with ERA, and in 21.5% of patients with established RA. In summary, from all anti-MCE abs positive samples, which were negative for anti-CCP IgG and for anti-MCV IgG, only 0.5% of patients with RA were detected in our cohort (Table IV). Of note, in patients with negative anti-CCP IgG, determination of anti-MCV IgG identified an additional proportion of 10% of ACPA positive cases with ERA and 2.6% of patients with established RA. In the same context, anti-MCE IgG ab were detectable in 5% of anti-CCP IgG seronegative patients with ERA and 0.5% of patients with established RA. A schematically summary of IgG positive cumulative

Table II. Mutated and citrullinated vimentin (MCV)-related peptides required for covering the reactivity of the whole recombinant protein.

MTP position	Sequence	Corresponding vimentin region and substitution
B1	SSSSY**XX**MFG**X**PGTAS**X**	Sequence 7 - 24, Arg12/13-Citr, Gly16-Citr
E1	S**X**PSSS**X**SYVTTST**X**TY	Sequence 22 - 41, Arg23 - Citr, Arg28 - Citr., Arg35 -Citr
H1	TYSLGSAL**X**PSTS**X**<u>**H**</u>LY	Sequence 37 - 54, Arg45-Citr, Arg50 - Citr, Ser51-His
C2	PG**X**VYAT**X**SSAV**X**L**X**SS	Sequence 57 - 74, Gly59-Citr, Arg64 - Citr., Arg69-Citr., Arg71-Citr
G5	NASLA**X**LDLE**X**KVESLQ	Sequence 211 - 228, Arg217 -Citr, Arg222- Citr
B8	DAL**X**QAKQESTEY**XX**QV	Sequence 307 - 324, Arg310-Citr., Arg320/321 - Citr

Six predominant MCV related peptides are required for covering the reactivity of the whole MCV recombinant protein. Bold "X" in the sequences denotes citrulline; bold and underlined "<u>X</u>" indicates mutated glycine residue in arginine and citrullinated.

Table III. Distribution of RA-associated autoantibody subclasses in early (ERA) and established RA in the Russian cohort.

ERA (0–12 months) n=39		Established RA (>12 months) n=191	
Positive samples absolute (relative) numbers	Titer Mean, U/ml	Positive samples absolute (relative) numbers	Titer Mean, U/ml
	CCP IgG (negative <30 U/ml)		
30 (76.9%)	2169.5	153 (80.1%)	1951.1
	MCE IgA (negative <20 U/ml)		
9 (23.1%)	99.8	51 (26.7%)	318.2
	MCE IgG (negative <20 U/ml)		
25 (64.1%)	687.4	122 (63.9%)	563.5
	MCE IgM (negative <20 U/ml)		
6 (15.4%)	378.4	25 (13.1%)	124.2
	RF IgA (negative <20 U/ml)		
28 (71.8%)	338.3	129 (67.5%)	364.7
	RF IgG (negative <20 U/ml)		
28 (71.8%)	359.3	138 (72.3%)	390.6
	RF IgM (ELISA, from Orgentec; negative <20 U/ml)		
15 (38.5%)	363.9	82 (43.0%)	228.9
	RF IgM (ELISA, from Medipan; negative <20 U/ml)		
10 (25.6%)	28.2	37 (19.4%)	44.5
	MCV IgG (negative <20 U/ml)		
25 (64.1%)	724.9	105 (55.0%)	532.5

Shown are the absolute and relative (in parenthesis) numbers, as well as mean titers of positive samples tested for the different parameters (CCP, MCV, MCE, RF) and immunoglobulin (Ig) isotypes (A, G, M) in a Russian cohort of patients with early (ERA) or established rheumatoid arthritis. Cut-off value for CCP was 30, for all other parameters always 20 Units/ml. CCP: cyclic citrullinated peptides; MCV: mutated and citrullinated vimentin; MCE: selected epitopes of MCV; RF: rheumatoid factor.

Table IV. Summary of combinatory analysis of RA-associated CCP/MCV/MCE auto-antibodies.

Parameter	ERA (n=39) absolute (relative) numbers	Established RA (n=191) absolute (relative) numbers
CCP+ / MCV+ / MCE+	17 (43.6%)	80 (41.9%)
CCP- / MCV- / MCE-	5 (12.8%)	32 (16.8%)
CCP+ / MCV -/ MCE-	3 (7.7%)	12 (6.3%)
CCP+ / MCV +/ MCE-	4 (10.3%)	20 (10.5%)
CCP+ / MCV -/ MCE+	6 (15.4%)	41 (21.5%)
CCP- / MCV +/ MCE+	2 (5.1%)	0 (0%)
CCP- / MCV -/ MCE+	0 (0%)	1 (0.5%)
CCP- / MCV +/ MCE-	2 (5.1%)	5 (2.6%)
CCP- / MCV- / MCE- IgG & MCE IgM- / MCE IgA-	4 (10.3%)	29 (14.7%)
CCP- / MCV- / MCE- IgG & MCE IgM ± / MCE IgA ±	1 (2.6%)	3 (1.6%)
MCE IgG- / IgA+ / IgM+	1 (2.6%)	0 (0%)
MCE IgG- / IgA+ / IgM-	2 (5.1%)	3 (1.6%)
MCE IgG- / IgA- / IgM+	2 (5.1%)	4 (2.1%)

Shown are the absolute and relative (in parenthesis) numbers of samples according to the combinations of CCP/MCV/MCE tested in a Russian cohort of patients with early (ERA) or established rheumatoid arthritis. CCP: cyclic citrullinated peptides; MCV: mutated and citrullinated vimentin; MCE: selected epitopes of MCV.

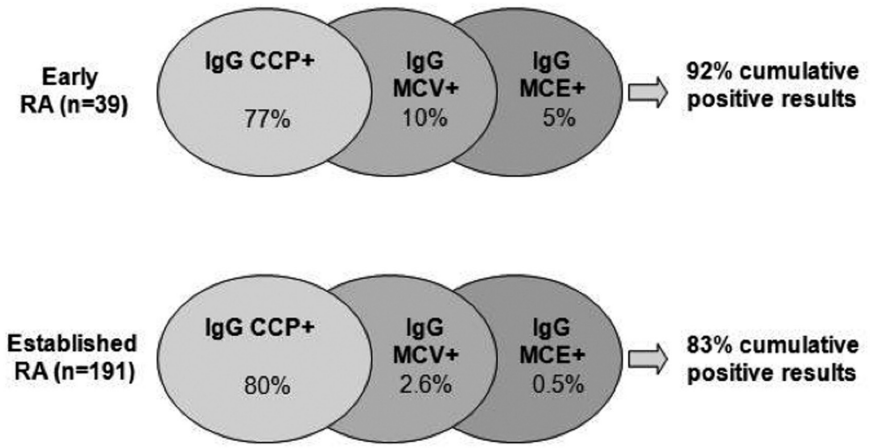

Fig. 1. Cumulative positive results of IgG CCP, MCV and MCE in patients with early and established arthritis.
Approximately 77% of early arthritis patients (ERA) and 80% of established RA patients were positive for CCP IgG. Additionally in CCP IgG negative patients, determination of anti-MCV IgG identified 10% ACPA further positive patients with ERA and 2.6% patients with established RA, and anti-MCE IgG ab were detectable in further 5% of ERA and 0.5% of patients with established RA.

results for these different parameters is presented in Figure 1. Taken together, anti-MCV IgG and anti-MCE IgG were found to be positive simultaneously in 15% of anti-CCP IgG negative patients with ERA and 3% with established RA, respectively.

Notable, none of the patients with hepatitis C virus infection (anti-HCV positive) showed a positive result for anti-MCE, while 3 samples (9.7%) were positive against CCP. Although the analysed number of samples was low, these results indicate that anti-MCE antibodies are not more frequent in patients with hepatitis C compared to anti-CCP antibodies.

In this cohort, no significant correlation was observed between the different autoantibody results and clinical (DAS28, functional class) as well as radiological stage (according to Steinbrocker). However, the number of completely seronegative cases was very low for ERA (8%) as well as established RA (6%), which does not allow appropriate comparison to the seropositive group.

Discussion

Since mutated and citrullinated vimentin (MCV) is another modified autoantigen in RA, containing approx. 45 potentially citrullinated sites, the aim of this study was to screen and select predominant MCV autoantigenic epitopes, which we called MCE, as possible new diagnostic targets, and to validate these selected epitopes through analysis of Ig subclass reactivity against them in detail. For this, we used a novel ELISA system for the detection of abs against MCE in a cohort of Russian patients with confirmed diagnosis of early or established RA in direct comparison with detection of the original MCV antigen and standard anti-CCP IgG assay. Taken together, we were able to show that the autoantigenic epitopes of MCV could be emulated by using cyclic citrullinated vimentin peptides.

Antibody reactivity against MCE included all Ig subclasses, with a predominance of IgG. Of note, the citrullinated cyclic vimentin peptides were recognised as autoantigens by abs from patients with early and established RA. The binding of anti-MCV IgG to the peptides was significantly influenced by citrullination of arginine residues as well as by cyclic formation of the epitopes. The main autoantibody subclass of anti-MCE abs was identified as IgG, followed by IgA and IgM subclasses.

According to our results, detection of anti-CCP IgG provided a higher sensitivity compared to anti-MCV IgG ab as well as anti-MCE IgG in Russian patients with early and established RA. Of note, the combination of anti-CCP IgG and anti-MCV IgG showed a higher sensitivity than the combination of anti-CCP IgG and anti-MCE IgG, especially in patients with early RA. Taken together, the standard anti-CCP ELISA provided the highest sensitivity for diagnostic purposes compared to both the anti-MCV as well as anti-MCE tests in this cohort of Russian ERA and RA patients. The observed differences are remarkable and have not been reported in a similar setting so far. In another study, a rather low sensitivity of 53.3% for anti-MCV abs has also been reported in a cohort of Russian patients with ERA (27). For full explanation of

these results, the molecular details of the antibody binding have to be elucidated in the future with respect to the published cross reaction of homocitrulline containing proteins/peptides with ACPAs. Although the comparability between anti-CCP and anti-MCV tests for diagnosis and prognosis of RA has been shown by different studies with a tendency for a higher predictive value and specificity of anti-CCP antibodies (6, 28-30), it should be also considered that the anti-MCV assay might provide inferior diagnostic sensitivity compared to anti-CCP at least in some European RA cohorts. In this context, a lower sensitivity for anti-MCV was also reported recently in cohorts of Italian patients with RA (31). In contrast, previous results from other European RA cohorts including own data showed a higher sensitivity of anti-MCV compared to anti-CCP antibodies in RA (6, 17, 32). Since our results revealed a higher sensitivity for anti-CCP in the present study, it is possible that different epitopes are linked to a stronger degree to the pathogenesis of RA in Russian patients. This could also explain the unusual high antibody titers detected here.

It has been reported that a broader ACPA recognition profile in patients with arthralgia seems to be associated with a subsequent progression to arthritis, while a serum conversion from ACPA positive to negative and vice versa was observed rather rarely (33-35). However, no significant epitope spreading of ACPAs has been described after disease onset. Furthermore, an extended ACPA reactivity profile was not associated with joint damage after a follow-up of seven years (36). In this RA cohort, we observed a higher sensitivity for anti-MCV IgG, anti-MCE IgG and IgM as well as for RF IgA in ERA compared to established RA. This can only be partially explained by the geographic origin of the cohort. Another striking finding in our study was the high prevalence of ACPA of the IgA subclass. Recently, a predictive value for the development of RA has been reported for anti-CCP IgG and IgA, while the prevalence of anti-CCP IgG and IgA subclass reactivities was also higher compared to IgM in different studies (37, 38).

Another study described that several citrullinated antigens are recognised only by IgG, whereas other are also recognised by IgM ACPA, suggesting that the IgM ACPA response is more restricted than that of IgG ACPA in the same patient, and that not all citrullinated antigens are able to activate new B cells, despite concurrent recognition by IgG ACPA (39). In our cohort, the prevalence of anti-MCE IgA was higher than anti-MCE IgM in ERA and established RA patients, which might be of relevance for the immunological mechanisms of the disease.

Surprisingly, the prevalence of RF IgM was confirmed to be very low in the investigated cohort of Russian patients with ERA as well as with established RA by using two independent ELISA tests. Even the comparison with the other RF subclasses revealed that prevalence of RF IgG was equal to IgA but higher than IgM in ERA, whereas prevalence of RF IgG was higher than IgA, and IgA higher than IgM in established RA. In fact, only 3 (7.7%) of patients with ERA and 4 (2.1%) of patients with established RA were positive for RF IgM without detectable RF IgG or IgA. This low frequency of IgM RF accompanied by a high frequency of IgG and IgA in this RA population is of special interest. Although the reason for this unusual finding is unclear, it seems that immunoglobulin class switch in IgM RF producing B cells/plasma cells is somehow enhanced to produce IgG and IgA isotypes in this cohort. Interestingly, this effect does not appear to correlate with duration of disease, since IgM RF frequency was almost equal low both in ERA and in established RA patients. Here, it can be speculated that an increased Ig class switching in these B cells is influenced by the genetic background (*e.g.* presence of shared epitopes), the immunological context (*e.g.* a prominent activation of the mucosal associated immune response), or by environmental factors (*e.g.* smoking, therapeutic strategy). The influence of these or other possible factors on RF formation and maintenance in the Russian population should be elucidated in a further longitudinal study.

Several studies provided strong evidence that ACPA positivity is associated with presence of RF and joint erosions at time of diagnosis, and that this autoantibodies correlate with radiologic progression in RA (40-42). Of note, we were not able to show any significant correlation between the different autoantibody reactivities, nor with clinical as well as radiological findings. In part, this can be explained by the very low number of completely seronegative cases for ERA as well as established RA, which does not allow for appropriate comparison to the seropositive group. Furthermore, the used Steinbrocker's score is also a rather non-sensitive method for the evaluation of radiological findings (43). However, a lack of correlation between antibody reactivity to MCV and clinical as well as radiological findings has also been reported in ERA patients from a Russian cohort (27).

Conclusions

So far, the combination of ACPA and RF has provided the best performance for the diagnosis of RA. In our study, the combined analyses of anti-CCP IgG, anti-MCV IgG and anti-MCE abs was sufficient to characterise the majority of patients (92% ERA and 94% of RA) as seropositive. Therefore, for anti-CCP IgG seronegative patients it can be recommended to test further ACPA, namely anti-MCV IgG as suggested elsewhere. Of note, the autoantigenic properties of the MCV antigen are also sufficiently displayed by using cyclic citrullinated vimentin epitopes. However, the MCE selected here did not provide an additional advantage or improved diagnostic performance compared to the CCP antigen. In this context, it is important to note that ACPAs can recognise different antigens, including citrullinated fibrinogen, alpha enolase, and collagen, but it is the citrulline moiety which forms the antibody binding site. Thus, the amino acids surrounding the citrulline are important for binding of ACPA with different fine specificities. Therefore, it might be possible to optimise the MCV peptide sequences identified here, to end up with better overall sensitivity, which is being investigated in a further ongoing project.

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