

Association of Anti-Cyclic Citrullinated Peptide Antibodies, Anti-Citrullin Antibodies, and IgM and IgA Rheumatoid Factors with Serological Parameters of Disease Activity in Rheumatoid Arthritis

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ABSTRACT: We evaluated the association of anti-cyclic citrullinated peptide (CCP) antibody titers with serological markers of disease activity. We also compared three different anti-CCP antibody ELISAs with an anti-citrullin ELISA and the IgM and the IgA rheumatoid factor (RF) in their performance of discriminating between rheumatoid arthritis (RA) and other rheumatic diseases. Sera from 333 consecutive patients of the Rheumazentrum der Medizinischen Poliklinik München, an outpatient clinic for rheumatic diseases, were collected and tested. Anti-CCP antibodies were assayed with three different commercially available ELISAs. Antifilaggrin antibodies were tested with a commercially available ELISA using *in vitro* deiminated recombinant rat filaggrin. IgA-RF was analyzed with an ELISA, whereas IgM-RF was measured by latex-enhanced turbidimetry. Rheumatoid arthritis (RA) was diagnosed in 87 patients according to the revised classification criteria of the American College of Rheumatology (ACR), probable RA was diagnosed in 23 patients in an early phase not (yet) fulfilling the ACR criteria, and 223 patients had other rheumatic diseases. Differences in sensitivity and specificity were calculated using McNemar's test. A measure of agreement (kappa statistic) was used to examine whether the tests tended to identify the same patients as positive or negative. Correlations between CCP titers and other tests were analyzed by Spearman nonparametric rank correlation. No significant differences in sensitivity and specificity were found between the tested CCP assays (80.0–80.9% and 97.3–98.1%, respectively). All three CCP tests were slightly but not significantly more sensitive and specific than the anti-citrullin assay (77% and 92%, respectively), comparably sensitive but significantly more specific compared with the IgM-RF (86% and 82%, respectively), and significantly more sensitive but comparably specific compared with the IgA-RF (63% and 94.4%, respectively) in detecting the patients with RA. There was no significant correlation between anti-CCP, anti-citrullin, or IgM-RF or IgA-RF antibody titers and C-reactive protein, erythrocyte sedimentation rate, or white blood cell count. A weak but significant linear correlation was found between anti-CCP titers and IgM-RF

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Ann. N.Y. Acad. Sci. 1050: 295–303 (2005). © 2005 New York Academy of Sciences.
doi: 10.1196/annals.1313.031

titers ($r = 0.2$, $P = 0.03$). We could not find a significant difference between the three tested anti-CCP assays and the anti-citrullin test in terms of sensitivity and specificity. Compared with the IgM-RF, all the anti-CCP assays were superior in specificity and comparable in sensitivity. Compared with the IgA-RF, they were more sensitive and comparably specific in the discrimination of patients with RA from other rheumatic diseases. No correlation of any tested autoantibody titer with serological parameters of inflammation was found.

KEYWORDS: autoantibodies; filaggrin; peptidyl-arginine deiminase; PADI

INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory joint disease and one of the most common autoimmune diseases, affecting 0.5–1% of the population in Western countries. RA is a systemic, chronic, inflammatory disease characterized by joint inflammation that often leads to joint destruction. Because of the highly variable and unpredictable course of the disease, current therapeutic strategies in RA are increasingly aggressive regimens early in the course of the disease. Therefore, diagnostic tests with high specificity are desirable for choosing the optimal treatment.¹ The rheumatoid factor, usually the IgM rheumatoid factor (RF), is currently used in the diagnosis of RA and constitutes one of the classification criteria proposed by the American College of Rheumatology (ACR).² However, IgM-RF positivity shows low diagnostic specificity because IgM-RF is present in patients with other autoimmune and infectious diseases, and even in a considerable percentage of normal healthy subjects, particularly in aging individuals.³ During recent years, various other circulating antibodies have been reported to be of potential diagnostic value, including antiperinuclear factor antibodies, antikeratin antibodies, and anti-RA33 with adequate specificity but rather low sensitivity for RA.^{4–6} These antibodies were later recognized to bind to citrullinated proteins, especially citrullinated filaggrin or fibrin. The use of cyclic synthetic peptides with a high content of citrullin as antigen turned out to improve sensitivity substantially without loss of specificity. These assays for anti-cyclic citrullinated peptide (CCP) antibodies are of great interest in the diagnosis of RA. They are reported to have a high specificity (91–98%) but wide variability in diagnostic sensitivity (41–80%).^{7–10} We therefore compared the sensitivities and specificities of three commercially available anti-CCP antibody tests of the second generation (CCP2) with those of an anti-citrullinated fibrin antibody ELISA and IgM-RF and IgA-RF. We evaluated the association of all methods with serological parameters of disease activity (erythrocyte sedimentation rate [ESR], C-reactive protein [CRP], and white blood cell [WBC] count) and disease duration. We also investigated the differences of anti-CCP-negative versus anti-CCP-positive patients with RA within these parameters.

PATIENTS AND METHODS

Patients and Controls

In a cross-sectional study, we recruited 333 patients with suspected rheumatic diseases in the outpatient rheumatology unit of the University Hospital of Munich

(median age, 54.8 years; range, 13–90 years); 87 patients had definite RA according to the 1987 revised ACR criteria² (median age, 58.6 years; range, 19–84 years); 75% were women; 23 patients had suspected early RA, not (yet) fulfilling the ACR criteria according to the clinical evaluation; and 223 non-RA control patients with other rheumatological diagnoses were included in the study. To analyze the sensitivity and specificity of the tests, we used the definite and early RA groups and for controls pooled data from the other 223 patients with degenerative or other inflammatory joint diseases, including psoriatic arthritis, reactive arthritis, crystal arthropathy, osteoarthritis, and spondylarthropathy. The patients were evaluated by clinical examination and laboratory tests. The final clinical diagnosis according to the ACR criteria served for the diagnosis of definite RA, and clinically suspicious RA patients not (yet) fulfilling the ACR criteria were classified as possible “early RA”. The examiner was blinded to the anti-CCP, anti-citrullin, and IgA-RF results at the time of diagnosis. Blood samples were obtained at first clinical presentation and stored at -20°C until assayed. Disease activity of patients with RA was assessed at their first visit according to clinical and serological parameters (ESR, CRP, and WBC count).

Autoantibody Assays

The IgM-RF was measured by turbidimetry on a latex-enhanced agglutination assay (Roche Integra, Penzberg, Germany). Results were expressed in units per mL. The IgM-RF was considered positive at values greater than 10 U/mL. The RF isotypes for IgA were measured by a commercially available ELISA (Orgentec, Heidelberg, Germany) according to the manufacturer’s instructions for use. The IgA-RF was considered positive at values greater than 20 U/mL. The anti-CCP antibodies were analyzed by three commercially available second-generation ELISAs (Menarini/INOVA, Florence, Italy; Euroimmun, Lübeck, Germany; Generic Assays/Euro-Diagnostica, Dahlewitz, Germany) and were conducted according to the manufacturers’ instructions for use. Results were expressed in arbitrary units. The samples were considered positive according to the manufacturer’s manual if the antibody titer was greater than 5 (Euroimmun), 20 (Menarini), or 25 (Generic Assays/Euro-Diagnostica) arbitrary units. For the purpose of this study, each company was assigned a letter (A–C). The anti-citrullinated fibrin antibody ELISA was provided by BIOZOL/Genesis (Eching, Germany). For statistical analysis, the results were analyzed as continuous and dichotomous variables. The serological disease activity parameters ESR, CRP, and WBC count were measured according to standard methods and used as continuous values. Statistic correlations between the autoantibody tests used, serological activity parameters, and the disease duration were determined by Spearman’s rank correlation. The sensitivity and specificity for each assay was determined with respect to the clinical diagnosis. Differences between the tests were calculated with the McNemar’s test. The concordance between the tests were assessed by coefficient kappa. In addition, receiver operating characteristic (ROC) analysis was performed to compare test characteristics independently of predefined cutoff points. Student’s *t*-test for continuous variables was used to examine the significance of differences between the different groups. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were done with Medcalc statistical software (Version 6.10; Belgium).

TABLE 1. Sensitivity and specificity of the tests, alone or combined, for the diagnosis of rheumatoid arthritis

	Sensitivity	Specificity
CCP (A)	0.80	0.97
CCP (B)	0.81	0.98
CCP (C)	0.81	0.98
Citrullin	0.77	0.92
IgA-RF	0.63 ^a	0.94
IgM-RF	0.86	0.82 ^b
CCP + IgM-RF	0.90	0.81 ^b

^aSignificantly less sensitive compared with all other tests according to McNemar's statistics.

^bSignificantly less specific compared with all other tests according to McNemar's statistics.

RESULTS

Sensitivity and Specificity of the Assays for the Diagnosis of RA

When individual tests were considered, sensitivity for RA was highest for IgM RF (86%), followed by anti-CCP antibodies (81%), anti-citrullin antibodies (77%), and IgA RF (63%). The difference between IgM RF and the three anti-CCP antibodies was not significant. Specificity was significantly greater for anti-CCP antibodies (98%) and IgA RF (94%) and anti-citrullin antibodies (92%) than for IgM RF (82%). Sensitivity for the diagnosis of RA could be further increased by a combination of the anti-CCP and IgM-RF test, which resulted in a respectably high sensitivity of 89.9% (TABLE 1). IgA-RF and anti-citrullin antibodies could not further increase the overall sensitivity. Furthermore, in our study cohort, three of the anti-CCP-positive patients in the "non-RA" group with the diagnosis of SLE, osteoarthritis, and undifferentiated spondylarthropathy presented with articular manifestations. They were positive in all three anti-CCP assays and also for IgM-RF, but negative for IgA-RF, and two of them were also negative for anti-citrullin antibodies. For further comparisons of the diagnostic value of each assay, we did an ROC analysis and calculated the area under the curve. The ROC analysis displays the pairs of sensitivity and specificity for different cutoff points of anti-CCP, anti-citrullin, IgA-RF, and IgM-RF concentrations. The area under the curve was best for the anti-CCP assays, at 0.94–0.95. The values for anti-citrullin, IgM-RF, and IgA-RF were 0.88, 0.88, and 0.89, respectively. It could be clearly shown that CCP ELISA provided the best combination of sensitivity and specificity for detecting RA with no significant difference between the three tested anti-CCP assays. We also analyzed the benefit of single or combined use of all four antibody assays. We found a significant additional diagnostic value of anti-CCP compared with the single use of IgM-RF alone. The combination with anti-citrullin antibodies or IgA-RF could not further increase the sensitivity. In 56.3% of the 87 definite RA patients investigated, all antibody assays were positive. However, in 10 patients (11.5%) with clinically diagnosed RA, the conventionally used RF (IgM-RF) and in 31 patients (35.6%) the IgA-RF were negative. In four (40%) of these IgM-RF-negative patients, all three anti-CCP tests were positive. In 9 (10.3%)

TABLE 2. Agreement between the assay expressed as the concordance coefficients of the kappa test (95% confidence interval)

	CCP (B)	CCP (C)	Citrullin	IgM	IgA
CCP (A)	0.91 (0.86–0.96)	0.93 (0.88–0.97)	0.76 (0.69–0.84)	0.60 (0.51–0.69)	0.58 (0.47–0.69)
CCP (B)		0.94 (0.89–0.98)	0.81 (0.74–0.88)	0.64 (0.56–0.73)	0.62 (0.52–0.73)
CCP (C)			0.80 (0.73–0.88)	0.63 (0.54–0.71)	0.62 (0.52–0.72)
Citrullin				0.57 (0.47–0.66)	0.53 (0.43–0.64)
IgM					0.54 (0.45–0.64)

TABLE 3. Difference in the serological activity parameter in the CCP-negative versus CCP-positive definite RA patients

	Leukocytes (g/L)	ESR (mm/h)	CRP (mg/L)
CCP-negative (<i>n</i> = 9)	8.4	26.3	16.9
CCP-positive (<i>n</i> = 78)	8.6	28.5	18.6
<i>P</i> value	n.s.	n.s.	n.s.

NOTE: n.s., not significant.

of the 87 patients with definite RA, anti-CCP was negative and in one of these nine patients anti-citrullin was positive, in two patients IgA-RF was positive, and in four patients IgM-RF was positive. For the comparison of the antibody assays, we used the kappa coefficient test, where we could show a very good agreement between the anti-CCP assays (>0.9). There was also a good agreement between the anti-CCP and the anti-citrullin test (>0.8), but only a medium agreement between the mentioned assays and the IgM-RF and IgA-RF (0.5–0.64). [See TABLE 2.]

Specific Antibodies as Markers for Disease Activity

The analysis of serological parameters of disease activity (ESR, CRP, and WBC count) between the anti-CCP-positive and -negative patients with RA showed no significant differences in any parameter (TABLE 3). There was also no correlation between disease duration and the anti-CCP antibody titers. We could find a small, but significant correlation between the IgM-RF titer and the anti-CCP antibody titer ($r = 0.2$, $P = 0.03$). There was no significant correlation between anti-CCP, IgM-RF, IgA-RF, or anti-citrullin antibody titers and ESR, CRP, or WBC count.

DISCUSSION

There is evidence that early intensive therapeutic intervention (“hit hard and early”) in patients with RA may stop disease progression and joint damage, resulting in a better prognosis. It therefore is important to differentiate between RA and other forms of arthritis early after the onset of symptoms.^{11,12} First reported by Schellekens *et al.*,¹³ there is now growing evidence that the diagnostic properties of the anti-CCP autoantibody in the specific diagnosis of RA outplays other available antibody tests, especially IgM-RF.^{14–16}

This study is to our knowledge the first to compare three commercially available second-generation anti-CCP antibody tests (CCP2) and an anti-citrullin antibody assay with the IgM-RF and IgA-RF tests. The patient groups tested in our study were biased, because all patients came into a university outpatient rheumatology unit. These patients all had “rheumatology” problems and most of them had been sent to the unit by an other physician. Therefore, the pretest probability for the diagnosis RA is higher than for other patients with joint problems, and the percentage of patients positive for any test was relatively high compared with other studies.^{17,18} On the other hand, the clinical situation of these patients may be less clear and the specificity of any test for the diagnosis of RA is challenged. Therefore, a very important finding from the data of our patient groups was that anti-CCP is a highly specific marker in the diagnosis of RA. Comparable with the results of some other studies using the CCP1 and CCP2 assay, we found a specificity of 98%.^{19,20} The somewhat lower specificity and sensitivity of some other studies using anti-CCP assays may reflect different cutoff levels and different patient populations. The sensitivity of anti-CCP has also been increased by the second test generation anti-CCP assays used in this study. Now there is no longer a significant difference in sensitivity to the IgM-RF. We found a high sensitivity of 80% as described by others.^{20,21} On the other hand, lower sensitivities of approximately 65% also had been described.^{22,23} The lower sensitivity in those study cohorts may reflect the presence of a relatively high percentage of early rheumatoid patients and higher cutoff levels. By combining the use of all antibodies (anti-CCP, anti-citrullin, and IgM/A-RF), only the combination of anti-CCP with IgM-RF could increase the sensitivity (to 90%). Like others,^{13,22,23} we could also find an additional diagnostic value of anti-CCP compared with IgM-RF; as in 40% of the sero-negative (IgM-RF–negative) RA patients, anti-CCP antibodies could be detected. Also interesting was the observation that all anti-CCP–positive patients in the control group had an articular disease manifestation. As it was shown that the anti-CCP antibody may precede the clinical manifestation of RA by many years, these patients may not have received false-positive results but may develop RA or have a clinically undiagnosed RA. Rantapaa-Dahlqvist *et al.* showed that anti-CCP and IgA-RF predict the development of RA, with anti-CCP having the highest predictive value of all tested antibodies (IgG-RF, IgA-RF, and IgM-RF and CCP2).²⁴ The value of anti-CCP and antifilaggrin antibodies and RF for predicting the outcome of RA, clinical signs of disease activity, and the severity of radiographic joint damage has been investigated recently. Bas *et al.* showed an association of IgA-RF and anti-CCP with clinical signs of disease activity.^{7,25} The high prevalence of anti-CCP in RA patients with extensive disease activity and severe radiological changes, and even more impressively in RA patients who are IgM-RF–negative, suggests that anti-CCP is more useful than the RF alone in the early prediction of

disease outcome and disease activity. Vencovsky *et al.* showed that patients with erosive RA were more likely to be anti-CCP-positive and IgM-RF-negative than RF-positive and anti-CCP-negative.²⁶ Using anti-citrullin antibody tests, some studies failed to predict joint destruction and radiological progression by a positive test result using an assay with purified human filaggrin²⁷ or citrullinated rat filaggrin but could show a high diagnostic value for an anti-citrullin antibody assay in the diagnosis of RA.^{28,29}

A special interest of our study was the correlation of the antibody titers of anti-CCP and anti-citrullin antibodies and IgM-RF and IgA-RF with the serological markers of disease activity, the ESR, CRP, and WBC count. ESR and CRP are, along with the number of swollen and painful joints, one of the major criteria for the “clinical” disease activity score and the ACR scores. First, we could not find an association of disease duration or patient’s age with any of the tested antibodies (data not shown). Furthermore, we could find no significant differences between anti-CCP-negative and anti-CCP-positive patients comparing ESR, CRP, and WBC count (TABLE 3). And finally, there was no correlation between anti-CCP antibody titers or any other antibody titer with ESR, CRP, or WBC count.

In conclusion, the IgM-RF is still mostly used as a screening marker in the diagnosis of RA. However, the second-generation anti-CCP antibody assays have a comparable sensitivity in the diagnosis of RA but a much higher specificity. To establish the diagnosis of RA in a preselected patient group, we would suggest the use of the highly specific anti-CCP antibody test in the first line. The use of IgM-RF and IgA-RF could be restricted to a few unclear cases of anti-CCP-negative patients. Especially in ambiguous cases or in RF-negative patients with suspected RA, the anti-CCP assay has proved to be very helpful and seems to now be the diagnostic marker of choice for the diagnosis of RA. In our study, the combined use of IgM-RF and anti-CCP assays reached a sensitivity of 90%, but loses specificity. Therefore, the use of anti-CCP is the best diagnostic tool and also a good prognostic marker and would allow the clinician to choose a more intensive disease-modifying antirheumatic therapy early in the course of the disease, and also in patients for whom the clinical and radiological findings are not conclusive or indicative for such a therapy.

ACKNOWLEDGMENTS

We thank Ms. B. Danne, Ms. J. Partzsch, and Ms. M. Siwy for excellent technical assistance. Part of this work is included in the doctoral thesis of Mr. Alexander Greiner at the Ludwig-Maximilians-University of Munich.

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